

Comparison of the Tecra VIA kit, Oxoid BCET-RPLA kit and CHO cell culture assay for the detection of *Bacillus cereus* diarrhoeal enterotoxin

R.L. BUCHANAN AND F.J. SCHULTZ. 1994. Two commercial serological kits (Oxoid BCET-RPLA and Tecra VIA) and a Chinese hamster ovary (CHO) cell cytotoxicity assay for the detection of *Bacillus cereus* diarrhoeal enterotoxin were compared. Eleven *B. cereus* strains and one enterotoxigenic *B. thuringiensis* strain were evaluated. Both kits and the CHO cell assay yielded positive toxin responses for cell-free culture filtrates from eight out of 11 diarrhoeal enterotoxigenic strains. An emetic enterotoxin producing strain was negative with all three assays. Two *B. cereus* strains were negative using the BCET-RPLA kit, but positive with the Tecra VIA kit and CHO cell assay. The BCET-RPLA indicated significant levels of enterotoxin after samples were boiled, whereas the CHO cell and Tecra assays were negative. Overall, the cell culture assay was the most sensitive. However, the Tecra VIA kit provided similar results and was better suited for the rapid detection of *B. cereus* diarrhoeal enterotoxin.

INTRODUCTION

There has been renewed interest in the role of *Bacillus cereus* as a foodborne pathogen with the identification and widespread isolation of psychrotrophic strains from a variety of foods, particularly pasteurized dairy products (Shehata and Collins 1971; Coghill and Juffs 1979; van Netten *et al.* 1990). A substantial portion of the isolates are positive for the heat labile *B. cereus* enterotoxin (Wong *et al.* 1988a,b; Christiansson *et al.* 1989; Griffiths 1990; van Netten *et al.* 1990), and isolates have been shown to produce the diarrhoeal toxin at temperatures as low as 4°C. Granum *et al.* (1993a) reported that 7% of *B. cereus* isolates from Norwegian dairy products were of particular food safety concern due to the combination of enterotoxigenicity and ability to grow at refrigeration temperatures.

In recent years, characterization of both mesophilic and psychrotrophic strains of enterotoxigenic *B. cereus* has been facilitated by the availability of cell culture assays and commercially-available serological kits for the heat-labile enterotoxin. However, in studies in this laboratory the Oxoid BCET-RPLA (Oxoid Ltd, Basingstoke, UK) kit failed to detect enterotoxin production by some strains of

B. cereus that were positive with cell culture assays (Buchanan and Schultz 1992). The kit also gave strong positive responses for boiled samples despite a complete loss of biological activity. Baker and Griffiths (1993) attempted to model the effects of environmental factors on formation of heat-labile enterotoxin by *B. cereus*, but found that different factors were significant depending on whether the Oxoid kit or a Vero cell assay were used for analysis. Recently the Tecra VIA kit (International BioProduct, Inc., Redmond, WA, USA), a second serological kit for *B. cereus* enterotoxin became commercially available. Availability of an accurate rapid assay for heat-labile enterotoxin would greatly facilitate both assessment of importance of *B. cereus* as a foodborne pathogen and elucidation of genetic factors responsible for toxin synthesis. Accordingly, the objective of the current study was to evaluate the efficacy of the Tecra VIA kit, and to compare its performance with that of the Oxoid kit and the Chinese hamster ovary (CHO) cell assay developed in our laboratory (Buchanan and Schultz 1992).

MATERIALS AND METHODS

Media

Brain heart infusion (BHI) broth and BHI agar were obtained from Difco Laboratories (Detroit, MI, USA).

Sample/strain	Response*		
	BCET-RPLA	Tecra-VIA	CHO cells
<i>B. thuringiensis</i>			
E-2	256*	43 (32-64)	427 (256-512)
<i>B. cereus</i>			
T-1	256	43 (32-64)	427 (256-512)
T-2	171 (128-256)	5 (4-8)	437 (32-1024)
Watertown	0	85 (64-128)	277 (64-512)
A-7	0	64	213 (128-256)
F45814/70	256	43 (32-64)	256
B4AC-1	256	75 (32-128)	1024 (512-2048)
B4AC-2	256	32	512
H-13	256	85 (64-128)	1024
5056	256	43 (32-64)	427 (256-512)
F4433/73	256	170 (128-256)	597 (256-1024)
F4552/75†	3 (0-8)	1 (0-2)	2
Positive controls			
Oxid toxin	85 (64-128)	0	0
Tecra toxin	0	+ ‡	53 (32-64)
Negative control (BHI)	0	0	0

*Average of reciprocal of the highest dilution showing positive response. Response ranges are shown in parentheses where applicable.

† Emetic enterotoxin producing strain.

‡ Tecra positive control (200 µl) was tested according to kit instructions and not diluted to determine titre.

Ham's F-12, Dulbecco's phosphate buffered saline (DPBS), 1 × trypsin-EDTA and fetal bovine serum (FBS) were obtained from ICN-Flow Biomedicals Inc. (Costa Mesa, CA, USA).

Micro-organisms

The 12 isolates used included a diarrhoeal enterotoxin producing strain of *B. thuringiensis* (E-2), an emetic entero-

<i>Bacillus</i> strain	Response*		
	BCET-RPLA	Tecra-VIA	CHO cells
<i>B. thuringiensis</i>			
E-2	112*(16-256)	0	1 (0-2)
<i>B. cereus</i>			
T-1	107 (64-256)	0	1 (0-2)
T-2	35 (8-64)	0	0
Watertown	0	2	0
A-7	0	0	0
F45814/70	32	0	0
B4AC-1	96 (32-256)	0	1 (0-2)
B4AC-2	136 (16-256)	0	0
H-13	256	0	1 (0-2)
5056	139 (32-256)	0	1 (0-2)
F4433/73	149 (64-128)	2 (0-4)	1 (0-2)
F4552/75†	0	0	0

* Titre based on the average of highest dilution showing positive response. Response ranges are shown in parentheses where applicable.

† Emetic enterotoxin producing strain.

Table 1 Analysis of cell-free culture filtrates of 24-h *Bacillus* cultures with three different detection techniques

Table 2 Titre of boiled cell-free culture filtrates of 24-h *Bacillus* cultures as determined with three different techniques

toxin producing *B. cereus* strain (F4552/75) and 10 *B. cereus* strains that produce the diarrhoeal enterotoxin (H-13, Watertown, F45814/70, F4433/73, B4AC (two subcultures designated B4AC-1 and B4AC-2), 5056, A-7 and T (two subcultures designated T-1 and T-2)). Stock cultures were maintained on BHI agar slants stored at 4°C and transferred monthly. Starter cultures were prepared by inoculating 50-ml Erlenmeyer flasks containing 20 ml BHI plus an additional 0.1% glucose (BHIG). These cultures were incubated on a rotary shaker (150 rev min) for 24 h at 37°C.

Culture conditions

Tubes containing 10 ml BHIG were inoculated with 0.1 ml of a 24 h starter culture and incubated 5 h at 37°C on a rotary shaker (150 rev min⁻¹). Cell-free supernatant fluids, were prepared by centrifuging cultures for 10 min using a clinical centrifuge (International Equipment Co., USA) followed by passage through 0.22 µm syringe filter (Nalgene Co., USA). Samples of denatured heat labile enterotoxin were prepared by placing tubes containing 5 ml of the culture filtrates in a boiling water bath for 10 min. All samples were kept on ice or at 4°C until assayed.

Assays for enterotoxigenic activity

The Oxoid BCET-RPLA and CHO cell assays were performed following cell-free supernatant fluid preparation. The Tecra VIA assays were performed on the following day. Preliminary studies indicated no change in toxigenic responses for samples maintained at 4°C for up to a week. Each complete comparison of the CHO cell, Oxoid BCET-RPLA and Tecra VIA assays was repeated three times. The positive toxin control from each kit was tested with its respective kit, CHO cell assay, and the other kit to assess cross reactivity. The Oxoid BCET-RPLA and Tecra VIA assays were performed according to the manufacturers' instructions.

The CHO assays were performed as previously described (Buchanan and Schultz 1992). Briefly, CHO cells (ATCC 61-CCL) were maintained in Ham's F-12 containing 10% FBS and incubated at 37°C, 95% RH, and 5% CO₂. Cells were washed with DPBS, treated with 1 × trypsin-EDTA, diluted to 10⁶ cells ml⁻¹, and 100 µl portions used to seed 96-well microtitre plates (flat-bottom, ICN-Flow) for cytotoxicity assays. The first two columns of the plate were reserved for dilutions of control cells to mimic 100, 50, 25 and 12.5% of total cells to help quantify cell concentrations. Plates were incubated for 18 h as described above. Cell-free supernatant fluids, boiled cell-free supernatant fluids, or uninoculated BHIG controls (100 µl per well) were added to wells and twofold serial dilutions were per-

formed across the plate using an 8-channel Finnpiptette (ICN-Flow). Plates were incubated at room temperature (approx. 22°C) for 18–24 h. Cells were examined with an inverted phase contrast microscope, and then stained as described previously (Buchanan and Schultz 1992). Reactions were considered positive when ≥ 50% of the cells had detached.

RESULTS

The titres of three separate trials for each of the three assays were averaged to provide an overall comparison for the 11 *B. cereus* strains and the one enterotoxigenic strain of *B. thuringiensis* (Table 1). Both kits and the CHO cell assay responded positively for cell-free culture filtrates from eight out of 11 diarrhoeal enterotoxigenic strains. The emetic enterotoxin producing strain (F4552/75) was negative with the kits and in the cell culture assay. Two strains, Watertown and A-7, were negative using the BCET-RPLA but positive with the Tecra VIA and CHO cell assays. One strain (T-2) produced only low titre responses with Tecra VIA, but was strongly positive with BCET-RPLA and the CHO cell assay. The enterotoxin controls provided with each kit did not cross-react. Only the positive control from the Tecra kit produced a cytotoxic response with CHO cells. The BHI negative control did not elicit a response with any of the three assays.

Cell-free extracts were boiled for 10 min to inactivate the diarrhoeal enterotoxin and determine the effect of denaturation on the effectiveness of the kits in relation to biological activity (Table 2). Heating eliminated activity in both the CHO cell assay and Tecra VIA kit. However, the strains originally positive with the BCET-RPLA kit continued to yield a strong, though somewhat reduced response.

DISCUSSION

The responses obtained in this study with the RPLA-BCET and CHO cell assays were similar to our earlier results (Buchanan and Schultz 1992), with the exception of strains T-2 and B4AC-2. Culture filtrates of B4AC-2 and T-2 did not previously display CHO cell activity, and T-2 showed only limited activity in the RPLA-BCET assay. The reasons for these differences were not investigated. These strains had been originally selected because of their past history of atypical response: strain B4AC has had a long history of being variable for enterotoxin synthesis (Turnbull 1986; Buchanan and Schultz 1992), and T-2 is a subclone of enterotoxin-negative reference strain T.

While the results of the three assays correlated for a number of the strains, it is apparent from the differences observed that the two kits detect different antigens. Particularly strong evidence is the lack of cross-reactivity between

the positive control included with each kit. The suggestion that the antigen used for the Oxoid BCET-RPLA kit is not directly correlated to enterotoxigenic activity is supported by lack of CHO cell activity for the BCET-RPLA positive enterotoxin control, as well as the kit's positive reaction after boiling had destroyed cell culture activity.

Several recent studies have also compared the effectiveness of the kits for detection of diarrhoeal enterotoxin production by *B. cereus*. Notermans and Tatini (1993) reported that the Oxoid test did not detect three of the nine strains that appeared to be positive for enterotoxigenicity by the Tecra kit. Comparing the two kits with a HEL cell culture assay, Christiansson (1993) reported that there was no correlation between the two kits, and that the Tecra kit more closely correlated with HEL cytotoxicity. Unlike Christiansson (1993), who found that the Tecra kit was more sensitive than either the BCET-RPLA kit or the HEL cell culture assay, the relative sensitivities for the current study were CHO cell assay > BCET-RPLA > Tecra.

Bacillus cereus enterotoxin appears to be co-produced with two other extracellular proteins, a haemolysin and a lecithinase (Shinagawa 1993). While there is some disagreement about the actual molecular weights of the three proteins, the relative molecular weights have been consistently reported as haemolysin > enterotoxin > lecithinase. Granum *et al.* (1993b) reported that the Oxoid kit was active against the largest protein, but not against the enterotoxin. Granum *et al.* (1993a) reported that only two of 85 isolates from Norwegian dairy products were misidentified by the Oxoid kit, and concluded that it was an effective means of detecting enterotoxigenic strains of *B. cereus*. Other investigations (Buchanan and Schultz 1992; Notermans and Tatini 1993; Christiansson 1993), including the current study, found higher false-negative rates for this kit.

Overall, the CHO cell assay provided the most accurate means for assaying cultures for *B. cereus* enterotoxin, both in terms of sensitivity and its direct relationship to biological activity. However, the use of cell culture techniques is not convenient unless they are performed on a routine basis. Of the two kits examined, the Tecra VIA kit was deemed to provide a better means for detecting enterotoxin due to its correlation with CHO cell cytotoxicity and its ability to distinguish enterotoxin that has been heat denatured.

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