

## Evaluation of the Oxoid BCET-RPLA Kit for the Detection of *Bacillus cereus* Diarrheal Enterotoxin as Compared to Cell Culture Cytotoxicity

### ABSTRACT

The relationship between detection of heat-labile enterotoxin production by *Bacillus cereus* using the Oxoid BCET-RPLA kit and the production of a cytotoxic response in HEp-2 and CHO cells was evaluated using 12 isolates. The strains were cultured in brain heart infusion + 0.1% glucose for 5 h at 37°C on a rotary shaker. Cell-free supernatants were prepared by filtration and tested using the two cell lines and the immunoassay. The primary biological response noted with the cell lines was cytotoxicity, with the CHO cells being consistently more sensitive than the HEp-2 cells. While there was general agreement between the cell culture tests and the immunoassay, differences were noted for specific strains. Further, while activity in cell cultures was lost when cell-free supernatants were heated to 80°C for 5 min, the BCET-RPLA kits continued to detect significant levels of enterotoxin. The results suggest that confirmation of *B. cereus* enterotoxin using a suitable biological response may be warranted until additional research can clarify the specificity of the BCET-RPLA assay.

*Bacillus cereus* is a well-recognized foodborne pathogen that causes two distinct types of food poisoning (diarrheal vs. emetic). Outbreaks have been most often associated with rice products, and the species occurs commonly in low numbers in a wide variety of food products and ingredients (5). While classically referred to as a spore-forming mesophile, interest in the species has increased recently with the determination that the microorganism occurs widely in pasteurized dairy products, and that a significant percentage of these isolates is capable of psychrotrophic growth. Shehata and Collins (6) isolated a number of psychrotrophic *Bacillus*, including *B. cereus*, from pasteurized milk that had spoiled after extended refrigeration. Coghill and Juffs (7) reported that 31% of 167 samples of pasteurized milk and cream from Queensland were positive for psychrotrophic bacilli, with *B. cereus* being the predominant species. Wong et al. (10) found that incidence of *B. cereus* in 253 samples of dairy products from retail markets in Taiwan ranged from 2% in pasteurized milk to 52% in ice cream. van Netten et al. (9) reported that 8% of 483 pasteurized milk samples were

positive for *B. cereus*, with approximately half of the isolates being capable of growth at 7°C. This group also observed that a large percentage of *B. cereus* isolated from refrigerated mousses/pates and convenience meals was psychrotrophic.

A number of groups have indicated that a significant percentage of the psychrotrophic *B. cereus* produces the diarrheal enterotoxin (2,4,9-11). Using cytotoxicity to cells in culture to assess diarrheal enterotoxin, Christiansson et al. (2) demonstrated that psychrotrophic *B. cereus* could produce the toxin at 8°C in agitated microbiological medium and milk or in whipped cream. However, enterotoxin production was greatly reduced or eliminated when the medium and milk were incubated without agitation. Griffiths (4) detected enterotoxin production in microbiological medium and reconstituted skim milk at temperatures as low as 6°C, using a reverse passive latex agglutination assay developed by Oxoid (BCET-RPLA kit, Oxoid Ltd., Basingstoke, England). However, aeration was not a requirement for production of enterotoxin in this study. Using the BCET-RPLA kit, van Netten et al. (9) observed enterotoxin production at temperatures as low as 4°C in microbiological medium, milk, minced meat, lasagne, and rice meal.

van Netten et al. (9) pointed out that the specificity of the BCET-RPLA had not been assessed, particularly in relation to enterotoxin production at refrigeration temperatures. An examination of the literature indicated that there has not been an evaluation of the BCET-RPLA kit in relation to its correlation with biological responses typically associated with enterotoxins. Accordingly, the objective of the current study was to employ 11 *B. cereus* and 1 *Bacillus thuringiensis* isolates to compare the response of the BCET-RPLA kit with the biological response in two cell lines, CHO cells and HEp-2 cells.

### MATERIALS AND METHODS

#### Media

Brain heart infusion (BHI) broth and BHI agar were obtained from Difco Laboratories (Detroit, MI). Ham's F-12, Dulbecco's Modified Eagle's Medium (DMEM), IX trypsin-EDTA, and fetal

bovine serum were obtained from ICN-Flow Biomedicals, Inc. (Costa Mesa, CA).

#### Microorganisms

Eleven strains of *B. cereus* [H-13, Watertown, F45814/70, F4433/73, B4AC (two subcultures designated B4AC-1 and B4AC-2), 5056, F4552/75, A-7, and T (two subcultures designated T-1 and T-2)] and an enterotoxigenic strain of *B. thuringiensis* (E-2) were used throughout the study. Stock cultures were maintained on BHI agar slants stored at 4°C and transferred monthly. Starter cultures were prepared by inoculating 125-ml Erlenmeyer flasks containing 50 ml of BHI broth and incubating on a rotary shaker (150 rpm) for 24 h at 37°C.

#### Culture techniques

Fifty-ml portions of BHI broth supplemented with an additional 0.1% glucose (BHIG) were sterilized by autoclaving for 15 min at 121°C in 125-ml Erlenmeyer flasks sealed with foam plugs. The flasks were then inoculated with 0.5 ml of a 24-h starter culture and incubated for 5 h at 37°C on a rotary shaker (150 rpm). After incubation, the test cultures were centrifuged for 10 min at 11,950 x g, and cell-free supernatants prepared by passing the supernatant through a 0.45-µm filter (#130-4045, Nalgene, Rochester, NY). An aliquot of each cell-free supernatant was boiled to inactivate the heat labile enterotoxin. All samples were then maintained on ice until used.

#### Cell culture assay for enterotoxin

Chinese hamster ovary (CHO) cells (ATCC 61-CCL) and HEP-2 (human epidermoid carcinoma, larynx; ATCC 23-CCL) were maintained in HAM's F-12 containing 10% fetal bovine serum and Dulbecco's Modified Eagle Medium with 10% fetal bovine serum, respectively, with both being incubated at 37°C in an atmosphere containing 5% CO<sub>2</sub> and relative humidity of 95%. Assays for enterotoxin activity were performed using 96-well microtiter plates (flat-bottom, ICN-Flow). Cell monolayers of each type were treated with IX trypsin-EDTA to suspend the cells and seeded into individual microtiter plates (100 µl/well) to achieve a level of approximately 10<sup>6</sup> cells/ml. Exceptions were columns 1 and 2 of each plate, which were reserved for controls. These wells were seeded in sets of four with either 100 µl of the original suspension or of 1/2, 1/4, and 1/8 dilutions. This allowed estimation of the extent of the monolayer. Plates were incubated overnight as described above and visually examined prior to sample application.

Cell-free culture supernatant (100 µl) or uninoculated BHIG (control) was added directly to appropriate wells in column 3, each containing 100 µl of tissue culture medium. Twofold serial dilutions were performed across the plate using an 8-channel Finnpiptette (ICN-Flow), with removal of 100 µl from each well in column 12 after mixing. Plates were incubated at room temperature (22°C) for 18-24 h. Preliminary trials indicated that activity was diminished if the plates were incubated at 37°C.

After microscopic examination to determine the type of biological response, attached cells were stained using a modification of the method of Gentry and Dalrymple (3). The plates were emptied and 100 µl of 2% formalin in 0.03 M phosphate buffered saline (pH 7.4) was added to each well for 1 min. This was removed by inverting the plate, and 100 µl of crystal violet (0.13%) in phosphate buffered saline with 5% ethanol and 2% formalin was added to each well. The plate was allowed to stand 20 min and then rinsed three times with distilled/deionized water. The plates were then either processed immediately or allowed to air-dry. Each well then received 100 µl of 50% ethanol, and the crystal violet was allowed to solubilize for 1-2 h. Absorbance was measured at 650 nm using a Bio-kinetics reader (Model EL-312, Bio Tek Instruments, Inc., Winooski, VT). Optical density mea-

surements allowed estimation of the remaining monolayer and calculation of titers. Reactions were considered positive when  $\geq 50\%$  of the cells had detached.

#### Enterotoxin assay using the oxid B. cereus enterotoxin passive latex agglutination BCET-RPLA kit

Sample preparation and plate set-up were performed as specified by the instructions provided by the manufacturer (Oxoid). Heated sample and BHIG were used as negative controls, and enterotoxin positive controls were provided with the kit. Two columns of the 96-well plate (V-well, ICN-Flow) were used per sample. Diluent was placed in the wells of row 8, undiluted sample was placed in rows 1 and 2, and 2-fold serial dilutions were performed from rows 2 to 7. After adding sensitized and nonsensitized (control) latex beads to the appropriate columns, the plates were covered and incubated for 18-24 h at room temperature.

## RESULTS

The results observed among replicate determinations performed on separate occasions were similar qualitatively; however, the results as represented by activity titers did differ quantitatively among replicates. Titers were averaged across several replicates to provide an overall quantitative comparison of the results with the BCET-RPLA kit and the cell culture assays (Table 1). The CHO cells and HEP-2 cells both responded to the active component in the cell-free culture supernatants by rounding-up and detaching from the microtiter plate (Fig. 1). The CHO cells were consistently more sensitive than the HEP-2 cells, with two strains, Watertown and F45814/70, being negative with HEP-2 cells but positive with CHO cells. Three strains, B4AC-2, T-2, and F4552/75, were negative with both cell lines. The latter strain is an emetic isolate and was used, in part, as a negative control. Two isolates of strains B4AC and T were available, with one isolate from each strain being biologically active and the other producing no response in the cell lines. The two B4AC cultures were chosen because of earlier reports of the strain losing its enterotoxigenicity (8). Strain T was included as a reference strain; however, it is apparent that there were differences in the enterotoxigenicity of T-1 and T-2.

There was general agreement between the results of the cell culture assays and those with the BCET-RPLA kit; however, there were a number of significant exceptions (Table 1). Strain A-7 was consistently negative with the immunoassay but had biological activity with both cell lines. Conversely, strain B4AC-2 gave a strong response with the kit but was negative in the cell lines. Likewise, strain T-2 consistently gave a low level response with the BCET-RPLA test but was negative in the cell lines. The Watertown strain, which was positive in CHO cells but negative with HEP-2 cells, was negative with the immunoassay.

In a separate set of assays, cell-free culture supernatants of four strains were boiled for 10 min to inactivate the diarrheal enterotoxin. There was a complete loss of biological activity in both cell lines (Table 2). Significant activity was still observed when boiled samples were assayed with the BCET-RPLA with three of the four tested strains. Again, the BCET-RPLA kit did not detect enterotoxin

TABLE 1. *Titers of cell-free supernatants of B. cereus cultures assayed using HEp-2 and CHO cells and the BCET-RPLA kit.<sup>a</sup>*

Isolate	Cell-culture assays		BCET-RPLA
	HEp-2	CHO	Kit
Watertown (J. Goepfert via FDA)	NA <sup>b</sup>	55	21
A-7 (J. Goepfert via FDA)	21	32	NA
F45814/70 (R. Gilbert via FDA)	NA <sup>c</sup>	27	64
H-13 (J. Goepfert via FDA)	32	55	64
5056 (T. Midura)	28 <sup>d</sup>	64 <sup>e</sup>	6 <sup>d</sup>
F4433/73 (R. Gilbert via F. Busta)	47	64	64
B4AC-1 (D. Mossel via F. Busta)	38	55	64
B4AC-2 (D. Mossel via J. Goepfert)	NA <sup>c</sup>	NA	34 <sup>c</sup>
T-1 (F. Busta)	37	48	48
T-2 (ERRC)	NA <sup>c</sup>	NA	7
F4552/75 <sup>f</sup> (R. Gilbert via F. Busta)	NA	NA	NA
E-2 <sup>g</sup> (J. Goepfert)	32	55	21
BHIG Control	NA	NA	NA

<sup>a</sup> Reciprocal of highest dilution yielding a positive response; average of four HEp-2 and three CHO and BCET-RPLA assays unless otherwise noted.

<sup>b</sup> No activity.

<sup>c</sup> Average of three assays.

<sup>d</sup> Average of two assays.

<sup>e</sup> Results of one assay with the other two assays being negative.

<sup>f</sup> Emetic toxin-producing isolate.

<sup>g</sup> *B. thuringiensis* isolate.

production by strain A-7, even though high levels of biological activity were observed with both cell lines.

TABLE 2. *Comparison of titers of untreated (U) and boiled (B) cell-free supernatants of four enterogenic isolates.<sup>a</sup>*

Isolate	Cell-culture assays				BCET-RPLA	
	HEp-2		CHO		Kit	
	U	B	U	B	U	B
H-13	36	NA <sup>b</sup>	72	NA	>64	>40
A-7	33	NA	49	NA	NA	NA
T-1	18	NA	40	NA	>64	16
E-2	25	NA	36	NA	>40	12
BHIG Control	NA		NA		NA	

<sup>a</sup> Reciprocal of highest dilution yielding a positive response; average of two independent trials.

<sup>b</sup> No activity.

## DISCUSSION

Cell culture assays have been used extensively to assess the production of enterotoxins by various species including the diarrheal toxin of *B. cereus* (2,7,10,11). The response observed by various investigators has included both cytotoxic and cytotoxic responses. The cytotoxic response observed in the current study suggests the production of an enterotoxin with biological activity similar to the type produced by *Vibrio cholerae* or enterotoxigenic *Escherichia coli*. The CHO cell line was consistently more sensitive than the HEp-2 cells indicating that it would be more effective for the routine assessment of biological activity of *B. cereus* isolates. This conclusion was reinforced by the inability of the HEp-2 cells to show activity in the Watertown and F45814/70 strains.

The comparison of the results achieved with cell culture assays and the BCET-RPLA assays indicates that additional research is needed to establish the specificity of the immunoassay. The inability of the assay to detect toxin production with two of the nine diarrheal toxin-producing strains represents a high rate of false negatives. An equal number of false positives were also noted based on the cell cultures responses. The results of thermal inactivation reinforce this conclusion, indicating that the epitope(s) detected by the immunoassay is more resistant to thermal denaturation than overall biological activity. The current study suggests that until the reasons underlying the differential response between cell culture and the BCET-RPLA assays can be established, it would be prudent to confirm the results achieved with the immunoassay with a suitable direct measure of biological activity.

The reasons underlying the differential responses observed between the cell culture and immunoassays will require additional research. One possibility is that there is serological heterogeneity among heat-labile enterotoxins produced by various isolates of *B. cereus*. Alternatively, the antibody used in the immunoassay may be reacting with an extracellular product that is associated with, but distinct from the component responsible for cytotoxicity in cell culture. For example, past research has indicated that the hemolysin produced by *B. cereus* is difficult to resolve from the enterotoxin (5).

Another area of potential interest is the differences observed with the subcultures of strains B4AC and T (Table 1). This suggests that the ability to synthesize the heat-labile enterotoxin can be lost and may represent an unstable genetic element. However, this supposition will require detailed confirmation that the subcultures are actually clones of the same original parent strains, differing only in their ability to produce enterotoxin.

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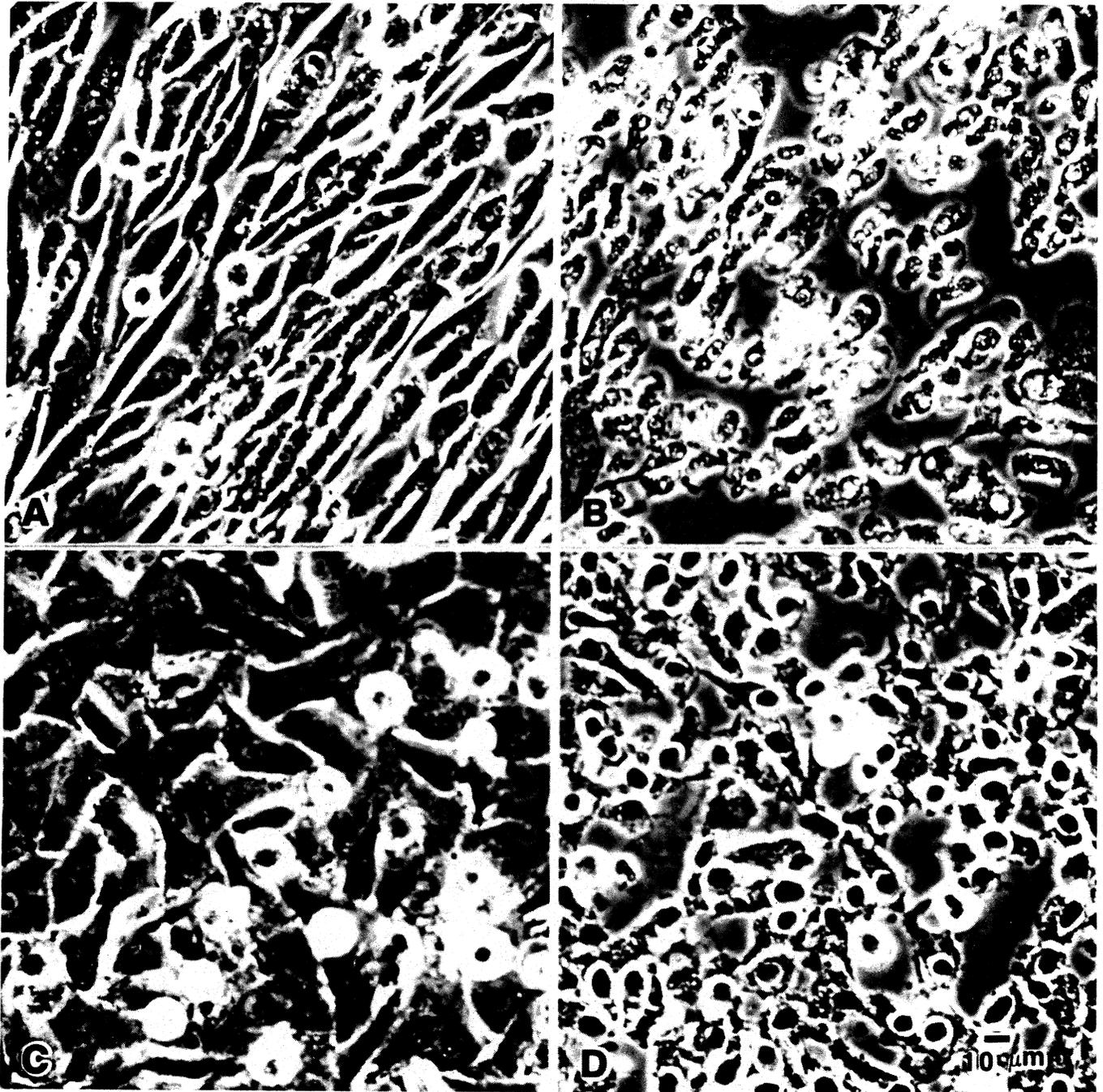


Figure 1. Cytotoxic response of CHO and HEP-2 cells to exposure to cell-free supernatants of diarrhegenic strains of *B. cereus*. A: CHO cells - control; B: CHO cells after 2.5 h exposure to supernatant; C: HEP-2 cells - control; D: HEP-2 cells after 2.5 h exposure to supernatant.

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