Research Note

Detection of *Bacillus cereus* Diarrheal Enterotoxin in Raw and Pasteurized Milk

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**ABSTRACT**

Raw and pasteurized milk samples submitted for routine quality analysis were screened for the presence of *Bacillus cereus* diarrheal enterotoxin (BDE) using the TECRA BDE Visual Immunoassay (VIA) kit. BDE was not detected in 298 raw milk samples tested by the TECRA VIA. *B. cereus* was isolated from 2 of 298 (0.7%) raw milk samples cultured. Culture supernatants from these isolates were positive for BDE in the TECRA VIA but negative in the Reverse Passive Latex Agglutination (RPLA) test for BDE. Forty-three of 112 (38.4%) pasteurized milk samples incubated at 10°C until their expiry dates were positive for BDE by the TECRA VIA. The same number of samples incubated at 4°C had no detectable levels of enterotoxin. *B. cereus* in the range of 103 to 106 CFU/ml was isolated from all BDE-positive pasteurized milk samples. BDE was detected in the culture supernatants of all the 43 isolates by TECRA VIA and in 30 of these isolates by RPLA. These results demonstrate that moderate temperature abuse of pasteurized milk may allow the growth of *B. cereus* and BDE production.

Key words: Enterotoxins, TECRA, *Bacillus cereus*

*Bacillus cereus* strains are known to produce one or two types of enterotoxins, a diarrheal toxin and an emetic toxin (22, 31). Enterotoxin-producing strains have been implicated in several food-borne-related illnesses (13, 15, 20, 21, 22, 32). The diarrheal-type illness is attributed to the production of heat-labile enterotoxin secreted during the vegetative growth of the organism, mainly in the late logarithmic phase (14, 17, 31). The number of reported cases of food-borne illness caused by *B. cereus* in Canada increased from 1 to 106 from 1975 to 1986 (29, 30).

Contaminated milk and dairy products are a common source of *B. cereus* (17, 22, 23, 32). Studies on psychrotrophic strains of *B. cereus* indicate that both raw and pasteurized milk frequently harbor this organism, with raw milk being arguably the main source of psychrotrophic *Bacillus* species in pasteurized milk (8, 11, 18, 19, 23). These organisms grow in milk with an average generation time of 17 h at 6°C (19) and can produce diarrheal toxin during growth in milk at low temperatures (10, 18, 32).

The Ontario Ministry of Agriculture, Food and Rural Affairs (OMAFRA), Agricultural and Food Laboratory Services Centre (AFLSC), Guelph, Ontario, Canada, routinely tests raw and pasteurized milk samples for quality. Enterotoxin tests are not part of the routine analysis performed on these samples. The purpose of this study was to determine the prevalence of *B. cereus* diarrheal enterotoxin (BDE) in raw and pasteurized milk samples submitted for routine quality tests at the AFLSC. A commercially available enzyme-linked immunosorbent assay, the TECRA BDE Visual Immunoassay was used for screening milk samples for the presence of diarrhoeal enterotoxin.

**MATERIALS AND METHODS**

*Bacillus cereus*

The BDE-positive strain of *B. cereus* used as a control in this study was a laboratory strain isolated previously from pasteurized milk.

**Milk samples**

A total of 298 raw milk samples stored for 72 h at 4°C were tested for BDE. One hundred and twelve pasteurized milk samples, (homogenized, 2%, 1%, and skim) were stored at 4°C and the same number of samples (112) was stored at 10°C until their expiry date (approximately two weeks) and then tested for BDE.

*B. cereus* diarrhoeal enterotoxin assay

Milk samples were assayed for BDE using the TECRA BDE Visual Immunoassay (VIA) (Bioenterprises Pty., Ltd., Roseville, NSW, Australia). The assay was performed following the manufacturer's instructions. The conjugate and substrate were incubated at 25°C for 45 min. Results were read on a microplate reader (Bio-Tec Instruments Inc., Winooski, Vt) at a wavelength of 405 nm.

*B. cereus* isolates obtained from raw and pasteurized milk samples were inoculated into 10 ml of brain heart infusion broth.
pH 7.4, and incubated at 32°C for 12 h on a shaker (New Brunswick Scientific Inc., NJ) at 250 cycles per min. The cultures were centrifuged at 900 × g for 20 min to pellet the cells and culture supernatants were tested for BDE by the TECRA VIA. *B. cereus* cultures were filtered using 0.45-μm-pore-size filters (Gelman Sciences, MI) and the filtrates tested for BDE by using the reverse passive latex agglutination (RPLA) kit from Oxoid (Unipath Ltd, Basingstoke, Hampshire, England) as per the manufacturer’s instructions.

Isolation and identification of Bacillus species

*Bacillus* species were isolated from raw and pasteurized milk samples by using phenylethyl alcohol agar with 5% sheep blood (PEA), and 5% sheep blood agar (BA). Colonies with morphologies typical of *Bacillus* species were tested for catalase production and examined for Gram stain characteristics and morphology. Suspect colonies of *Bacillus* species were identified by using the Microbial Identification System (MIDI, Inc., Newark, DE).

RESULTS AND DISCUSSION

BDE was not detected in the 298 raw milk samples tested in this study (Table 1). Although one of the raw milk samples showed a borderline-positive result for BDE on the TECRA kit, the sample tested negative on repeat tests on the TECRA and RPLA kits for BDE and *B. cereus* was not recovered from the sample. *B. cereus* was isolated in low numbers (<10² CFU/ml) from 2 of 298 (0.7%) raw milk samples (Table 2) and other *Bacillus* species were isolated from 4 of 298 (1.3%) raw milk samples cultured. There is a low prevalence of BDE and of vegetative cells of *B. cereus* in raw milk because this organism is mostly in the spore state in raw milk and germination is initiated by stress conditions such as pasteurization temperatures (23). Growth of vegetative *B. cereus* cells in foods including dairy products is required for the production of detectable levels of BDE (18, 27). *Bacillus* spores can survive pasteurization, germinate, and grow under conditions of temperature abuse and even at refrigeration temperatures (10, 18, 23, 32). The raw milk samples tested in this study were 72-h samples stored at 4°C. Sutherland (27) showed that the lag phase of *B. cereus* spores in dairy desserts is >14 d. Vegetative cells of psychrotrophic *B. cereus* strains have a lag time of 78 h and a generation time of 17 h at 6°C (19). Thus temperatures higher than 6°C are required to accelerate growth of the organism.

BDE was detected in 43 of 112 (38.4%) pasteurized milk samples incubated at 10°C until their expiry dates (approximately 2 weeks) by using the TECRA BDE VIA, while the same number of pasteurized milk samples cultured at 4°C had no detectable levels of BDE (Table 1). Lack of production of BDE by toxigenic and psychrotrophic dairy isolates of *B. cereus* in cream and dairy products maintained at 6°C has been reported (27). van Netten et al. (32) showed that growth of and enterotoxin production by psychrotrophic *B. cereus* could be prevented at temperatures below 4°C. *B. cereus* in the range of 10³ to 10⁸ CFU/ml was isolated from all the BDE-positive milk samples cultured. There are reports indicating that relatively high numbers of *B. cereus* (10⁵ to 10⁸ CFU/g) are required to produce detectable levels of BDE in food (22, 27).

All 43 isolates tested positive for BDE production by the TECRA VIA and 30 of these isolates tested positive for BDE by the RPLA test (Table 2). The two *B. cereus* isolates obtained from raw milk also tested positive for BDE production by TECRA VIA but negative on the RPLA system. The BDE-producing strain of *B. cereus* used as a positive control tested positive in both kits. Previous studies comparing these two commercial kits for the production of BDE by *B. cereus* cultures showed no correlation between results obtained by these kits (1, 9, 12, 24). The TECRA VIA results had a better correlation with results of a vero cytotoxicity assay (1) and results of human embryonic lung cell cytotoxicity titers (9) than the RPLA system results. Noterman and Tatini (24) found nine *B. cereus* strains implicated in food-borne disease outbreaks showed positive results for BDE production by TECRA VIA and three of these isolates were negative in the RPLA test. In a similar report (12), 12 *B. cereus* isolates associated with diarrheal disease were positive for BDE by TECRA VIA and six of these isolates were negative in the RPLA test system. One strain positive in monkey-feeding experiments was positive for BDE production by the TECRA VIA but negative in the RPLA test. Buchanan and Schultz (6) questioned the specificity of the RPLA assay for BDE when two of nine diarrheal toxin-producing *B. cereus* strains were negative for BDE in RPLA tests, and they found differences between the results of the RPLA and cytotoxicity tests. The lack of correlation between the TECRA VIA and RPLA results is because these two kits detect different antigens (5, 7, 12, 16). In the present study the toxin control provided with the TECRA VIA kit tested negative in the RPLA test and the toxin control provided with the RPLA kit tested negative in the TECRA VIA test. This finding is in agreement with previous reports (9, 12). The antibody used in the TECRA system was raised to antigen 577, believed to be the diarrheal enterotoxin (7).
and the RPLA antibody was raised against a nontoxic component (58 kDa) of the “enterotoxin complex” (16). Granum et al. (16) found that two enterotoxigenic \textit{B. cereus} strains lacking the 58 kDa protein were negative in the RPLA system.

There are reports that BDE is a multicomponent toxin (2, 5, 28). Beecher and Macmillan (2, 3) described a tripartite hemolysin (hemolysin BL) produced by \textit{B. cereus} and showed that purified hemolysin BL consists of three proteins, designated B, L1, and L2. A combination of these three components was required for the hemolytic and vascular permeability activities of hemolysin BL (3) and for its diarrheal activity in the ligated rabbit ileal loop assay (4). The characteristics of this toxin were consistent with those described for BDE (28). The RPLA kit was shown to detect the L2 component of the hemolysin BL and the TECRA VIA detected two apparently nontoxic proteins (5). Other studies showed that the protein responsible for enterotoxic activity is a single 45-kDa protein (25, 26). This protein showed vascular permeability activity and mouse lethal toxicity and caused fluid accumulation in ligated mouse intestinal loops, but it had no hemolytic and lecithinase activities (25). There are conflicting reports regarding the identity of BDE and the reliability of the RPLA and TECRA kits in detecting BDE in food and \textit{B. cereus} cultures. Nevertheless, in the present study only pasteurized milk samples stored at 10°C contained material positive in the TECRA VIA which may be BDE. Pasteurized milk samples stored at this temperature also contained high numbers of \textit{B. cereus} cells. The majority of \textit{B. cereus} isolates were shown to be enterotoxin producers by both kits. These results demonstrate that moderate temperature abuse of pasteurized milk can promote the growth of \textit{B. cereus} and subsequent BDE production. Thus raw milk with high levels of \textit{B. cereus} spores is a potential source of \textit{B. cereus} and BDE-contaminated pasteurized milk, especially under temperature abuse storage conditions.

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