Plasmid, Serotypic, and Enterotoxin Analysis of *Bacillus cereus* in an Outbreak Setting

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Bacillus cereus is a recognized agent of food-borne disease. In this report we describe an outbreak of *B. cereus* gastroenteritis associated with consumption of beef stew among patients and staff at a Rhode Island nursing home. The beef had been improperly stored after preparation. The predominant symptoms of the illness were cramps and diarrhea; it lasted an average of 16 h. No deaths occurred. The organism was recovered from 10 of 23 stools collected from ill patients and 1 of 21 stools collected from controls ($P = 0.0044$, Fisher's two-tailed exact test). All isolates had the same biotype and serotype, newly designated H.26; all elaborated the diarrheal *B. cereus* enterotoxin when tested in rabbits by the vascular permeability reaction; and all had identical plasmid profiles, which differed from those of *B. cereus* strains selected randomly from other outbreaks. Plasmid analysis may prove to be a useful new tool in investigating outbreaks of *B. cereus* food poisoning.

Bacillus cereus has been recognized as an agent of food-borne disease for the past several decades. It causes both a diarrheal and emetic syndrome, each associated with a distinct toxin (30). The emetic syndrome, characterized by upper intestinal tract symptoms such as nausea and vomiting, has been described most often in association with Chinese fried rice dishes and has an incubation period of 1 to 6 h (23). The diarrheal syndrome has been linked to meats, vegetable dishes, desserts, sauces, and soups and has a longer incubation period of 10 to 12 hours (4, 9, 11). Serotyping *B. cereus* strains serves to link the organism epidemiologically to a food-borne outbreak even when it cannot be isolated from incriminated foods (12). We report what we believe to be the first instance in which plasmid profiles were used to fingerprint and epidemiologically trace an outbreak strain of *B. cereus*. The plasmid analyses were compared with serotyping and enterotoxin analyses. Demonstration of diarrheagenic enterotoxin production by the isolates confirmed their potential pathogenicity.

MATERIALS AND METHODS

On 20 May 1985 the nursing director of a nursing home in Newport County, Rhode Island, called the Rhode Island Department of Health to report an outbreak of a gastrointestinal illness among patients and staff which had begun on Saturday, 18 May. At the request of the facility, the Rhode Island Department of Health carried out an epidemiologic investigation.

The outbreak investigation began with a visit to the facility where a tour was conducted and nursing personnel described the illness. Questionnaires inquiring about food and beverage consumption and illness were issued to patients and staff. A sanitarian from the Division of Food Protection and Sanitation reviewed food preparation techniques and inspected the kitchen of the nursing home on 22 May.

A case patient was defined as any individual who resided or worked at the nursing home with new onset of diarrhea (more than three loose stools in a 12-h period) occurring within a 36-h period from the morning of Saturday, 18 May, to Sunday evening, 19 May.

To evaluate the possibility of person-to-person spread of the illness, cases were mapped out by room location and staff assignments, and an epidemic curve was constructed.

Stool samples were collected from 21 May through 5 June and plated on blood agar and selective media for *Salmonella*, *Shigella*, and *Campylobacter* species. Standard previously described techniques for culture were utilized (15, 22). Standard previously described laboratory techniques for isolation of *Clostridium perfringens* were used (1).

Blood agar plates on which stool was directly plated were examined, and suspicious *B. cereus* colonies were picked, streaked onto a second blood agar plate, and incubated at 35°C (5). Standard biochemical tests for identification of *B. cereus* organisms were utilized (10, 16). The isolates were stored on nutrient agar slants at room temperature. *B. cereus* isolates were referred to the Central Public Health Laboratory in London, subjected to biochemical analysis, and serotyped by the scheme of Taylor and Gilbert (25). *B. cereus* enterotoxin analysis was also performed at the Central Public Health Laboratory by vascular permeability reaction testing as described by Turnbull et al. (27, 28). Plasmid DNA from each isolate of *B. cereus* was obtained by generating protoplasts with penicillin G (10 µg/ml) in a hypertonic medium as previously described (24). Protoplasts were then subjected to rapid alkaline hydrolysis by the method of Kato and Liu (14). Plasmid DNA was then separated by vertical agarose gel electrophoresis (20), strained with ethidium bromide, and photographed under UV light (Fotodyne, Inc., New Berlin, Wis.) with Polaroid type 47 film. Plasmid profiles were then compared with those of 15 randomly selected isolates of *B. cereus* obtained from other outbreaks caused by different serotypes. These strains were kindly provided by Stanley Harmon of the Food and Drug Administration, Washington, D.C.

Statistical associations between food consumption and

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illness were tested by the methods of Mantel and Haenszel (18), the Fisher exact test, and the χ^2 test for significance.

RESULTS

Outbreak investigation. Questionnaires were completed for 121 (93%) of 130 patients at the nursing home and all 33 staff members who worked on the 7:00 a.m.-to-3:00 p.m. and 3:00 p.m.-to-11:00 p.m. shifts. Illness was not reported by staff who worked on the 11:00 p.m.-to-7:00 a.m. shift and they were unavailable to complete questionnaires. Thirty-eight patients (31%) and eight staff (24%) met the case definition, yielding an overall attack rate of 24%. All staff who became ill worked on the 7:00 a.m.-to-3:00 p.m. shift. The median age of ill patients was 86 years, and that of ill staff was 22 years. The illness was similar in the patients and staff and consisted of the sudden onset of cramps followed by profuse watery diarrhea. The median duration of illness was 16 h (range, 12 to 72 h). It was self-limited and required no therapy. There were no reports of fever, bloody stool, headache, nausea, or vomiting. The median incubation period was 9 h (range, 2 to 13 h). The first case was reported to have occurred at 2:00 p.m. on Saturday, and the last case began at 1:00 a.m. on Sunday. No new cases occurred thereafter. There were no complications from the illness among patients and staff, no secondary cases, and no fatalities.

The cases were equally distributed among patients and staff throughout the wards on which regular diets were served. However, on one ward in which only a puree diet (which consisted of different foods) was served, no one became ill.

Food-specific attack rates were calculated for each food served during the weekend and demonstrated that beef stew and rice, which were served at the Saturday noon meal, were significantly associated with illness. When controlled for rice consumption, beef stew alone was significantly associated with illness (Mantel Haenszel $\chi^2 = 17.64$; $P < 0.0002$). Conversely, when controlled for beef stew consumption, rice alone was not significantly associated with illness (Mantel Haenszel $\chi^2 = 0.12$; $P = 0.729$).

Review of the food preparation technique by the Rhode Island Department of Health food sanitarian disclosed that beef for the stew was defrosted for 3 days at 42°F (ca. 5.6°C). At 24 h before the meal the meat was cooked with frozen vegetables and boiled for 4 to 5 h and then cooled uncovered on a stove at room temperature for 3 to 5 h. The stew was then transferred into hotel pans (metal pans, 12 by 18 in. [ca. 30 by 45 cm]), which were left uncovered in a refrigerator overnight. The next morning, the stew was transferred to smaller hotel pans and placed in a large steamer, where it was warmed but not reboiled and served to the patients and staff. None was saved for microbiological analysis.

Isolation of B. cereus. During the period of 21 May through 5 June, a total of 44 stool samples were collected. Stool samples were obtained from 23 ill persons and 21 randomly selected well persons. Large numbers of B. cereus colonies were isolated directly from blood agar plates from the stool of 10 ill persons and 1 well person ($P = 0.0044$, Fisher two-tailed exact test). It was not known whether the one well person with a positive stool consumed the beef stew. B. cereus were isolated from two separate stool samples that were submitted by one ill person 15 days apart. These two stool samples were considered as one for epidemiologic purposes given the time frame of collection. C. perfringens was isolated from the stool of one ill person and seven well persons ($P = 0.032$, Fisher two-tailed exact test). Salmo-
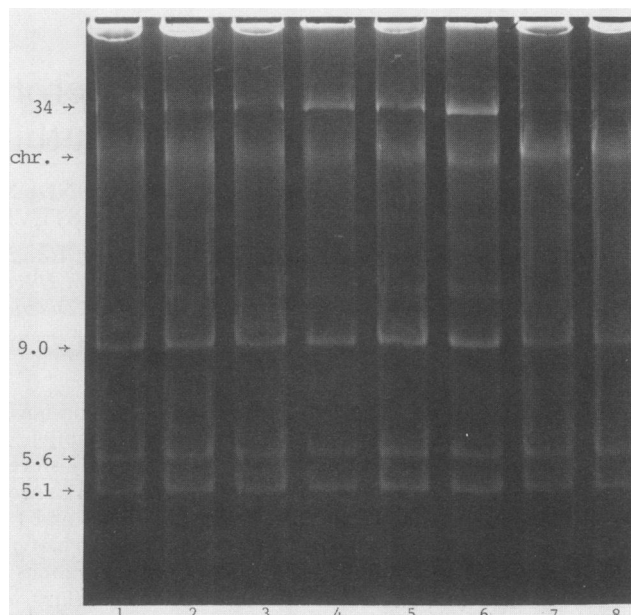

FIG. 1. Agarose gel electrophoresis of plasmid DNA from eight *B. cereus* isolates (lanes 1 to 8) from the outbreak with identical plasmid bands. Lanes: 1, isolate 035 (Table 1); 2, 036; 3, 037; 4, 046; 5, 047; 6, 126; 7, 127; 8, 128. *Escherichia coli* V517 used as a molecular mass standard. The molecular mass is given in megadaltons. chr., Chromosomal DNA.

nella, Shigella, and Campylobacter species were not isolated from any stool samples.

Characterization of B. cereus isolates. All 11 *B. cereus* isolates had identical biochemical profiles; however, none was serotypable initially against the routine set of antisera from the United Kingdom. Rabbit antisera subsequently raised to the outbreak strain showed all isolates to be identical antigenically. This new strain has been designated *B. cereus* serotype H.26.

Enterotoxin assays for each isolate were carried out by using the vascular permeability reaction test in rabbits. All strains elaborated a diarrheagenic toxin. Interestingly, however, testing revealed the existence of two hitherto unsuspected clusters of isolates distinguishable according to the degree of vascular permeability and necrotic activity exhibited in the rabbit model. Using the criteria of Turnbull (27) for the categorization of the skin test response, six isolates elaborated toxin at a category 3 (intermediate) level, and five isolates elaborated toxin at a level of category 5 (very strong). The additional isolate produced two morphological variants on subculture, and these were tested individually. Both strains gave similar biochemical profiles and were found to be of the same antigenic type, H.26. In the enterotoxin assay, however, one of the strains gave a category 3 vascular permeability response and the other gave a category 5 response.

The plasmid profiles of the 11 isolates from this outbreak revealed an identical plasmid profile, with all isolates having four plasmids ranging in molecular mass from 34 to 5.1 megadaltons. One strain (*B. cereus* 027) obtained from an ill patient who ate the beef stew, had an additional plasmid band at 26 megadaltons (Fig. 1). None of 15 reference strains of *B. cereus* isolates from other outbreaks had a similar plasmid profile (Table 1).

TABLE 1. Characteristics of *B. cereus* strains

Strain	Source	Toxin type[a]	Serotype[b]	Plasmid profile (megadaltons)
F4433/1973	R. Gilbert[c]	D	H.2	32, 8.9
F4552/1975	R. Gilbert	E	H.3	8.0
F2769/1977	R. Gilbert	D	NT	42, 13
F4165/1975	R. Gilbert	E	1	38
F3463/1976	R. Gilbert	E	H.1	8.0
F3605/1973	T. J. Lipton[c]	E	H.3	None
F4810/1972	R. Gilbert	E	H.1	32, 8.9
F3502/1973	T. J. Lipton	E, D	H.5	None
F2426/1976	T. J. Lipton	D	H.1	10.2
S247	Itoh, Japan	D	ND	10.1
038-1	R. Bennett[d]	D	ND	None
038-2	R. Bennett	D	ND	None
22	R. Bennett	ND	ND	None
2224	R. Bennett	ND	ND	None
027	Current outbreak	D	H.26	34, 26, 9, 5.6, 5.1
035	Current outbreak	D	H.26	34, 9, 5.6, 5.1
036	Current outbreak	D	H.26	36, 9, 5.6, 5.1
037	Current outbreak	D	H.26	36, 9, 5.6, 5.1
046	Current outbreak	D	H.26	36, 9, 5.6, 5.1
047	Current outbreak	D	H.26	36, 9, 5.6, 5.1
126	Current outbreak	D	H.26	36, 9, 5.6, 5.1
127	Current outbreak	D	H.26	36, 9, 5.6, 5.1
128	Current outbreak	D	H.26	36, 9, 5.6, 5.1
130	Current outbreak	D	H.26	36, 9, 5.6, 5.1
134	Current outbreak	D	H.26	36, 9, 5.6, 5.1
146	Current outbreak	D	H.26	36, 9, 5.6, 5.1

[a] D, Diarrheagenic; E, emetic; ND, not determined (food isolate).
[b] NT, Nontypable; ND, not determined.
[c] United Kingdom.
[d] United States.

DISCUSSION

The incubation period, symptoms, duration of illness, vehicle of infection, stool culture results, serotype data, and plasmid analyses demonstrate that this was a food-borne outbreak of gastroenteritis caused by a single strain of *B. cereus*. There was no evidence for person-to-person or secondary spread of the illness, based on geographic mapping of cases and the pattern of the epidemic curve.

B. cereus is a gram-positive, facultatively anaerobic, endospore-forming bacillus that causes two distinct types of food poisoning, an emetic and a diarrheal syndrome, each associated with a separate toxin (30). The diarrheal syndrome has been widely reported in Europe; implicated foods include meat, cooked vegetables, soups, sauces, stews, poultry and desserts (4, 9, 11). In the outbreak that we report, the gastrointestinal illness probably resulted from consumption of beef stew containing *B. cereus,* which was served at the Saturday noon meal. The spores are relatively heat resistant compared with vegetative cells and therefore survive boiling and then germinate and multiply during cooling at room temperature. In this episode, the beef stew was boiled with stock and vegetables and then cooled on a stove at room temperature, probably allowing heat-resistant *B. cereus* spores in the meat to germinate. Storage of foods at room temperature for several hours has been previously described as a situation which lends itself to *B. cereus* multiplication and subsequent food poisoning (12). Steaming the contaminated food before serving, as occurred in this outbreak, does not serve to eliminate the remaining spores. In this outbreak, as in others previously reported, toxin was produced by organisms growing in the digestive tracts of individuals who ingested the spores (6, 19, 26, 30).

It is unclear how the organism entered the stew, but *B. cereus* is common in soil and on vegetation and has been isolated from a wide variety of foods (9). Most likely, organisms were present in the meat before it was ever cooked and the organisms survived food preparation.

One patient who was not ill had a stool specimen that was positive for *B. cereus*. This is not unusual; the organism has been found in the feces of healthy adults. In one survey, 20 to 43% of single fecal specimens from healthy adults and children in the general population were found to contain the organism. Twenty-three serotypes were represented among the strains found, and 50% were nontypable (29). This correlates with the distribution found in various foods and suggests that *B. cereus* can be carried transiently in the gut according to an individual's diet (9). Therefore, an individual such as the well person in this outbreak with a positive stool culture could harbor the organism in his digestive tract without having it cause disease. This may occur because the organism can function as a commensal in the gastrointestinal tract of certain individuals, or there may be a critical inoculum that one must ingest to produce disease. Some authors believe that large numbers of organisms must be present in the ingested food to result in the diarrheal form of *B. cereus* food poisoning (2, 3, 7, 13, 21). In this outbreak, *B. cereus* organisms were recovered in large numbers from direct culture of blood plates, suggesting that a sizable inoculum was present in the stew and/or ingested by those persons who became ill.

To help distinguish background carriage of the organism from infection, one can attempt to serotype *B. cereus* isolates found in stools of ill persons involved in a food-borne outbreak. If the organisms are present because of background carriage, one would expect to find multiple *B. cereus* serotypes represented in stool isolates. However, the finding of serotypic homogeneity among the *B. cereus* strains isolated from stools collected during this food poisoning

episode documents this as a common-source outbreak, despite the unavailability of samples of incriminated food for microbiological analysis. Here, the presence of *B. cereus* in the stools was due to ingestion of contaminated food and not due to background carriage.

The enterotoxin analysis of the isolates in which the presence of a diarrheal toxin was confirmed by the vascular permeability response test demonstrates that all strains isolated from this outbreak elaborated the diarrheagenic toxin. Strains were either very strong or intermediate toxin producers, with corresponding manifestations of vascular permeability and necrosis in the rabbit model. We found therefore that enterotoxin production did not correlate with serotype of plasmid composition in this outbreak, since serotypically identical *B. cereus* isolates with identical plasmid profiles produced toxin in various degrees.

The genetics of *B. cereus* enterotoxin production are not fully understood; however, the ability of serotypically identical *B. cereus* isolates to produce toxins in various degrees raises questions regarding the possibility of genetic mediation of toxin production through episomes or plasmids (8).

The results of this study indicate that plasmid fingerprinting may be of value in investigating outbreaks of *B. cereus* food poisoning. Similar analyses have proven useful in food-borne outbreaks of *C. perfringens* (17). To our knowledge, this technique has not been previously utilized in epidemiologic studies of *B. cereus*. Such an analysis is relatively simple to perform and may prove quite useful in outbreak investigations where isolates are not typable by the usual serotyping system.

ACKNOWLEDGMENTS

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