APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Mar. 1988, p. 699–702
0099-2240/88/030699-04$02.00/0

Vol. 54, No. 3

Incidence and Characterization of *Bacillus cereus* Isolates Contaminating Dairy Products

HIN-CHUNG WONG,* MAN-HUEI CHANG, AND JIN-YUAN FAN
Department of Microbiology, Soochow University, Taipei, Taiwan 11102, Republic of China

Received 2 November 1987/Accepted 19 December 1987

A total of 293 dairy products purchased from local markets were examined to determine the incidence of and characterize *Bacillus cereus*. Isolations were made on mannitol-egg yolk-polymyxin B agar medium and confirmed by several staining and biochemical tests. *B. cereus* occurred in 17% of fermented milks, 52% of ice creams, 35% of soft ice creams, 2% of pasteurized milks and pasteurized fruit- or nut-flavored reconstituted milks, and 29% of milk powders, mostly in fruit- or nut-flavored milk mixes. The average population of *B. cereus* in these dairy products was 15 to 280 CFU/ml or CFU/g (range, 5 to 800). The characteristics of these *B. cereus* isolates in terms of heat resistance, biochemical reactions, and antibiotic susceptibility were similar to previously reported data except for a higher utilization of sucrose. Some isolates were especially resistant to carbenicillin, nalidixic acid, streptomycin, and tetracycline. The MICs for the isolates were also determined. All of the tested isolates lysed rabbit erythrocytes; 98% showed verotoxicity, 68% showed cytotonic toxicity for CHO cells, and 3 of 11 selected isolates that showed strong hemolysin activity killed adult mice.

Bacillus cereus is a widely distributed bacterium, having been isolated from rice, spices, meat, and egg and dairy products (13). Food poisoning caused by *B. cereus* is mostly associated with the consumption of rice products (3, 16). *B. cereus* is recognized as causing diarrheal and emetic types of food-poisoning outbreaks (13). *B. cereus* has a high incidence in dairy products (7). As reported by Ahmed et al. (1), 9% of raw milk, 35% of pasteurized milk, 14% of cheese, and 48% of ice cream samples were contaminated with *B. cereus*; conversely, no fermented milk was found to be contaminated. However, food-poisoning outbreaks caused by the contamination of dairy products by *B. cereus* are rare (13). Nevertheless, the growth of *B. cereus* significantly spoils the quality of dairy products, causing sweet curdling and "bitty" cream (7). To estimate the role of a microorganism as a food contaminant and pathogen in dairy products, one must know the incidence, growth, germination, and toxin production of that microorganism in these products. Although *B. cereus* has been frequently isolated from dairy products, its role as a food pathogen in these products has not been well studied.

In recent years, the consumption of dairy products has increased rapidly in Taiwan, and high quality is demanded by consumers. Fermented milk, soft ice cream, fruit- or nut-flavored milk mixes, and pasteurized fruit- or nut-flavored reconstituted milk in addition to other conventional milk powders and ice cream have also large markets in Taiwan. Since some of these items may not be popular in Western societies, we systematically studied the *B. cereus* problem in dairy products in Taiwan to estimate the significance of this organism as a food pathogen in these products. This paper reports the incidence of *B. cereus* in various dairy products purchased from local markets and the characteristics of the *B. cereus* organisms isolated in terms of heat resistance, biochemical reactions, antibiotic susceptibility, and toxigenicity.

* Corresponding author.

MATERIALS AND METHODS

Samples. Various dairy products were obtained from local markets in each of the four seasons, i.e., August, November, February, and May, in 1985 to 1986. All samples except milk powders were transported to the laboratory in an icebox.

Enumeration of *B. cereus*. The milk powders were rehydrated by soaking 10-g samples separately in 90 ml of sterile distilled water and shaking them at 160 rpm for 30 min at room temperature. Ice creams and soft ice creams were liquified at 4°C. Serial dilutions of all products were made in sterile distilled water. The dilutions (0.1 ml) were spread on mannitol-egg yolk-polymyxin B agar and incubated at 30°C for 24 h. Typical *B. cereus* colonies (with a dry, rough surface; red-purple with a white precipitate) were counted, transferred to nutrient agar slants, and confirmed by microscopic and biochemical characterization, including Gram stain, spore stain, motility, gelatin hydrolysis, the Voges-Proskauer test, anaerobic utilization of glucose, and nitrate reduction (10, 18). The means of four determinations were used to summarize the basic results.

Heat resistance. Twenty-five isolates were randomly selected. Spores were obtained by culturing on fortified nutrient agar plates incubated upright at 30°C for 24 h and then inverted for an additional 24 h at 30°C. The plates were then incubated at 4°C for 24 h (14). The spores were washed, collected by centrifugation, diluted to 10^8 spores per ml, and stored at 4°C. Spore solution (0.1 ml) was suspended in 0.025 M phosphate buffer at pH 7 and heated at 100°C for various times. Survival rates were determined by plating the spores on tryptic soy agar (Difco Laboratories), and the $D_{100°C}$ values were then calculated.

Biochemical tests. Ninety-one randomly selected *B. cereus* isolates were tested for different biochemical characteristics by the methods outlined in the *Bacteriological Analytical Manual* (10). Tests done included hydrolysis of gelatin; the Voges-Proskauer test; anaerobic utilization of glucose; catalase; hydrolysis of starch; utilization of sucrose, xylose, mannitol, salicin, and arabinose; alkaline peptonization of litmus milk; and nitrate reduction.

Antibiotic susceptibility. Antibiotic susceptibility was determined by the disk agar diffusion method in accordance

TABLE 1. Contamination of dairy products by *B. cereus*

Product	% Contamination	CFU/ml or CFU/g	
		Range	Avg
Milk powder	27	5–450	74
Pasteurized milk	2	280	280
Fruit-flavored reconstituted milk	2	15	15
Fermented milk	17	5–115	45
Ice cream	52	5–250	52
Soft ice cream	35	5–800	159

with the instructions of the antibiotic disk supplier (Difco). The MICs of some selected resistant isolates were also determined by the agar dilution method (22).

Hemolysin assay. Blood agar plates were prepared by adding 5 ml of defibrinated rabbit blood to 100 ml of basal medium (11), inoculated with the *B. cereus* isolates, and incubated at 30°C for 48 h. The dimensions of the blood lysis halo zones were measured.

Mouse lethal-toxin test. Fresh *B. cereus* cultures were inoculated separately into 20 ml of brain heart infusion broth (Difco) in 250-ml Erlenmeyer flasks and cultured at 35°C for 10 h in a shaking incubator set at 280 rpm. The culture filtrates subjected to the mouse lethal-toxin test and animal cell culture assays were prepared by filtering the culture broth through a 0.2-μm-pore membrane filter (Millipore Corp.) (21). These culture filtrates (0.5 ml) were separately injected into the caudal veins of four adult ICR mice. Death within 30 min was considered to be a positive response (19–21).

Animal cell culture assays. Fresh culture filtrates were used in these tests. Vero cells and Chinese hamster ovary cells (CHO cells) were cultured in Eagle minimum essential medium (GIBCO Laboratories) and McCoy 5A medium (GIBCO), both supplemented with 10% fetal calf serum (GIBCO), respectively, in wells of microdilution plates. The culture filtrates (200 and 15 μl) were added to each well of Vero cells and CHO cells, respectively, and observed for 24 h. Disruption of the monolayer of Vero cells was regarded as a positive test for verotoxin. Alteration of at least 30% of the CHO cells to an abnormal shape was regarded as a positive test for cytotonic toxin (4, 6, 21).

In the mouse lethality test and the animal cell culture assays, two clinical isolates, *B. cereus* F837 and F4433, obtained from the Central Public Health Laboratory, London, England, were used as positive references, and culture medium was used as the blank control.

RESULTS

Incidence of *B. cereus* in dairy products. About 29% of 94 milk powders were contaminated by *B. cereus*; however, the population was usually low (Table 1). Among the milk powders, the fruit- and nut-flavored milk mixes were contaminated at a higher rate than were regular whole-fat milk or nonfat milk powders (Table 2). Among the imported regular milk powders, the aluminum foil-packed samples were contaminated at a much higher rate than were products canned in iron containers, while *B. cereus* was not isolated from the domestic aluminum foil-packed samples (Table 3).

Only a few of the pasteurized milk and pasteurized nut- or fruit-flavored reconstituted milk products were contaminated (Table 1). Seventeen percent of fermented milk samples were also contaminated by *B. cereus* (Table 1). Ice cream samples were highly contaminated with *B. cereus* (52%). Higher incidences were found in summer and fall in both domestic and imported samples (Table 1). The soft ice creams were obtained from various stations in the city of Taipei, and the contamination rate was high (35%), especially in summer (Table 1).

Heat resistance of *B. cereus*. The $D_{100^\circ C}$ values determined for 25 randomly selected isolates ranged from 2.0 to 5.4 min. These isolates originated from milk powders, ice creams, and soft ice creams. The heat resistances of the isolates from different sources were nearly identical (Table 4).

Biochemical characteristics. The biochemical characteristics of the isolates were determined and compared with those described in the *Bacteriological Analytical* manual (10) and with those reported for isolates from rice by Chung and Sun (5). The results were similar in all cases, except for a few tests. Hydrolysis of starch was lower than with isolates from rice samples; however, utilization of sucrose was higher (Table 5).

Antibiotic susceptibility of *B. cereus* isolates. The isolates were highly resistant to penicillins and susceptible to chloramphenicol, erythromycin, gentamicin, kanamycin, nalidixic acid, streptomycin, and sulfamethoxazole-trimethoprim (Table 6). Some isolates were especially resistant to carbenicillin, nalidixic acid, streptomycin, and tetracycline; the MICs were 256, 256, 256, and 32 μg/ml, respectively.

Toxigenicity of *B. cereus* isolates. The results of toxigenicity assays are summarized in Table 7. All of the 183 isolates tested showed weak to strong hemolysin activity. Of 11

TABLE 2. Contamination of milk powders by *B. cereus*

Product	Sample no.	% Contamination	CFU/g	
			Range	Avg
Regular milk powder[a]				
Imported	46	13	5–100	43
Domestic	16	19	10–50	37
Milk mix[b]	32	56	10–450	91

[a] Including whole-fat, nonfat, and instantly soluble products.

[b] Including apple-, fruit-, almond-, chocolate-, and coffee-flavored products.

TABLE 3. Contamination of whole-fat and nonfat milk powders by *B. cereus*

Product	Packaging	Sample no.	% Contamination	CFU/g	
				Range	Avg
Domestic	Iron container	12	25	10–50	17
	Foil	4	0	0	0
Imported	Iron container	25	4	50	50
	Foil	17	29	5–100	42

TABLE 4. Heat resistance of *B. cereus* isolates from dairy products

Source	No. of isolates	$D_{100^\circ C}$ (min)	
		Range	Avg
Milk powder	11	2.2–5.4	3.6
Ice cream	5	2.0–4.6	3.6
Soft ice cream	9	2.5–4.1	3.4
Total	25	2.0–5.4	3.5

TABLE 5. Biochemical characteristics of 91 *B. cereus* isolates

Test	No. (%) positive in our study	Result in reference:	
		10	5 (%)
Gram stain reaction	91 (100)	90–100%	
Spore stain reaction	91 (100)		
Motility	91 (100)	50–90%	100
Hydrolysis of gelatin	91 (100)	+	100
Voges-Proskauer reaction	91 (100)	+	100
Anaerobic utilization of glucose	91 (100)	+	
Catalase	91 (100)	+	100
Hydrolysis of starch	30 (33)	±	77
Acid produced from:			
Mannitol	7 (8)	−	0
Sucrose	91 (100)		55
Xylose	15 (17)		
Salicin	53 (58)		41
Arabinose	11 (12)		11
Alkaline peptonization of litmus milk	91 (100)		97
Nitrate reduction	90 (99)	+	96

isolates having strong hemolysin activity, 3 killed adult ICR mice. Although other isolates did not kill the mice, they significantly lowered the physical activity of the tested animals. A total of 50 isolates were tested in animal cell culture assays. Ninety-eight percent of the isolates had verotoxin activity, while 68% had cytotonic toxin activity. The two clinical isolates, *B. cereus* F837 and F4433, also killed adult mice and showed positive reactions for hemolysin, verotoxin, and cytotonic toxin.

DISCUSSION

B. cereus is becoming an important food-poisoning organism because of its cosmopolitan distribution in nature. Its presence in dairy products may be traced back to the environment or mastitic bovine sources (7). *B. cereus* could be transmitted to other foods when the contaminated dairy products are used as constituents in the processing of these foods. This study also revealed a high incidence of *B. cereus* in dairy products. *B. cereus* organisms were isolated from 29, 17, 52, 35, 2, and 2% of milk powder, fermented milk, ice cream, soft ice cream, pasteurized milk, and pasteurized fruit- or nut-flavored reconstituted milk samples, respectively. Since the incidence of *B. cereus* in whole-fat milk and nonfat milk powders was low, the high incidence of *B. cereus* in fruit- or nut-flavored milk mixes may be due to the carry-over of flavoring or coloring additives.

TABLE 6. Antibiotic susceptibility of 195 *B. cereus* isolates

Antibiotic	(μg/disk)	No. (%) that were:	
		Resistant	Susceptible
Ampicillin	(10)	101 (52)	39 (20)
Carbenicillin	(100)	182 (93)	2 (1)
Cephalothin	(30)	83 (43)	41 (21)
Chloramphenicol	(30)	5 (3)	186 (95)
Erythromycin	(15)	5 (3)	182 (93)
Gentamicin	(10)	4 (2)	190 (97)
Kanamycin	(30)	2 (1)	182 (93)
Nalidixic acid	(30)	3 (2)	184 (96)
Penicillin G	(10)	154 (79)	0 (0)
Streptomycin	(10)	18 (9)	173 (89)
Sulfamethoxazole-trimethoprim	(23.75/1.25)	36 (19)	153 (78)
Tetracycline	(30)	7 (4)	36 (19)

TABLE 7. Toxigenicity of *B. cereus* isolates from dairy products

Test	No. (%) with the following reaction:	
	Negative	Positive
Hemolysis	0 (0)	183 (100)
Mouse lethality	8	3
Verotoxin[a]	1 (2)	49 (98)
Cytotonic toxin[b]	16 (32)	34 (68)

[a] Determined by Vero cell culture assay.
[b] Determined by CHO cell culture assay.

The incidence of *B. cereus* in pasteurized milk was much lower than that reported in the United States (1); this may be due to the overpasteurization generally practiced by the local manufacturers.

Various organic acids, peroxides, and antibacterial agents are produced by lactic acid bacteria during fermentation (15, 17), so it is reasonable to find much less *B. cereus* in fermented milk. Ahmed et al. (1) reported no contamination in fermented milk. However, 4 of 24 of our fermented milk samples were contaminated with *B. cereus*; this may also be carry-over in the form of endospore contamination from additives.

Contamination of ice cream and soft ice cream with *B. cereus* was higher in summer than in other seasons. The incidence in ice cream was similar to that reported by Ahmed et al. (1). *B. cereus* may be present in higher proportions in the raw materials or may have a considerably higher growth rate during the manufacturing process in summer than in other seasons. In addition, the display cabinets for ice cream or soft ice cream machines may be more contaminated by *B. cereus* in summer. Soft ice creams are sold at stations all over the city, and semiprocessed materials are transported to these stations and processed into final products. The sanitary conditions of these stations are generally below standard; however, *B. cereus* organisms were isolated from only 10% of the swabs from outlets of soft ice cream machines (unpublished data).

The heat resistance of *B. cereus* isolated from dairy products was similar to that reported previously (5, 14); $D_{95°C}$ values ranged from 1.2 to 36.0 min, with a z value of 9.2°C (14). The $D_{100°C}$ of the isolates determined in this study ranged from 2.0 to 5.4 min. The heat resistance of these isolates did not seem to be related to their origin. However, the abilities of these isolates to use starch and sucrose were different from the abilities of isolates which originated from rice (5). Natural selection of *B. cereus* by the substrates may have occurred.

There are only a few papers reporting on the drug resistance of *B. cereus* (2, 5, 13). It is highly susceptible to nisin, aureomycin, dehydrostreptomycin, terramycin, bacitracin, oxytetracycline, chloramphenicol, and gentamicin and slightly susceptible to neuromycin, cloxacillin, ampicillin, and penicillin (13). In this study, the *B. cereus* isolates were highly susceptible to chloramphenicol, erythromycin, gentamicin, kanamycin, and nalidixic acid; the isolates also were susceptible to streptomycin and sulfamethoxazole-trimethoprim. Isolates especially resistant to carbenicillin, streptomycin, and tetracycline were identified. Certain *B. cereus* isolates may acquire these drug resistance phenotypes, as antibiotics are frequently used in animal feeds and in chemotherapy. Penicillin, tetracycline, chloramphenicol, streptomycin, and erythromycin have been used frequently in Taiwan and are occasionally detected in local meat products (12). As shown by Bernhard et al. (2), the tetracycline resistance of *B. cereus* is associated with a plasmid which could be transformed and maintained in *B. subtilis*. The drug resistance patterns determined in this study were different from those of isolates originating from rice (5).

Although *B. cereus* has been isolated from a wide range of food products, these isolates have not been well characterized, especially in terms of toxigenicity. A number of methods have been used to assay toxin production by *B. cereus*, for example, rabbit ileal loop ligation (19), vascular permeability (9), and animal tissue cultures (21). The suckling mouse assay is a convenient method for detecting enterotoxin and is valid for examining enteropathogenic *Escherichia coli* and some other food-poisoning microorganisms (8). However, all *B. cereus* isolates and reference strains reacted negatively in this assay (data not shown). Hemolysin, mouse lethal-toxin, verotoxin, and cytotonic toxin assay methods were valid for examining the toxigenicity of *B. cereus*. An assay with a CHO cell culture conveniently demonstrated the cytotonic toxin-producing activity of *B. cereus*. Diarrheal toxin and emetic toxin are produced by *B. cereus* (13). The emetic toxin production of the isolates was not characterized in this study.

The incidence of contamination of dairy products by *B. cereus* is fairly high. Almost all of the isolates used in this study showed hemolysin and cytotoxic toxin activities. Enterotoxin activity may have been characteristic of at least 68% of the isolates, so it is clear from this study that the risk of food poisoning caused by *B. cereus* in dairy products should not be neglected.

ACKNOWLEDGMENTS

This work was supported by grants (DOH75-0401-44 and DOH76-0401-37) from the Department of Health, Central Government of the Republic of China.

We thank M. R. Thompson of the Central Public Health Laboratory, London, England, for providing the clinical isolates of *B. cereus*. We also thank Deam H. Ferris for critical reading and valuable suggestions.

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