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Detection of toxigenic strains of *Bacillus cereus* and other *Bacillus* spp. with an improved cytotoxicity assay

S.H. Beattie and A.G. Williams
Hannah Research Institute, Ayr, UK

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S.H. BEATTIE AND A.G. WILLIAMS. 1999. An improved qualitative cell cytotoxicity assay for the detection of *Bacillus cereus* emetic and enterotoxin is described. The presence of toxin in culture supernatant fluids was detected by measurement with the tetrazolium salt MTT, as it adversely affects the metabolic status of cultured CHO cells. Psychrotrophic *B. cereus* isolates (65) were assessed for toxin production using the cytotoxicity assay, and 91% of culture supernatant fluids were cytotoxic. Toxin assessment using BCET-RPLA and ELISA immunoassays indicated that 51% and 85% of the cultures, respectively, were toxigenic. There were pronounced strain differences in the amount of toxin produced by the *B. cereus* isolates. Some isolates of *B. circulans*, *B. laterosporus/cereus*, *B. lentus*, *B. licheniformis*, *B. mycoides*, *B. subtilis* and *B. thuringiensis* were also toxigenic.

INTRODUCTION

Bacillus cereus is a potential problem to the food industry because it causes spoilage and because it is also associated with two distinct types of food poisoning. The diarrhoeal syndrome is characterized by abdominal cramps and watery diarrhoea which lasts for 12–24 h. It has been associated with the consumption of a wide range of foodstuffs including milk, sauces, pasta, desserts and cakes (Kramer and Gilbert 1989). There are two distinct enterotoxin complexes (Beecher and Wong 1994; Lund and Granum 1996), and a single enterotoxic protein coded for by the *bceT* gene has also been implicated (Agata *et al*. 1995a). More than one enterotoxin may be produced by a strain of *B. cereus* (Ombui *et al*. 1997).

The emetic syndrome is commonly associated with farinaceous foods and is caused by a heat-stable dodecadepsipeptide (Agata *et al*. 1995b). The intoxication is characterized by nausea and vomiting, which is often accompanied by diarrhoea, although this is not the major symptom (Kramer and Gilbert 1989; Granum 1994).

The characterization of the toxigenic potential of *Bacillus* isolates, and safety requirements for toxin detection in food products, is dependent upon the availability of reliable assays. Cytotoxicity methods for enterotoxin detection are based on a subjective microscopic assessment of toxin-induced cell damage (Hughes *et al*. 1988; Szabo *et al*. 1991); commercially available antibody-based kits are component-specific and therefore recognize only one of the enterotoxin complexes (Granum and Lund 1997). The emetic toxin induces damage in Hep-2 cells (Agata *et al*. 1994; Isobe *et al*. 1995) and can be detected in a bioassay using boar semen (Andersson *et al*. 1998).

In this work, a modified cytotoxicity assay for detection of both diarrhoeal and emetic toxins is described. Assessment of cytotoxic effect was made by measuring total cellular metabolic activity using the tetrazolium salt 3-(4,5,-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT). The assay was used to monitor the ability of *B. cereus* and isolates of other *Bacillus* spp. from raw milk or the farm environment to produce toxins. Enterotoxin formation was also monitored using commercially available systems.

MATERIALS AND METHODS

Culture of *Bacillus* spp.

Reference strains *B. cereus* NCTC 11145 (diarrhoeagenic) and *B. cereus* NCTC 11143 (emetic) were used for evaluation of the cytotoxicity method. The isolates of *B. cereus* and other *Bacillus* spp. were isolated from either raw milk, vegetarian Cheddar cheese or the farm environment (Beattie 1997). They were identified by morphological and biochemical characteristics. Single colonies were selected from pure cultures grown on milk agar, and inoculated into 10 ml brain heart infusion broth (BHI) for overnight culture. An aliquot (250 μl) of the overnight culture was inoculated into 25 ml

Correspondence to: Dr S.H. Beattie, Hannah Research Institute, Ayr KA6 5HL, UK.

BHI or 10% sterile skim milk (SMP; Oxoid), and incubated at 30 °C for 18 h with shaking (200 rev min^{-1}) in an orbital incubator (L.H. Engineering Co. Ltd, Stoke Poges, UK). Cultures were harvested by centrifugation (5000 *g*, 4 °C for 30 min) and residual cells were removed by filtration (0·45 μm porosity filters, Millipore). Supernatant fluids were immediately assessed for toxicity. Assays for the presence of emetic toxin were conducted on filter-sterilized culture supernatant fluids that had been heated for 10 min at 100 °C to inactivate the enterotoxin.

Cell cytotoxicity assay

Chinese hamster ovary (CHO) cells were maintained in RPMI 1640 media (Gibco, Life Technologies Ltd) supplemented with 5% (v/v) foetal bovine serum (FBS), 2 mmol l^{-1} glutamine and 1% (v/v) penicillin (streptomycin). Cells (30 ml) were cultured at 37 °C in a humid atmosphere containing 5% CO_2, and sub-cultured every 3–4 d. CHO cells were harvested with trypsin, and suspensions containing 5×10^4 cells ml^{-1} were prepared. The assay was performed in 96 well, flat-bottomed, gamma-irradiated, microtitre plates with lids (InterMed, Denmark). Filter-sterilized, cell-free bacterial culture supernatant fluid (50 μl) was serially diluted 256-fold. Negative controls (25 μl phosphate-buffered saline) and positive controls (25 μl Triton X-100) were included. The wells were inoculated aseptically with 5×10^3 cells, and the plates were incubated at 37 °C in a humid atmosphere containing 5% CO_2 for 72 h. After the incubation period, 50 μl of an aqueous MTT solution (2 mg ml^{-1}) were added to each well, and the plates incubated for a further 4 h at 37 °C in a humid atmosphere containing 5% CO_2. After this time, the liquid medium in the wells was removed, 100 μl dimethyl sulphoxide (DMSO) was added to each well, and the absorbance at 540 nm determined in a microplate reader (BioRad). The toxic effect of the cell free bacterial culture supernatant fluids on the CHO cell line was calculated from the following equation:

$$1-(OD_{\text{Negative Control}}-OD_{\text{Test}}) \times 100\%.$$

Samples were considered to be toxic if the optical density of the test well was $\geqslant 20\%$ less than that detected in the negative control wells.

Enterotoxin was also measured using commercially available BCET-RPLA (Oxoid) and ELISA (Tecra) kits. Assays were performed, with positive and negative controls, according to the manufacturers instructions.

RESULTS AND DISCUSSION

Cell cytotoxicity assay

Bacillus cereus enterotoxin has a cytostatic effect on cultured cells (Mikami *et al*. 1994), and this inhibition of cell proliferation forms the basis of the assay described. The assay conditions were optimized in a series of preliminary experiments which examined the CHO cell inoculation level, the incubation period for the assay, the effect of storage conditions on the toxicity of samples, and the inhibitory effect of media. Cell lines differ in their sensitivities to *B. cereus* toxins (Szabo *et al*. 1991); CHO cells were selected because of their high level of sensitivity, although other cell lines (INT 407) may also be used.

Cultures of *B. cereus* were found to be consistent in their production of toxins under standardized conditions, as had been found previously (Jackson 1989). Measurements of toxicity were found to be reproducible (Fig. 1a,b), and there was high correlation between metabolic and microscopic toxicity assessment methods (Beattie 1997). Skim milk (10% w/v) and BHI media caused no detectable toxic effect at a twofold

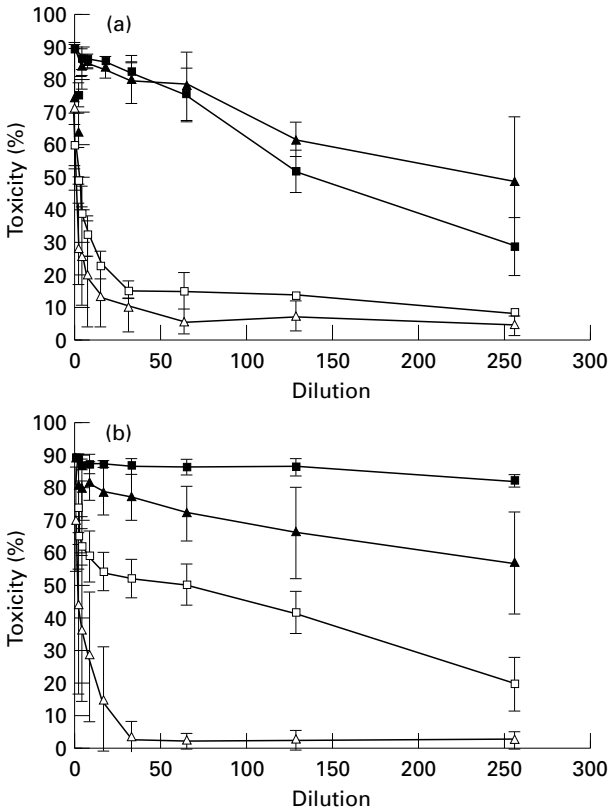

Fig. 1 Cytotoxic effect of *Bacillus cereus* (a) enterotoxigenic strain (NCTC 11145) and (b) emetic strain (NCTC 11143) cell-free culture supernatant fluids on CHO cells, after growth of *B. cereus* strain in skim milk (10% w/v), or brain heart infusion broth, with and without heat treatment of supernatant fluids. Skim milk (10% w/v) with no heat treatment (□); skim milk (10% w/v) with heat treatment (■); BH without heat treatment (△); BHI with heat treatment (▲). ($n = 6$). Standard deviation indicated by marker bar

dilution. Reduction in the metabolic activity of the cell line was therefore considered to be caused by the toxic effect of the cell-free culture supernatant fluids when it occurred at or beyond a fourfold dilution. Toxicity was reduced in samples stored at 4 °C for 24 h; therefore, samples were assessed immediately for toxin production.

The cytotoxicity assay described is not dependant on a subjective assessment of cell damage and enables a qualitative measurement of toxicity of culture supernatant fluids (i.e. % inhibition at given dilution); strain differences and culture effects may therefore be compared directly. The assay can be used to detect toxins of both emetic and enterotoxigenic strains of *B. cereus*. Figure 1(a,b) shows the responses of CHO cells to cell-free culture supernatant fluids of an enterotoxigenic strain (NCTC 11145) and an emetic strain (NCTC 11143) of *B. cereus*. The media in which the *B. cereus* isolates were grown appeared to influence the toxicity exhibited by the organism; for example, the emetic *B. cereus* strain NCTC 11143 produced greater amounts of heat-stable toxin when grown in SMP rather than BHI (Fig. 1a,b).

Assessment of the metabolic status of tissue culture cells using MTT has been made previously (Mosmann 1983). The tetrazolium ring of MTT is cleaved in the mitochondria of metabolically active cells, resulting in a colour change from pale yellow to purple (Mosmann 1983). Only viable cells can produce this formazan reaction product, which makes MTT a sensitive compound for assessment of living cells (Mosmann 1983). Tetrazolium salts have been used to assess *Pasterurella haemolytica* A1 leukotoxin (Vega *et al*. 1987; Craig *et al*. 1990) and the cytotoxin of *Campylobacter jejuni* (Coote and Arain 1996), and they were recently used to investigate *B. cereus* toxicity (Seidel *et al*. 1996; von Dietrich *et al*. 1997).

Toxin production by isolates of *B. cereus* from raw milk and the farm environment

Toxin was detectable by at least one assay method in the cell-free culture supernatant fluids of all of the psychrotrophic *B. cereus* isolates from raw milk (five), the farm environment (51) and vegetarian Cheddar cheese (nine). The majority of isolates (91%) tested positive with the cytotoxicity assay, and 36% gave positive results in all tests (Table 1). In this and other studies (Christiansson *et al*. 1989; Buchanan and Schultz 1994), a higher proportion of toxigenic strains was detected by cytotoxicological methods. The apparent lower detection rate with immunological methodologies (Tecra 85%, Oxoid 51%) is attributable to their specificity for individual components in the toxin complexes. The results of the immunological detection methods indicated that there were pronounced inter-strain differences in the enterotoxin profiles of the isolates studied; 38% of isolates appeared to produce more than one enterotoxin. The cell-free culture supernatant fluids of 40% of the *B. cereus* isolates retained a cytotoxic effect after heat treatment (100 °C, 10 min). None of these isolates originated from raw milk or vegetarian Cheddar cheese samples.

The individual isolates varied considerably in the amount of toxin produced (9% of samples were borderline for cytotoxic effect, while 15% of samples retained cytotoxic effect at or beyond 256 times dilution); earlier work also found

Table 1 toxicity of *Bacillus* spp. isolates

Species	Isolates tested	Tecra (% positive)	Oxoid (% positive)	Cytotoxicity N/H* (% positive)	Cytotoxicity H† (% positive)
B. brevis	6	0	0	33	17
B. cereus	65	85	51	91	40
B. circulans	7	14	14	29	29
B. lacterosporous/ cereus	2	50	50	50	0
B. lentus	6	67	50	100	17
B. licheniformis	4	33	33	50	25
B. polymyxa	2	0	0	0	0
B. mycoides	15	N/T	100	100	93
B. sphaericus	2	0	0	0	0
B. subtilis	10	N/T	0	70	60
B. thuringiensis	13	100	54	92	77

N/T, not tested.

*Unheated cell-free culture supernatant fluids.

†Heated (100 °C, 10 min) cell-free culture supernatant fluids.

wide variation in the amounts of toxin produced by different isolates (Dufrenne *et al*. 1995). Although a high number of *B. cereus* isolates are capable of producing toxin, it has been suggested that only a small number is able to produce large enough amounts of diarrhoeal enterotoxin to cause food poisoning (Ronner and Andersson 1995). Of the *B. cereus* isolates tested, 15% were cytotoxic to levels comparable with *B. cereus* NCTC 11145, which was isolated initially from meat loaf as the causative micro-organism in a food poisoning outbreak in the USA (Midura *et al*. 1970). These results suggest, therefore, that there is a potential health risk arising from the contamination by *B. cereus* of milk and dairy products. Nevertheless, despite a high incidence of *B. cereus* in milk, there are few reports of associated food poisoning (Christiansson 1995).

Toxin production by *Bacillus* spp. other than *B. cereus*

Isolates of *B. mycoides, B. thuringiensis, B. subtilis, B. lentus, B. circulans, B. licheniformis* and *B. laterosporus/cereus* also produced detectable toxin (Table 1). Positive detection of toxic effect was most frequently made using the cell cytotoxicity assay, but culture supernatant fluids of *B. thuringiensis, B. circulans, B. licheniformis, B. lentus* and *B. laterosporus/cereus* reacted positively with the Tecra assay. Supernatant fluid from isolates of *B. circulans, B. laterosporus/cereus, B. lentus, B. licheniformis, B. mycoides* and *B. thuringiensis* reacted positively in the RPLA assay. This reaction of toxins formed by other *Bacillus* spp. with Tecra and Oxoid antibodies indicates that these isolates were producing protein toxins which were very similar to those of *B. cereus*, and that these species may also represent a potential hazard in food products.

Bacillus subtilis, *B. licheniformis, B. pumilus* and *B. thuringiensis* have been implicated in outbreaks of food-borne disease (Kramer and Gilbert 1989). Griffiths (1990) also demonstrated, using the RPLA assay, that isolates of *B. mycoides, B. thuringiensis*, *B. circulans, B. lentus, B. pumilus, B. polymyxa* and *B. carotarum* were toxigenic.

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REFERENCES

Agata, N., Mori, M., Ohta, M., Suwan, S., Ohtani, I. and Isobe, M. (1994) A Novel dodecadepsipeptide, cereulide, isolated from *Bacillus cereus* causes vacuole formation in Hep-2 cells. *FEMS Microbiology Letters* **121**, 31–34.

Agata, N., Ohta, M., Arakawa, Y. and Mori, M. (1995a) The bcet gene of *Bacillus cereus* encodes an enterotoxin protein. *Microbiology* **141**, 983–988.

Agata, N., Ohta, M., Mori, M. and Isobe, M. (1995b) A novel dodecadepsipeptide, cereulide, is an emetic toxin of *Bacillus cereus*. *FEMS Microbiology Letters* **129**, 17–19.

Andersson, M.A., Mikkola, R., Helin, J., Andersson, M.C. and Salkinoja-Salonen, M. (1998) A novel sensitive bioassay for detection of *Bacillus cereus* emetic toxin and related depsipeptide ionophores. *Applied and Environmental Microbiology* **64**, 1338–1343.

Beattie, S.H. (1997) Incidence and Importance of *Bacillus cereus* Species in Raw Milk and the Dairy Environment. PhD Thesis, Glasgow University.

Beecher, D.J. and Wong, A.C.L. (1994) Improved purification and characterisation of haemolysin BL, a haemolytic dermonecrotic vascular permeability factor from *Bacillus cereus*. *Infection and Immunity* **62**, 980–986.

Buchanan, R.L. and Schultz, F.J. (1994) Comparison of the TECRA VIA kit, OXOID BCET-RPLA kit and CHO cell culture assay for the detection of *Bacillus cereus* diarrhoeal enterotoxin. *Letters in Applied Microbiology* **19**, 353–356.

Christiansson, A. (1995) The significance of potentially pathogenic species of sporeformers, other than *Bacillus cereus* in milk and milk products. *Bulletin of the International Dairy Federation* **302**, 2–3.

Christiansson, A., Naidu. A.S., Nilsson, I., Wadstrom, T. and Pettersson, H.E. (1989) Toxin production by *Bacillus cereus* dairy isolates in milk at low temperatures. *Applied and Environmental Microbiology* **55**, 2595–2600.

Coote, J.G. and Arain, T. (1996) A rapid, colorimetric assay for cytotoxin activity in *Campylobacter jejuni*. *FEMS Immunology and Medical Microbiology* **13**, 65–70.

Craig, F.F., Dalgleish, R., Sutherland, A.D. *et al.* (1990) A colorimetric, microplate assay for the leucotoxin of *Pasteurella haemolytica*. *Veterinary Microbiology* **22**, 309–340.

Dufrenne, J., Bijwaard, M., te Giffel, M., Beumer, R. and Notermans, S. (1995) Characteristics of some psychrotrophic *Bacillus cereus* isolates. *International Journal of Food Microbiology* **27**, 175–183.

Granum, P.E. (1994) *Bacillus cereus* and its toxins. *Journal of Applied Bacteriology Symposium* **76** (Suppl.), 61S–66S.

Granum, P.E. and Lund, T. (1997) Mini review: *Bacillus cereus* and its food poisoning toxins. *FEMS Microbiology Letters* **157**, 223–228.

Griffiths, M.W. (1990) Toxin production by psychrotrophic *Bacillus* species present in milk. *Journal of Food Protection* **53**, 790–792.

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 Microbiology Letters* **52**, 7–12.

Isobe, M., Ishikawa, T., Suwan, S., Agata, N. and Ohta, M. (1995) Synthesis and activity of cereulide, a cyclic dodecadepsipeptide ionophore as emetic toxin from *Bacillus cereus*. *Bioorganic and Medical Chemistry Letters* **5**, 2855–2858.

Jackson, S.G. (1989) Development of a fluorescent immunodot assay for *Bacillus cereus* enterotoxin. *Journal of Immunological Methods* **120**, 215–220.

Kramer, J.M. and Gilbert, R.T. (1989) *Bacillus cereus* and other

Bacillus species. In *Foodborne Bacterial Pathogens* ed. Doyle, M.P. pp. 21–70. New York: Marcel Dekker.

Lund, T. and Granum, P.E. (1996) Characterisation of a non-haemolytic enterotoxin complex from *Bacillus cereus* isolated after a foodborne outbreak. *FEMS Microbiology Letters* **141**, 151–156.

Midura, T., Gerber, M., Wood, R. and Leonard, A.R. (1970) *Public Health Report* **85**, 45–48.

Mikami, T., Horikawa, T., Murakami, T. *et al.* (1994) An improved method for detecting cytostatic toxin (emetic toxin) of *Bacillus cereus* and its application to food samples. *FEMS Microbiology Letters* **119**, 53–58.

Mosmann, T. (1983) A rapid colorimetric assay for cellular proliferation and survival: application to proliferation and cytotoxicity assays. *Journal of Immunological Methods* **65**, 55–63.

Ombui, J.N., Schmieger, H., Kagiko, M.M. and Arimi, S.M. (1997) *Bacillus cereus* may produce two or more diarrhoeal enterotoxins. *FEMS Microbiology Letters* **149**, 245–248.

Ronner, U. and Andersson, A. (1995) Emerging microbial risks in processes case: how serious is *Bacillus cereus*? *New Shelf Life Technologies and Safety Assessments Symposia* **148**, 193–194.

Seidel, K.E., Pfisterer, C., Hormansdorfer, S. and Bauer, J. (1996) Zum Nachweis toxinogener *Bacillus cereus* Stämme. *Journal of Veterinary Medicine* **43**, 421–428.

Szabo, R.A., Spiers, J.I. and Akhtar, M. (1991) Cell culture detection and conditions for production of a *Bacillus cereus* heat-stable toxin. *Journal of Food Protection* **54**, 272–276.

Vega, M.V., Maheswaran, S.K. and Leininger, J.R. (1987) Adaptation of a colorimetric microtitration assay for quantifying *Pasteurella haemolytica* A1 leukotoxin and antileukotoxin. *American Journal of Veterinary Research* **48**, 1559–1564.

von Dietrich, R., Mauersberger, K. and Märtlbauer, E. (1997) Zur anwendbarkeit des MTT zytotoxizitatstests zum nachweis von *B. cereus* enterotoxin. *Archiv für Lebensmittelhygiene* **48**, 73–96.