JOURNAL OF CLINICAL MICROBIOLOGY, Dec. 2005, p. 5865–5872 Vol. 43, No. 12
0095-1137/05/$08.00+0 doi:10.1128/JCM.43.12.5865–5872.2005

Rapid Ped-2E9 Cell-Based Cytotoxicity Analysis and Genotyping of *Bacillus* Species

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Received 1 April 2005/Returned for modification 22 May 2005/Accepted 25 September 2005

***Bacillus* species causing food-borne disease produce multiple toxins eliciting gastroenteritis. Toxin assays with mammalian cell cultures are reliable but may take 24 to 72 h to complete and also lack sensitivity. Here, a sensitive and rapid assay was developed using a murine hybridoma Ped-2E9 cell model. *Bacillus* culture supernatants containing toxins were added to a Ped-2E9 cell line and analyzed for cytotoxicity with an alkaline phosphatase release assay. Most *Bacillus cereus* strains produced positive cytotoxicity results within 1 h, and data were comparable to those obtained with the standard Chinese hamster ovary (CHO)-based cytotoxicity assay, which took about 72 h to complete. Moreover, the Ped-2E9 cell assay had 25- to 58-fold-higher sensitivity than the CHO assay. Enterotoxin-producing *Bacillus thuringiensis* also gave positive results with Ped-2E9 cells, while several other *Bacillus* species were negative. Eight isolates from food suspected of *Bacillus* contamination were also tested, and only one strain, which was later confirmed as *B. cereus*, gave a positive result. In comparison with two commercial diarrheal toxin assay kits (BDE-VIA and BCET-RPLA), the Ped-2E9 assay performed more reliably. Toxin fractions of >30 kDa showed the highest degree of cytotoxicity effects, and heat treatment significantly reduced the toxin activity, indicating the involvement of a heat-labile high-molecular-weight component in Ped-2E9 cytotoxicity. PCR results, in most cases, were in agreement with the cytotoxic potential of each strain. Ribotyping was used to identify cultures and indicated differences for several previously reported isolates. This Ped-2E9 cell assay could be used as a rapid (~1-h) alternative to current methods for sensitive detection of enterotoxins from *Bacillus* species.**

Bacillus cereus is one of the major food-borne pathogens and is a spore-forming aerobic bacterium sometimes causing severe vomiting in 1 to 5 h and/or diarrhea within 8 to 16 h following ingestion of contaminated food. The difference in onset is due to the type of toxin the bacteria produce. In the former case, ingestion of the preformed emetic toxin manifests immediate symptoms, while for diarrhea, toxicoinfection results in enterotoxin production, thus requiring longer to induce gastrointestinal disorder (19, 50). While the emetic syndrome is generally associated with cereal foods including rice and pasta, diarrheal toxins are found in many foods, including milk, vegetables, and meat products (19, 31). *B. cereus* can grow over wide temperature and pH ranges, at high salt concentrations (31), and under aerobic or anaerobic conditions (19) and can also survive harsh food-processing treatments through spore formation; therefore, *B. cereus* is a serious concern in foods (36). Several other *Bacillus* species, such as *B. thuringiensis*, *B. subtilis*, *B. licheniformis*, *B. pumilus*, *B. mojavensis*, *B. fusiformis*, and *B. sphaericus*, are known to produce enterotoxin and could be food safety concerns (5, 16, 17, 30).

In addition to emetic or diarrheal enterotoxins, *B. cereus* also produces hemolysins, phospholipases, and other enterotoxins (30) (summarized in Table 1). The hemolysin HBL complex, composed of a B component and two L components (L_1 and L_2), is thought to be the primary virulence factor for *B. cereus* because it is hemolytic, cytotoxic, dermonecrotic, and increases vascular permeability (6, 7, 14, 33). While all three subunits are required for maximal activity, the L_1 subunit alone is cytotoxic (7). Another three-component enterotoxin produced by *B. cereus* is the nonhemolytic enterotoxin (NHE), composed of the NHE-A, NHE-B, and NHE-C proteins (20, 33). As with the HBL complex, all three components must be present for full activity (34). Other enterotoxins produced by *B. cereus* include enterotoxin T (BcET), enterotoxin FM (EntFM), and cytotoxin K (CytK). BcET has either an unknown type of enterotoxic action or none at all (1, 12) but is generally found in more than half of outbreak-associated strains. EntFM, like BcET, has not been implicated as the enterotoxin responsible for a food poisoning outbreak, but EntFM is present in most outbreak-associated strains and is actually the most prevalent enterotoxin gene for all *B. cereus* strains (25, 52). Unlike EntFM and BcET, CytK has been implicated in *B. cereus*-related deaths due to necrotic enteritis (32).

Traditional plating and biochemical assays are time-consuming and do not indicate *Bacillus* toxin production capabilities (31). In order to assess biological activity, a number of eukaryotic-cell-based assays have been employed (5, 11, 27). The emetic toxin is traditionally assessed using a HEp-2 tissue culture assay by observing vacuole formation (3, 26, 51) or colorimetrically, by using the 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) metabolic staining assay (15, 47). Another bioassay, based on the loss of motility of boar spermatozoa, has also been developed (4). Recently, an assay has been developed for emetic toxin based on its mitochondrial respiratory uncoupling activity on rat liver mitochondria (28). A number of cell lines have been employed to detect diarrheal

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TABLE 1. Toxins produced by *Bacillus cereus*

Toxins	Mol mass (kDa)	Activity	Reference(s)
Emetic toxin	~1.2	Emesis (vomiting)	30, 51
Diarrheal toxin	38–43	Diarrhea	30, 50
Hemolysin (HBL)		Hemolytic, enterotoxic, dermonecrotic	6, 7, 14, 33
B component	37.8		
L component (L_1)	38.5		
L component (L_2)	43.5		
NHE		Enterotoxic	20, 33, 34, 40
NHE-A	41		
NHE-B	39.8		
NHE-C	36.5		
BcET	41	Unknown	1, 12
EntFM	45	Enterotoxic, induces vascular permeability	25, 52
CytK	34	Necrotic enteritis	32

enterotoxin activity, including the McCoy cell line (16, 27), where cytotoxicity was determined by microscopic observation. In the Vero cell line, enterotoxin activity was evaluated by measuring protein synthesis inhibition and decreased cell proliferation (14, 17, 35, 42, 56). Similar analysis was also done with the Caco-2 cell line (24, 47). A CHO-based assay was developed for detection of both the emetic and the diarrheal toxin through cytotoxic staining and measurement of metabolic activity (5, 25, 40). The drawback of these cell-based assays is the length of time required to complete the assays, 24 h to 72 h (5, 38, 54).

The PCR is generally used as a tool for toxin and strain characterization (18, 22, 25, 29, 41–43). PCR-based detection gives a high level of accuracy but does not reveal pathogenic potentials of strains, since phenotypic expression of genes is not always warranted. Two commercially available kits, the *Bacillus* Diarrheal Enterotoxin Visual Immunoassay (BDE-VIA; Tecra) and *Bacillus cereus* Enterotoxin-Reversed Passive Latex Agglutination (BCET-RPLA; Oxoid) kits, are used by the industry or clinicians (48). The drawback of these tests is that the BDE-VIA kit detects only the 41-kDa subunit of NHE, while the BCET-RPLA kit detects only the L_2 subunit of HBL. Since it is known that different strains of *B. cereus* may produce both, only one, or perhaps neither of these subunits, the tests could easily produce false negatives (16) in a complex medium such as food.

Previously, a cytotoxicity assay based on a B-cell hybridoma, Ped-2E9, was developed that measures *Listeria monocytogenes*-induced cell damage in 1 to 2 h either by trypan blue staining or by an alkaline phosphatase (AP) release assay (8–10, 37, 53, 57). Recently a B-cell line-based biosensor assay was developed to sensitively detect *Bacillus anthracis*, *Yersinia pestis*, and *Escherichia coli* O157:H7 (45). In this study, we developed a Ped-2E9 cell-based assay as an alternative to the current *B. cereus* enterotoxin assays. Quantitative cytotoxic assessment was made by an alkaline phosphatase release assay, and results were compared to those of the traditional CHO assay and the commercial BDE-VIA and BCET-RPLA kits. Toxin gene profiles were determined by PCR, and culture identifications were done by ribotyping.

MATERIALS AND METHODS

Bacterial cultures and ribotyping. The *Bacillus* cultures used in this study are listed in Table 2. *Bacillus* cultures were also isolated from nine ready-to-serve pasta and rice dishes procured from local restaurants (Table 2). Within 1 h of arrival, 11 g of each sample was added to 99 ml of 0.85% saline, dilutions were plated onto mannitol-egg yolk-polymyxin agar (Difco Laboratories, Sparks, MD) and incubated at 37°C for 24 h, and suspected *Bacillus* colonies were collected as natural isolates (44) for use with the cytotoxicity assay in a blind format.

All cultures were grown in Luria-Bertani (LB) broth for 18 h at 37°C and streaked onto LB agar (LB containing 1.5% agar) for isolation of single colonies. Isolated colonies were used for cytotoxicity and PCR assays and were ribotyped (21) using an automated Riboprinter (Qualicon, Inc.) with the EcoRI restriction enzyme. Ribopatterns were compared with the RiboPrinter database for culture identification.

Hemolysin and lecithinase activity. Cultures were tested for hemolytic and lecithinase activity by spot inoculation onto 5% sheep blood agar plates (Difco) and 10% egg yolk agar plates (44), respectively.

Toxin preparation. Crude toxins were prepared from bacterial-cell-free culture supernatants. Test strains were grown in LB for 18 h at 37°C in a shaker incubator (New Brunswick Scientific Co.) at 140 rpm and centrifuged (10,000 × *g* for 10 min at 4°C). The supernatants were passed through a 0.45-μM-pore-size filter (Nalgene), designated crude toxin preparation, and either used for cytotoxicity assays immediately or stored at refrigeration temperature (4°C) for no more than 48 h. The protein concentration of the crude toxin preparation was determined by using a Bio-Rad protein assay kit.

To determine the effect of heat, cell-free culture supernatants (toxin preparations) from *B. cereus* MS1-9, ATCC 14579, A926, CA-1, HS23-11, and F4810 were held at 58°C for 40 min (13) or at 70°C for 15 min, and cytotoxicity was determined on Ped-2E9 cells as described below.

Cell cytotoxicity assays. The Ped-2E9-based cytotoxicity assay was modified slightly from the previous methods (8, 9). Ped-2E9 murine hybridoma lymphocytes were maintained in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% (vol/vol) fetal bovine serum (Atlanta Biologicals, Norcross, GA). Cells were cultured at 37°C under a humid atmosphere containing 7% CO_2 and were subcultured every 2 to 4 days. Ped-2E9 cells were harvested, counted by trypan blue staining (0.4%), centrifuged (300 × *g* for 10 min), and resuspended to 1×10^6 to 2×10^6 cells/ml in serum-free medium (Sigma). The assay was performed in sterile 1.5-ml Eppendorf tubes with 1 ml Ped-2E9 cell suspension and 100 μl of toxin preparation. Negative controls of LB (100 μl) and positive controls of 1% Triton X (100 μl) in 20 mM phosphate-buffered saline, pH 7.0, were included. All tubes were incubated at 37°C for 1 h, and cytotoxicity for Ped-2E9 cells was determined by the AP release assay (9).

Cytotoxicity profiles of selected *Bacillus* cultures were also compared with the standard CHO cell-based MTT metabolic staining assay (5, 40) using an MTT assay kit (Roche Applied Science, Indianapolis, IN). Briefly, CHO (CHO-K1; ATCC, Manassas, VA) cell monolayers in T-25 flasks were trypsinized, and viable-cell concentrations were determined by trypan blue staining. About 4×10^4 viable cells were added to each well of a 96-well tissue culture plate. After an overnight incubation at 37°C in 7% CO_2 under humidified conditions, the medium was replaced with 100 μl of the toxin preparation/well in triplicate, and the cells were incubated for an additional 24 h. Ten microliters of MTT labeling reagent (final concentration, 0.5 mg/ml) was added per well and incubated (37°C, 7% CO_2) for an additional 4 h, followed by the addition of 100 μl/well of solubilization buffer. The plates were again incubated overnight or until the purple formazan salts were dissolved, and the absorbance was measured in a microplate reader (Bio-Rad) at 595/655 nm.

To compare the sensitivities of the Ped-2E9- and CHO-based assays, successive twofold dilutions of the crude toxin preparations from *B. cereus* MS1-9 and ATCC 14579 were tested with both cell lines by using AP release from Ped-2E9 cells (9) and an MTT assay with CHO cells (5). The protein concentration of each toxin preparation was determined by using a protein assay kit from Bio-Rad.

Determination of cytotoxic action of fractionated toxin preparations. To identify the toxin components that are responsible for cell cytotoxicity, a crude toxin preparation from *B. cereus* MS1-9 was used. The crude toxin preparation was fractionated based on the molecular size by using molecular weight cutoff membranes in protein concentrators (Millipore-Amicon). Membranes with molecular cutoff values of 10, 30, and 50 kDa were used to yield fractions of 10 to 30 kDa (fraction a), 30 to 50 kDa (fraction b), and >50 kDa (fraction c). The protein concentration of each fraction was estimated by using the Bio-Rad protein assay kit and adjusted to a uniform concentration of about 40 μg/ml. All toxin fractions were tested for cytotoxicity on Ped-2E9 cells as described above.

PCR. Individual cultures of *Bacillus* species were resuspended in nuclease-free water, and the genomic DNA was prepared by boiling for 10 min. Samples

TABLE 2. Ped-2E9 cell-based cytotoxicity analysis and characterization of *Bacillus* species

Culture (mo-day-yr) or food isolate	Riboprinter identification (Dupont ID)	Source[a]	Hemolysin[b]	Lecithinase[b]	Cytotoxicity (%)[c]	Presence of the following gene[d] by PCR analysis:					
						bceT	*cytK*	*hblA*	*nheA*	*nheB*	*nheC*
Cultures											
B. cereus PU1	*B. cereus* DUP-6001	Our collection	+	+	20 ± 2.1	−	−	+	+	−	+
B. cereus NRL 569	*B. cereus* DUP-13212	A. Aronson	+	+	67 ± 5.3	+	−	+	+	+	+
B. cereus T	*B. thuringiensis* DUP-6040	A. Aronson	−	−	13 ± 1.1	+	−	+	+	+	+
B. cereus ATCC 3432	*B. cereus* DUP-6078	B. Applegate	+	+	55 ± 3.3	−	−	+	+	−	+
B. cereus CA1	*B. cereus* DUP-6092	B. Applegate	+	+	0.2 ± 1.3	+	−	−	+	−	−
B. cereus CA6	NT	B. Applegate	+	+	36 ± 1.3	+	−	+	+	+	+
B. cereus ATCC 33018	*Bacillus* sp., no matching	A. Wong	+	+	43 ± 2.0	−	−	−	+	+	+
B. cereus F837/76	*B. cereus* DUP-6079	A. Wong	+	+	17 ± 1.3	−	−	+	−	−	−
B. cereus A926	*B. cereus* DUP-6082	A. Wong	+	+	88 ± 2.1	+	+	+	+	+	+
B. cereus F4810	*B. cereus* DUP-6012	A. Wong	+	+	60 ± 0.9	−	−	−	+	+	+
B. cereus AS4-12	*B. cereus* DUP-12561	J. Handlesman	+	+	78 ± 2.2	−	−	+	+	+	+
B. cereus MS1-9	*B. cereus* DUP-12561	J. Handlesman	+	+	80 ± 2.0	−	−	+	+	+	+
B. cereus HS23-11	*B. cereus* DUP-12561	J. Handlesman	+	+	72 ± 2.0	−	−	+	+	+	+
B. cereus UW85	*B. cereus* DUP-11561	J. Handlesman	+	+	69 ± 1.4	−	−	+	+	+	+
B. cereus 1230-88	*B. cereus* DUP-12561	J. McKillip	+	+	82 ± 3.0	NT	NT	NT	NT	NT	NT
B. cereus ATCC 14579	*B. cereus* DUP-6082	J. McKillip	−	−	64 ± 3.0	+	+	+	+	+	+
B. subtilis	*B. subtilis* DUP-12546	Our collection	−	+	1 ± 1.2	−	−	−	−	−	−
B. subtilis PY79	*B. subtilis* DUP-12546	G. Siragusa	−	−	−	NT	NT	NT	NT	NT	NT
B. subtilis ATCC 6633	*B. subtilis* DUP-12551	J. McKillip			−	NT	NT	NT	NT	NT	NT
B. megaterium MA	*B. thuringiensis* DUP-6044	A. Aronson	+	+	53 ± 1.2	+	−	+	+	+	+
B. megaterium DSM 319	*B. subtilis* DUP-9501	J. McKillip	−	−	−	NT	NT	NT	NT	NT	NT
B. megaterium ATCC 9885	*B. megaterium* DUP-16973	G. Siragusa	−	−	−	+	−	−	−	−	−
B. thuringiensis H073	*B. thuringiensis* DUP-6034	A. Aronson	−	−	37 ± 1.3	+	−	+	+	+	+
B. thuringiensis subsp. *kurstaki* #4	*B. cereus* DUP-13204	J. McKillip	±	+	74 ± 1.1	NT	NT	NT	NT	NT	NT
B. thuringiensis subsp. *kurstaki* #7	*B. oleronius* DUP-17021	J. McKillip	−	−	−	NT	NT	NT	NT	NT	NT
B. thuringiensis subsp. *berliner*	*B. cereus* DUP-6082	J. McKillip	+	+	66 ± 2.8	NT	NT	NT	NT	NT	NT
B. lentimorbis #11	*B. thuringiensis* DUP-6040	J. McKillip	+	+	79 ± 3.7	NT	NT	NT	NT	NT	NT
B. licheniformis #15	*B. pumilus* DUP-6073	J. McKillip	−	−	−	NT	NT	NT	NT	NT	NT
B. polymyxa B719 W (3-18-76)	*Paenibacillus polymyxa* DUP-11063	K. Hayes	−	−	−	NT	NT	NT	NT	NT	NT
B. polymyxa B719 X (1-20-65)	*Paenibacillus polymyxa* DUP-11063	K. Hayes	−	−	−	−	−	−	−	−	−
B. polymyxa B719 V (8-20-69)	*Paenibacillus polymyxa* DUP-11063	K. Hayes	−	−	−	NT	NT	NT	NT	NT	NT
Food isolates											
Baclo 2b	*B. subtilis* DUP-12544	Lo mein (veg)	−	+	−	NT	NT	NT	NT	NT	NT
Baclo 2d	*B. subtilis* DUP-12551	Lo mein (veg)	−	+	−	NT	NT	NT	NT	NT	NT
Baclo 2e	*B. subtilis* DUP-6098	Lo mein (veg)	−	−	−	NT	NT	NT	NT	NT	NT
Baclo 3a	*B. cereus* DUP-6002	Lo mein	+	−	15 ± 6.5	−	−	−	+	+	+
BacSR4a	*B. subtilis* DUP-6098	Steamed rice	−	−	−	NT	NT	NT	NT	NT	NT
BacSpR8a	*Bacillus* sp., no matching	Spinach rice	−	−	−	NT	NT	NT	NT	NT	NT
BacSpR8b	*B. subtilis* DUP-12546	Spinach rice	−	−	−	NT	NT	NT	NT	NT	NT
BacSpR8c	*B. subtilis* DUP-12544	Spinach rice	−	−	−	NT	NT	NT	NT	NT	NT

[a] Sources of cultures are as follows: J. Handlesman, Department of Plant Pathology, University of Wisconsin; A. Wong, Food Research Institute, University of Wisconsin; B. Applegate, Department of Food Science, Purdue University; A. Aronson, Department of Biology, Purdue University; J. McKillip, Department of Biology, Ball State University; G. Siragusa, USDA-ARS, Russell Research Center, Athens, GA; K. Hayes, Department of Food Science, Purdue University. Veg, vegetable.

[b] Hemolysin activity was determined on 5% sheep blood agar plates, and lecithinase activity was determined on plate count agar containing egg yolk. +, strongly positive; ±, weakly positive; −, negative.

[c] Cytotoxicity values are averages from at least two experiments analyzed in triplicate. Values above 1% were considered positive based on the values produced by a negative-control strain, *B. subtilis*. −, no cytotoxicity.

[d] +, presence of PCR product; −, absence of PCR product; NT, not tested.

were centrifuged (930 × *g*, 10 min), and the supernatants were decanted into sterile Eppendorf tubes. One microliter of each supernatant was used as the template for PCR amplification (see Table 5). PCR products were amplified using Ready-To-Go PCR beads (Amersham Biosciences, Piscataway, NJ), and all reactions were carried out in a PTC-100 Programmable Thermal K-6 Controller (MJ Research, Inc., Watertown, MA). The *hblA*, *bceT*, and *cytK* genes were amplified individually, while the *nheA*, *nheB*, and *nheC* genes were multiplexed according to the PCR conditions given in Table 5. Amplified PCR products were analyzed by agarose (1 to 1.2%) gel electrophoresis and visualized by ethidium bromide staining.

Commercial assay kits. Two commercial immunological kits, BCET-RPLA (Oxoid) and BDE-VIA (Tecra), were used with cell-free culture supernatants to assess enterotoxin production according to the manufacturer's directions. The BCET-RPLA is based on agglutination, which detects antibody reaction to the L_2 subunit of HBL. The BDE-VIA is a visual assay based on the enzyme-linked immunosorbent assay that detects the 41-kDa subunit of NHE.

Statistical analysis. Data were analyzed using SAS software 8.02 (Cary, NC) for analysis of variance and residual plots. The differences in mean values were determined by Tukey's test; a P value of <0.05 was considered significant.

RESULTS

Characterization of *Bacillus* cultures. Thirty-one previously isolated cultures obtained from various researchers were used in this study; these included 16 *B. cereus*, 4 *B. thuringiensis*, 3 isolates each of *B. subtilis*, *B. megaterium*, and *B. polymyxa*, and 1 isolate each of *B. lentimorbis* and *B. licheniformis* (Table 2). All *B. cereus* strains were identified as *B. cereus* by ribotyping except for *B. cereus* T, CA6, and ATCC 33018. T was identified as *B. thuringiensis*, CA6 was not typed, and ATCC 33018 did not give any matching with any known *Bacillus* ribopatterns in the RiboPrinter database. All *B. cereus* strains except ATCC 14579 were hemolytic and showed positive lecithinase activity. All *B. subtilis* and *B. polymyxa* strains were correctly identified. Of three *B. megaterium* strains tested, only one was identified as *B. megaterium*, while two others were identified as *B. thuringiensis* and *B. subtilis*, respectively. Of four *B. thuringiensis* strains tested, only one was identified as *B. thuringiensis*, while two were identified as *B. cereus* and one as *B. oleronius*. *B. lentimorbis* and *B. licheniformis* were identified as *B. thuringiensis* and *B. pumilus*, respectively. Besides *B. cereus*, only two of four *B. thuringiensis* strains were hemolytic and lecithinase positive. None of the other *Bacillus* isolates was hemolytic or lecithinase positive except for one strain of *B. subtilis*, which was lecithinase positive (Table 2).

A total of eight suspected *Bacillus* cultures were isolated from four of nine prepared, ready-to-serve rice and pasta dishes (Table 2). Six of those isolates were identified by ribotyping as *B. subtilis*, one (Baclo3a) was identified as *B. cereus*, and the remaining strain, BacSpR8a, was unknown. Although strain BacSpR8a was identified as a *Bacillus* sp. by ribotyping, no strain designation was assigned, because the fingerprinting similarity values were well below the acceptable range (<90%). Of the six *B. subtilis* strains, only two were positive for lecithinase activity and none were hemolytic. The *B. cereus* strain Baclo3a was hemolytic but lecithinase negative.

Cytotoxicity assay. In all cytotoxicity assays, a strain was considered positive when its cytotoxicity value was above the value produced by a negative-control *B. subtilis* strain for that experiment. During initial screening (Table 3), cytotoxic effects of eight *B. cereus* isolates and one *B. subtilis* isolate were compared with Ped-2E9 and CHO cells. The *B. subtilis* strain in this set showed 16.4% ± 19.8% cytotoxicity with CHO cells and only 1% ± 1.2% with Ped-2E9 cells. Residual plots were generated with cytotoxicity values of all *Bacillus* isolates with CHO and Ped-2E9 cells (Table 3) separately to eliminate false negatives among test strains; this analysis allowed the cutoff to be set at 21% for the CHO assay and at 4% for the Ped-2E9 assay. All *B. cereus* strains from this set appeared to be cytotoxic to both cell lines, and the difference between the positive strains and the *B. subtilis* negative control in both the CHO and Ped-2E9 assays appeared to be significant at a P value of <0.05 (Table 3). These data indicated that the Ped-2E9 cell assay could potentially be used as a substitute for the CHO-based assay to test for *B. cereus* toxins.

Further testing of additional previously isolated *Bacillus* cultures with Ped-2E9 cells revealed that only *B. cereus* and *B. thuringiensis* strains were positive with this cell line, while other species were negative. Our ribotyping results differed in several cases from previous identifications (Table 2). Taking those into account, in our final estimate, of 17 *B. cereus* strains, only 1 (CA1) was negative while values for the remaining 16 ranged from 27 to 88%. All four *B. thuringiensis* strains were positive, with cytotoxicity values of 13 to 79% (Table 2). Heat treatment (58°C or 70°C) of the toxin preparations from several *B. cereus* strains resulted in significant loss ($P < 0.05$) of activity, suggesting that the Ped-2E9-specific component(s) is heat sensitive (Table 4).

TABLE 3. Comparison of the Ped-2E9-based cytotoxicity assay with other cell culture-based and commercial assay kits

Strain	Result by:			
	Commercial assay[a]		Tissue culture assay	
	BDE-VIA	BCET-RPLA	CHO diarrheagenic assay[b,c]	Ped-2E9 cytotoxicity[b,d]
B. cereus				
F837/76	+++	+++	94.2 ± 5.57^{A}	17 ± 1.3^{F}
ATCC 33018	+	−	28.5 ± 10.34^{E}	43 ± 2.0^{E}
A926	+/++	+++	62.4 ± 4.27^{C}	88 ± 2.1^{A}
F4810	+++	−	24.9 ± 11.3^{E}	60 ± 0.9^{D}
AS4-12	+	+++	43.8 ± 12.69^{D}	78 ± 2.2^{B}
MS1-9	+	+++	41.4 ± 12.87^{D}	80 ± 1.9^{B}
HS23-11	+	+++	82.0 ± 8.18^{B}	72 ± 2.0^{C}
UW85	+	+++	107.6 ± 0.95^{A}	69 ± 1.4^{C}
B. subtilis	−	−	16.4 ± 19.8^{F}	1.0 ± 1.2^{G}

[a] The BDE-VIA is NHE specific, while the BCET-RPLA is HBL specific. Results were scored visually as positive (+, ++, or +++) or negative (−) according to the manufacturers' directions.

[b] Cytotoxicity is expressed as a percentage ± standard deviation. Data are averages from two experiments analyzed in duplicate. Values in the same column that are followed by different superscript letters (A through G) are significantly different ($P < 0.05$).

[c] Cytotoxicity was determined using a 1:2 dilution of the toxin, and the assay needed at least 72 h to complete. Cytotoxicity values above 16.4%, the level produced by a negative-control strain (*B. subtilis*), were considered positive for diarrheagenic activity with the CHO cell-based assay.

[d] Positive samples resulted in >1% cytotoxicity. Cytotoxicity values were obtained after a 1-h incubation.

The ability of this Ped-2E9-based assay to detect cytotoxicity of nascent food isolates was investigated. Eight suspected *Bacillus* cultures obtained from four of nine food samples were tested with Ped-2E9 cells without any prior knowledge of their "species" identity. Only one strain (Baclo3a) gave positive cytotoxicity (15%); the remaining strains did not show any cytotoxicity (Table 2). Upon ribotyping, the cytotoxic strain (Baclo3a) was identified as *B. cereus* DUP-6002 and the remainder of the isolates were identified as *B. subtilis* or a *Bacillus* sp. Although a small number of natural isolates were tested, this Ped-2E9 assay accurately detected the cytotoxic potential of a single *B. cereus* strain.

The sensitivities of Ped-2E9 cells to toxins of two diarrheagenic strains of *B. cereus*, MS1-9 and ATCC 14579, were compared with the MTT-based CHO assay (Fig. 1A and B). When *B. cereus* MS1-9 toxin was used with Ped-2E9 cells, 50% cytotoxicity was observed at a toxin concentration of 4.4 μg/ml, whereas 35 μg/ml was needed to show 50% cytotoxicity ($P < 0.05$) in the CHO cell assay. Cytotoxicity titration end points revealed that a minimum toxin concentration of 0.3 μg/ml

TABLE 4. Cytotoxicity analysis of heat-treated toxin preparations from *Bacillus cereus*

Bacillus cereus toxin preparation (treatment)	% Cytotoxicity (avg ± SD)[a]
F 4810[b] (none)	66.56 ± 0.11[A]
F 4810 (58°C, 40 min)	0.75 ± 0.01[D]
HS23-11 (none)	45.59 ± 0.15[B]
HS23-11 (58°C, 40 min)	0.94 ± 0.02[D]
CA1[c] (none)	4.13 ± 0.04[D]
CA1 (58°C, 40 min)	2.46 ± 0.07[D]
A926 (none)	51.56 ± 2.35[B]
A926 (58°C, 40 min)	8.20 ± 1.02[C]
A926 (70°C, 15 min)	2.48 ± 0.05[D]
ATCC 14579 (none)	63.89 ± 3.01[A]
ATCC 14579 (58°C, 40 min)	11.04 ± 5.58[C]
ATTC 14579 (70°C, 15 min)	5.06 ± 0.04[D]

[a] Data are averages from two experiments analyzed in duplicate. Values followed by different superscript letters (A through D) are significantly different ($P < 0.05$). In this experiment, the cytotoxicity value for the negative-control strain (*B. subtilis*) was about 2.24%.
[b] Emetic-toxin-producing strain.
[c] Considered a noncytotoxic strain (see Table 2).

caused positive cytotoxicity in Ped-2E9 cells while a concentration of >17.5 μg/ml was required to show positive cytotoxicity in CHO cells, indicating that the Ped-2E9 assay is about 58-fold more sensitive than the CHO assay (Fig. 1A). A similar pattern of cytotoxicity was observed for another *B. cereus* strain, ATCC 14579 (Fig. 1B), where 50% cytotoxicity in Ped-2E9 cells was observed at a protein concentration of about 6.6 μg/ml, whereas >13 μg/ml of toxin was needed to show 50% cytotoxicity in CHO cells. In this case, a minimum toxin concentration of 0.4 μg/ml caused positive cytotoxicity in Ped-2E9 cells, while a concentration of ~10 μg/ml was required to show positive cytotoxicity in CHO cells, indicating that the Ped-2E9 assay is 25-fold more sensitive than the CHO assay (Fig. 1B). Cytotoxicity titration curves for Ped-2E9 cells with toxin preparations from both *B. cereus* strains revealed a gradual decline in cytotoxicity values with decreasing concentrations of toxins (0.3 to 0.4 μg/ml), whereas examination of the same curves for CHO cells revealed a sudden drop in cytotoxicity values, approaching zero percent, at toxin concentrations between 17.5 and 6.6 μg/ml. In this experiment, the cytotoxicity titration end point was established in comparison with a negative-control (*B. subtilis*) strain that gave a cytotoxicity value of ~1%. Taken together, these data indicate that the Ped-2E9 assay is 25- to 58-fold more sensitive than the CHO assay based on the lowest concentrations of toxins that gave positive cytotoxicity values on both cell lines.

Identification of toxin fractions with cytotoxic effects on Ped-2E9 cells. The total protein concentrations for *B. cereus* MS1-9 crude toxin fractions a, b, and c were adjusted to ~40 μg/ml and were analyzed by the cytotoxicity assay with Ped-2E9 cells. Fraction c (>50 kDa) showed the maximum cytotoxicity (>100%) in the Ped2E9 assay, followed by fraction b (30 to 50 kDa) with 70% activity, levels that were significantly different ($P < 0.05$) from that of fraction a (10 to 30 kDa), with 19.2% activity, or the toxin preparation from the negative-control *B. subtilis* strain, with 6.5% activity (Fig. 2). This study demonstrated that the majority of cytotoxicity effects are associated with high-molecular-weight toxin components (>30 kDa).

PCR analysis of toxin genes. PCR was performed to screen various *Bacillus* isolates for the target genes listed in Table 5.

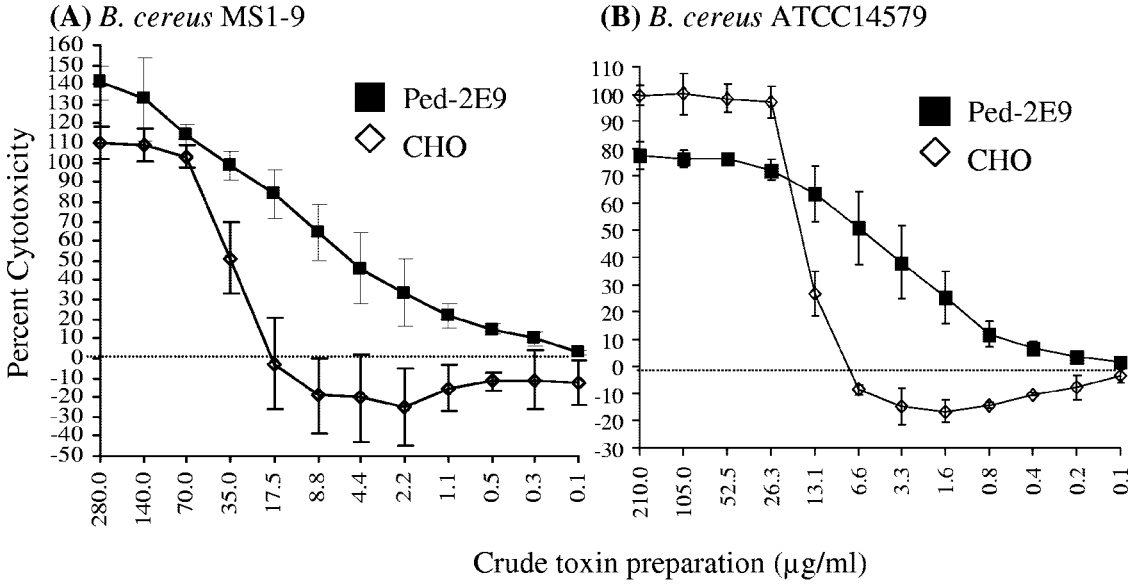

FIG. 1. Determination of sensitivities of the Ped-2E9-based assay and the CHO-based assay with toxin preparations from *Bacillus cereus* MS1-9 and ATCC 14579. Protein concentrations of each toxin preparation were determined, and preparations were serially diluted (twofold dilutions) in LB broth and tested on the CHO and Ped-2E9 cells. All dilutions were tested with Ped-2E9 at 2×10^6 hybridoma cells/ml, and cytotoxicity values were determined by the AP release assay at 1 h. All dilutions were also tested on confluent CHO cell monolayers in a 96-well plate, and cytotoxicity values were determined by the MTT assay after 72 h. Data are averages for three experiments analyzed in duplicate. Negative values indicate higher CHO viability in the diluted toxin samples than in the negative control.

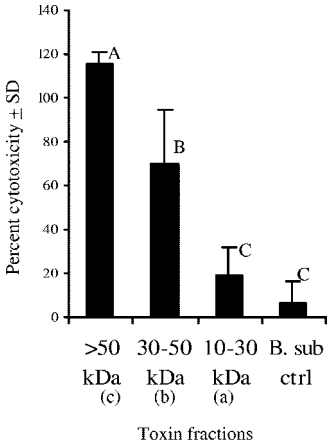

FIG. 2. Cytotoxicity analyses of toxin fractions of *B. cereus* MS1-9. Toxin fractions were obtained after separation by using specific-molecular-weight cutoff membranes (see the text for details), and the protein concentration for each fraction was adjusted to ~40 μg/ml. Fractions were designated a (10 to 30 kDa), b (30 to 50 kDa), c (>50 kDa), and *B. subtilis* toxin preparation (B. sub ctrl) and were tested with 2×10^6 hybridoma cells/ml. Results are averages for three experiments. SD, standard deviation. Bars marked with different letters (A, B, C) are significantly different ($P < 0.05$).

The *hblA* gene, coding for hemolysin BL, was found in all strains except for *B. cereus* ATCC 33018, F4810, and Baclo3a. Phenotypically, these three strains showed zones of hemolysis on blood agar plates. This is probably because the PCR primers were targeted only toward the B component of the hemolysin gene, which is absent in these strains, but the two L components of HBL may be present in strains showing positive hemolysis (6, 7). Incidentally, these strains were also phospholipase (lecithinase) positive, which may aid in the hemolytic activity. In contrast, two *B. thuringiensis* strains and a *B. cereus* strain were *hblA* positive but did not show hemolysis on blood agar, indicating a possible lack of expression of HBL in these strains. Furthermore, these strains were also negative for lecithinase activity (Table 2).

Genes coding for nonhemolytic components A, B, and C (*nheA*, *nheB*, and *nheC*) were uniformly distributed in all *B. cereus* strains tested except for F837/76. The *cytK* gene was observed in only one *B. cereus* strain (A926), and *bceT* was found in six *B. cereus* strains and in *B. megaterium* and *B. thuringiensis* strains (Table 2).

Comparison with commercial assay kits. Eight *B. cereus* strains used in this experiment gave positive enzyme-linked immunosorbent assay results with the BDE-VIA, which detects the 41-kDa subunit of NHE (Table 3). However, in the BCET-RPLA assay (HBL L_2 subunit expression), all but two *B. cereus* strains (F4810 and ATCC 33018) were negative (Table 3). These data agree with the PCR data, where these two strains were found to be negative for the *hblA* gene (Table 2).

DISCUSSION

Sensitive and rapid detection of enterotoxin from *B. cereus* is highly desirable in order to protect consumers from food-associated illness and to promote food safety and food biosecurity. A Ped-2E9 cell-based assay to detect cytopathogenic *Listeria* species has been described (8, 9), and later the same cell line was found to be sensitive to *B. cereus* toxins (53). This cell line was used to measure cytotoxicity by assaying AP release spectrophotometrically. Cell-free culture supernatants from 31 previously isolated *Bacillus* cultures were tested, of which 17 were *B. cereus* and the remaining 14 belonged to other *Bacillus* species. All but one *B. cereus* strain gave positive cytotoxicity results, and a majority were positive for the *hblA*, *nheA*, *nheB*, and *nheC* genes. Furthermore, decreased cytotoxicity or no cytotoxicity was seen in strains in which all or some of the subunits of the NHE complex were absent. It has been shown that some subunits of the NHE toxin complex have some biological activity, but all are required for maximal activity (33). *B. cereus* CA1 is a food isolate and contained genes for *bceT* and one of the NHE subunits (*nheA*). This strain did not cause cytotoxicity (0.2%). This may be due to the lack of other subunits of NHE or the absence of a protein expression system. HBL is also an important cytotoxic factor; however, it may not be essential for Ped-2E9-mediated cytotoxicity, since *hblA*-negative *B. cereus* strains F4810 and ATCC 33018 exhibited positive cytotoxicity with Ped-2E9 cells (Table 3). Among the non-*B. cereus* cultures, only *B. thuringiensis* strains were positive with the Ped-2E9 assay, and those strains were also positive for the *hbl* or *nhe* gene (41). The cytotoxicity of these cultures was in agreement with published reports (5, 33, 46, 49). *B. megaterium* is also known to produce heat-stable emetic toxin (55). In this study, although we used three strains, only one was

TABLE 5. Nucleotide sequences of specific primers and product sizes for toxin genes of *B. cereus*

Toxin gene	Primer sequence (5′–3′)	PCR conditions[a]	Product size (bp)	Reference
hblA	GCTAATCTAGTTTCACCTGTAGCAAC AATCATGCCACTGCGTGGACATATAA	35 cycles of 94°C for 20 s, 58°C for 40 s, and 72°C for 40 s	874	25
bceT	TTACATTACCAGGACGTGCTT TGTTTGTGATTGTAATTCAGG	30 cycles of 94°C for 15 s, 55°C for 45 s, and 72°C for 2 min	428	1
cytK	AACAGATATCGGTCAAAATGC CGTGCATCTGTTTCATGAGG	30 cycles of 94°C for 1 min, 52°C for 1 min, and 72°C for 1 min	623	32
nheA	TACGCTAAGGAGGGGCA GTTTTTATTGCTTCATCGGCT	Multiplex reaction	499	20
nheB	CTATCAGCACTTATGGCAG ACTCCTAGCGGTGTTCC	30 cycles of 94°C for 15 s, 55°C for 45 s, and 72°C for 2 min	769	
nheC	CGGTAGTGATTGCTGGG CAGCATTCGTACTTGCCAA		581	

[a] Preceded by initial denaturation at 94°C for 5 min and followed by a final extension at 72°C for 7 min.

confirmed by ribotyping to be *B. megaterium* (ATCC 9885), and it showed no cytotoxicity effect on Ped-2E9 cells. This strain was also determined to be nonhemolytic and lecithinase negative, and PCR data indicated the absence of the *hbl* and *nhe* genes.

Among the eight nascent *Bacillus* isolates from food, only one strain was cytotoxic, and it was later confirmed by ribotyping to be *B. cereus*. This strain also contained *nhe* genes but lacked *hblA*, reiterating the significance of *nhe* gene sequences in Ped-2E9 cytotoxicity. Although a limited number of food samples or isolates were tested, this assay accurately detected the cytotoxic potential of a single *B. cereus* strain.

In order to elucidate the molecular weights of the toxins responsible for cytotoxic actions on Ped-2E9 cells, toxin preparations were fractionated and analyzed for activity. Fractions with M_rs of 30,000 and higher showed the most cytotoxic effects. As mentioned before, *B. cereus* produces multiple toxins, including hemolysins (HBL; 37.8, 38.5, and 43.5 kDa) (33) and NHE (39, 45, and 105 kDa) (40). Based on the protein analysis data, PCR results, and hemolysis on blood agar plates, we speculate that cytotoxic action on Ped-2E9 cells could be due to the NHE and/or HBL components, since these toxins were also found to be involved in cytotoxic activities by using other cell lines (39). As indicated above, HBL possibly enhances Ped-2E9 cytotoxicity induced by NHE but is not essential. Heat treatment significantly reduced the activity of the toxin on Ped-2E9 cells, suggesting the possible involvement of NHE (diarrheal enterotoxins) in cytotoxicity, since these toxins are heat labile (6, 46). The emetic toxin is thermostable and consists of low-molecular-weight cereulide (2, 55). The association of cytotoxic activity with a high-molecular-weight compound and its thermal inactivation strongly imply the involvement of NHE (diarrheal enterotoxins) in Ped-2E9 cytotoxicity. Furthermore, the heat-inactivated toxin preparation from a known emetic strain (*B. cereus* F4810) (23) did not show any cytotoxicity with the Ped-2E9 cells, providing circumstantial evidence that this cell line is not sensitive to emetic toxin.

The Ped-2E9-based assay was also compared with the standard CHO-based assay, which is sensitive to both diarrheagenic and emetic toxins (5, 40). In this study, CHO cells were sensitive to the enterotoxin-producing *B. cereus* strains and a *B. subtilis* strain. The latter strain is known to produce emetic toxin (25), and the CHO cell line is sensitive to emetic toxin. The Ped-2E9 assay did not show any cytotoxicity with *B. subtilis*, confirming the possible lack of response of Ped-2E9 cells to emetic toxin.

The sensitivity of the Ped-2E9-based assay was compared with that of the CHO cell assay by using crude toxin preparations from *B. cereus* MS1-9 and ATCC 14579. The Ped-2E9 assay was 58-fold more sensitive than the CHO assay for the toxin preparation from *B. cereus* MS1-9 and about 25-fold more sensitive for that from *B. cereus* ATCC 14579, based on the lowest concentrations of toxins showing positive cytotoxicity effects on both cell lines. Cytotoxicity titration curves for Ped-2E9 cells revealed a gradual decline in cytotoxicity values with decreasing concentrations of toxins (0.3 to 0.4 μg/ml), while the cytotoxicity titration curve for CHO cells revealed a sudden drop in cytotoxicity values, approaching zero percent at toxin concentrations of 17.5 μg/ml for *B. cereus* MS1-9 and >6.6 μg/ml for *B. cereus* ATCC 14579 (Fig. 1). These data indicate that CHO cells presumably require an optimum concentration of toxins, below which the lethal effects of toxins are attenuated, as indicated by a sudden drop in the curve. In contrast, Ped-2E9 cells are highly sensitive to a very low concentration of toxin, thus producing a linear cytotoxicity curve. Further, the Ped-2E9 assay is considerably faster (taking only 1 h to complete after addition of toxin) than the CHO assay, which required 72 h to complete (40).

The procedures of the commercially available toxin assay kits were relatively simple to perform; however, these assays are toxin subunit specific and have high likelihoods of false negatives, since strains do not always possess all subunits. Also, both kits must be used, since they each react with different toxin complexes, and some strains may possess only one or the other. The results of the BDE-VIA and BCET-RPLA did correlate with PCR results; however, they did not give any indication of the strain's pathogenic potential.

In summary, a sensitive cytotoxicity assay was developed for enterotoxin-positive *Bacillus* species using Ped-2E9 cells. This assay is considerably faster than other methods, showing a cytotoxic effect in just 1 h, saving at least a day compared to other cell-based assays. The Ped-2E9 assay is also relatively simple to perform and gives quantitative results through a simple enzyme-based colorimetric assay, as opposed to a metabolism-based MTT assay for other cell lines. Since the Ped-2E9 assay is sensitive to enterotoxin, it could be used for any *Bacillus* species capable of causing food-borne illnesses.

ACKNOWLEDGMENTS

Sincere thanks to J. Handlesman (Department of Plant Pathology, University of Wisconsin), A. Wong (Food Research Institute, University of Wisconsin), B. Applegate and K. Hayes (Department of Food Science, Purdue University), A. Aronson (Department of Biology, Purdue University), J. McKillip (Department of Biology, Ball State University), and G. Siragusa (USDA-ARS, Russell Research Center, Athens, GA) for providing the cultures. Also our sincere thanks to Bruce Craig and Benjamin Tyner for assistance with statistical analyses and John McKillip and Jennifer Cutraro for critical reading of the manuscript.

This research was supported through a cooperative agreement with the Agricultural Research Service of the U.S. Department of Agriculture (project 1935-42000-035) and the Center for Food Safety and Engineering at Purdue University.

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