APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Dec. 1994, p. 4614–4616
0099-2240/94/$04.00+0

Vol. 60, No. 12

Identification and Analysis of the Antigens Detected by Two Commercial *Bacillus cereus* Diarrheal Enterotoxin Immunoassay Kits

DOUGLAS J. BEECHER AND AMY C. LEE WONG*

Department of Food Microbiology and Toxicology, University of Wisconsin–Madison, Madison, Wisconsin 53706

Received 12 July 1994/Accepted 14 September 1994

The usefulness of two commercial immunoassays for the detection of diarrheal enterotoxin of *Bacillus cereus* is unclear because the identity of the enterotoxin(s) has not been proven and the kits detect different proteins. We found that the *Bacillus cereus* Enterotoxin-Reversed Passive Latex Agglutination kit (Oxoid) detects the L_2 component from hemolysin BL, and the *Bacillus* Diarrhoeal Enterotoxin Visual Immunoassay (Tecra) detects two apparently nontoxic proteins.

Bacillus cereus causes diarrheal and emetic food-poisoning syndromes, each caused by distinct toxins. Two commercial immunoassays are available for the detection of the diarrheal enterotoxin of *B. cereus*: the *Bacillus* Diarrhoeal Enterotoxin (BDE) Visual Immunoassay from Tecra and the *Bacillus cereus* Enterotoxin-Reversed Passive Latex Agglutination (BCET-RPLA; TD950) kit from Oxoid. Both kits are in general use (8–10, 13, 16, 18, 19, 24, 25, 29). However, the value of these kits is questionable because the diarrheal enterotoxin has been identified only provisionally and equivocally. Further, these kits detect different antigens (11). No data that characterize the respective antigens or demonstrate that they have diarrheal activity have been supplied by either kit manufacturer.

Two, apparently distinct, toxins have been purified and characterized as enterotoxins. One is hemolysin BL (HBL), a tripartite hemolytic, cytotoxic, dermonecrotic, vascular permeability factor (1–4) that is apparently identical to the tripartite enterotoxins described by Thompson et al. (26) and Bitsaev and Ezepchuk (7). We have recently confirmed that HBL causes fluid accumulation in the rabbit ileal loop test and is thus an enterotoxin (5). The components of HBL are designated B, L_1, and L_2. The individual components do not possess any of the known HBL activities, each of which requires all three components for maximal activity.

The other reported *B. cereus* enterotoxin, consisting of a single protein, was described by Shinagawa et al. (20–23). It possessed vascular permeability, rabbit ileal loop, mouse ileal loop, and cytotoxic activities and activities that are lethal to mice.

It was claimed recently (6) that the *B. cereus* enterotoxin is composed of only one of the three antigens described as enterotoxin components by Thompson et al. (26). However, there was no toxic activity (diarrhea in monkeys) in partially purified preparations of that antigen, indicating that it is likely not an enterotoxin.

Our aims in this study were to (i) identify the antigens detected by both kits, (ii) determine if these antigens are components of HBL, and (iii) determine if these proteins have enterotoxic activity.

* Corresponding author. Mailing address: Dept. Food Microbiology and Toxicology, Food Research Institute, University of Wisconsin—Madison, 1925 Willow Drive, Madison, WI 53706. Phone: (608) 263-1168. Fax: (608) 263-1114. Electronic mail address: amywong@macc.wisc.edu.

The methods used in this study for protein purification, protein determination, electrophoresis, protein blotting, N-terminal sequence analysis, spectrophotometric hemolysis assay, and rabbit vascular permeability assay have been described previously (4). The purified HBL components used here were the same as those described previously (4) and appeared homogeneous on electrophoresis gels stained with silver. Recombinant component B of HBL (expressed in *Escherichia coli*) was prepared and quantitated as described previously (17). The commercial immunoassays were performed per the instructions of the respective manufacturers.

Preparations of the three purified HBL components were assayed with the BCET-RPLA kit (Oxoid). The lowest concentration of each component producing a positive response was 625 ng/ml for B, 2,500 ng/ml for L_1, and 0.6 ng/ml for L_2. A negative response occurred in preparations containing 15,000 ng of recombinant component B per ml. These observations indicate that the BCET-RPLA kit is specific for the L_2 component of HBL at the sensitivity claimed by the manufacturer (1 ng/ml in the test wells). The reactivity with purified component B was apparently due to low-level contamination with L_2.

We also tested the hemolytic activity of the positive enterotoxin control provided with the BCET-RPLA kit. The enterotoxin control was not lytic alone or when combined with B or B plus L_2. However, it did cause lysis when combined with B and L_1, which identifies it as the L_2 component of HBL. Granum et al. identified the same protein as the target of the BCET-RPLA kit but did not recognize it as a component of HBL (14, 15).

The BDE kit (Tecra) is a double-sandwich enzyme immunoassay. To identify the antigens detected by this kit, we used the detecting antibody-enzyme conjugate to probe Western blots (immunoblots) of culture supernatant from *B. cereus* F837/76, a highly enterotoxigenic isolate (28). Blots were probed overnight at 37°C with the BDE conjugate supplemented with 0.05% Tween 20.

Bands were visible on the Western blot at positions corresponding to ca. 40, 41, 56, 100, 108, and 114 kDa (data not shown). The darkest bands were at 40 and 41 kDa. Proteins corresponding to the two smaller sizes coeluted from an anion-exchange column used to purify HBL (see reference 4, Fig. 2, lanes 18 and 20), and the fractions containing them were strongly positive in the BDE assay. They were further purified on a Mono-Q column (Pharmacia, Piscataway, N.J.), which

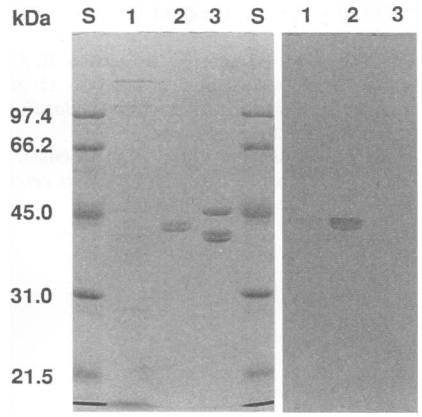

FIG. 1. Identification of proteins detected by BDE visual immunoassay (Tecra). (Left) Sodium dodecyl sulfate-polyacryalamide gel; (right) Western blot probed with detecting antibody-enzyme conjugate from BDE visual immunoassay. Lanes: S, molecular weight standards; 1, supernatant (5 μl) from a 5-h culture of *B. cereus* F837/76; 2, partially purified protein preparation strongly positive in the BDE visual immunoassay; 3, pooled HBL components, B, L_1, and L_2 (bottom to top). Each band in lanes 2 and 3 contains ca. 0.2 μg of protein. A low protein load was used for the culture supernatant to identify only the major antigens detected by the BDE antibodies.

separated them completely from contaminating proteins and partially from one another. Fractions in which the 40-kDa protein was purified to apparent homogeneity were obtained, but the 41-kDa protein was not completely separated from the 40-kDa protein. The detection limit of the BDE assay was 5 ng/ml for the pure 40-kDa preparation and ca. 2 ng/ml for the 40- and 41-kDa protein mixture. The N-terminal sequence of the 40-kDa protein was A·Q·N·V·I·A·P·N·T·L·S. The Western blot shown in Fig. 1 confirmed that (i) both of the purified proteins reacted with the BDE antibodies, (ii) they are the major antigens detected, and (iii) HBL components are not detected by the BDE antibodies.

No dermal reactions occurred in the vascular permeability assay with duplicate injections of either 10 μg of the pure 40-kDa protein or a combination of 2.5 μg (each) of the 40- and 41-kDa proteins (Fig. 1, lane 2). In contrast, injection of 0.04 μg per component of HBL caused edema and bluing; 0.16 μg per component also caused dermonecrosis (4). Dermonecrotic and vascular permeability activities are strongly correlated with *B. cereus*-induced diarrhea (7, 12, 26–27).

The above observations indicate that the Oxoid BCET-RPLA kit detects the L_2 component of HBL and the Tecra BDE visual immunoassay detects two apparently nontoxic proteins. The relevance of detecting the L_2 component alone is not yet clear, because it is still not known whether all of the HBL components are coregulated.

We cannot rule out the possibility that the BDE-reactive proteins participate in human diarrhea or that detection of the L_2 component alone is useful in detecting the enterotoxin. However, our observations clearly demonstrate a need for rigorous validation of these kits before they can legitimately assume general usage. Until then, prudence should be exercised when drawing conclusions based on data from these kits.

This work was supported by grant 93-37201-9194 from the National Research Initiative Competitive Grants Program of the U.S. Department of Agriculture.

We thank James D. Macmillan of Rutgers University, New Brunswick, N.J., for supplying the *E. coli* strain that expresses recombinant B component. The BDE Visual Immunoassay was a gift from International BioProducts, Inc., Redmond, Wash.

REFERENCES

1. **Beecher, D. J.** 1990. Isolation and characterization of hemolysin BL: a novel three-component diarrheagenic hemolysin from *Bacillus cereus*. Ph.D. thesis. Rutgers University, New Brunswick, N.J.
2. **Beecher, D. J., and J. D. Macmillan.** 1990. A novel bicomponent hemolysin from *Bacillus cereus*. Infect. Immun. **58:**2220–2227.
3. **Beecher, D. J., and J. D. Macmillan.** 1991. Characterization of the components of hemolysin BL from *Bacillus cereus*. Infect. Immun. **59:**1778–1784.
4. **Beecher, D. J., and A. C. L. Wong.** 1994. Improved purification and characterization of hemolysin BL: a hemolytic dermonecrotic vascular permeability factor from *Bacillus cereus*. Infect. Immun. **62:**980–986.
5. **Beecher, D. J., and A. C. L. Wong.** 1994. Unpublished results.
6. **Bennett, R. W., G. Murthy, L. Kaylor, S. Cox, and S. H. Harmon.** 1993. Biological characterization and serological identification of *Bacillus cereus* diarrhoeal factor. Neth. Milk Dairy J. **47:**105–120.
7. **Bitsaev, A. R., and Y. V. Ezepchuk.** 1987. The molecular nature of the pathogenic effect induced by *B. cereus*. Mol. Genet. Mikrobiol. Virusol. **7:**18–23.
8. **Buchanan, R. L., and F. J. Schultz.** 1992. Evaluation of the Oxoid BCET-RPLA kit for the detection of *Bacillus cereus* diarrheal enterotoxin as compared to cell culture cytotonicity. J. Food Prot. **55:**440–443.
9. **Carlson, C. R., D. A. Caugant, and A.-B. Kolstø.** 1994. Genotypic diversity among *Bacillus cereus* and *Bacillus thuringiensis* strains. Appl. Environ. Microbiol. **60:**1719–1725.
10. **Carlson, C. R., and A.-B. Kolstø.** 1993. A complete physical map of a *Bacillus thuringiensis* chromosome. J. Bacteriol. **175:**1053–1060.
11. **Christiansson, A.** 1993. Enterotoxin production in milk by *Bacillus cereus*: a comparison of methods of toxin detection. Neth. Milk Dairy J. **47:**79–87.
12. **Glatz, B. A., W. M. Spira, and J. M. Goepfert.** 1974. Alteration of vascular permeability in rabbits by culture filtrates of *Bacillus cereus* and related species. Infect. Immun. **10:**299–303.
13. **Granum, P. E., S. Brynestad, and J. M. Kramer.** 1993. Analysis of enterotoxin production by *Bacillus cereus* from dairy products, food poisoning incidents and non-gastrointestinal infections. Int. J. Food Microbiol. **17:**269–279.
14. **Granum, P. E., S. Brynestad, K. O'Sullivan, and H. Nissen.** 1993. Enterotoxin from *Bacillus cereus*: production and biochemical characterization. Neth. Milk Dairy J. **47:**63–70.
15. **Granum, P. E., and H. Nissen.** 1993. Sphingomyelinase is part of the 'enterotoxin complex' produced by *Bacillus cereus*. FEMS Microbiol. Lett. **110:**97–100.
16. **Griffiths, M. W.** 1990. Toxin production by psychrotrophic *Bacillus* spp. present in milk. J. Food Prot. **53:**790–792.
17. **Heinrichs, J. H., D. J. Beecher, J. D. Macmillan, and B. A. Zilinskas.** 1993. Molecular cloning and characterization of the gene encoding the B component of hemolysin BL from *Bacillus cereus*. J. Bacteriol. **175:**6760–6766.
18. **Luby, S., J. Jones, H. Dowda, J. Kramer, and J. Horan.** 1993. A large outbreak of gastroenteritis caused by diarrheal toxin-producing *Bacillus cereus*. J. Infect. Dis. **167:**1452–1455.
19. **Notermans, S., and S. Tatini.** 1993. Characterization of *Bacillus cereus* in relation to toxin production. Neth. Milk Dairy J. **47:**71–77.
20. **Shinagawa, K., K. Ichikawa, N. Matsusaka, and L. Sugii.** 1991. Purification and some properties of a *Bacillus cereus* mouse lethal toxin. J. Vet. Med. Sci. **53:**469–474.
21. **Shinagawa, K., K. Sato, H. Konuma, N. Matsusaka, and S. Sugii.** 1991. Fluid accumulation in mouse ligated intestine inoculated with the vascular permeability factor produced by *Bacillus cereus*. J. Vet. Med. Sci. **53:**167–171.
22. **Shinagawa, K., J. Sugiyama, T. Terada, N. Matsusaka, and S. Sugii.** 1991. Improved methods for purification of an enterotoxin produced by *Bacillus cereus*. FEMS Microbiol. Lett. **80:**1–6.
23. **Shinagawa, K., S. Ueno, H. Konuma, N. Matsusaka, and S. Sugii.** 1991. Purification and characterization of the vascular permeability factor produced by *Bacillus cereus*. J. Vet. Med. Sci. **53:**281–286.

24. **Sutherland, A. D.** 1993. Toxin production by *Bacillus cereus* in dairy products. J. Dairy Res. **60:**569–574.
25. **Sutherland, A. D., and A. M. Limond.** 1993. Influence of pH and sugars on the growth and production of diarrhoeagenic toxin by *Bacillus cereus*. J. Dairy Res. **60:**575–580.
26. **Thompson, N. E., M. J. Ketterhagen, M. S. Bergdoll, and E. J. Shantz.** 1984. Isolation and some properties of an enterotoxin produced by *Bacillus cereus*. Infect. Immun. **43:**887–894.
27. **Turnbull, P. C. B.** 1986. *Bacillus cereus*, p. 397–448. *In* F. Dorner and J. Drews (ed.), Pharmacology of bacterial toxins. Pergamon Press, Oxford.
28. **Turnbull, P. C. B., J. M. Kramer, K. Jørgensen, R. J. Gilbert, and J. Melling.** 1979. Properties and production characteristics of vomiting, diarrheal, and necrotizing toxins of *Bacillus cereus*. Am. J. Clin. Nutr. **32:**219–228.
29. **van Netten, P., A. van de Moosdijk, P. van Hoensel, and D. A. A. Mossel.** 1990. Psychrotrophic strains of *Bacillus cereus* producing enterotoxin. J. Appl. Bacteriol. **69:**73–79.