Use of Pulsed-Field Gel Electrophoresis To Investigate a Pseudo-Outbreak of *Bacillus cereus* in a Pediatric Unit

PETER YUK-FONG LIU,^{1*} SE-CHIN KE,² AND SHUN-LIANG CHEN²

Section of Infectious Diseases¹ and Laboratory of Clinical Microbiology,²
Shalu Tungs' Memorial Hospital, Shalu, Taiwan, Republic of China

Received 8 January 1997/Returned for modification 25 February 1997/Accepted 15 March 1997

Bacillus cereus is a well-known cause of food poisoning. It also causes rare systemic infections, usually in immunocompromised patients. Dissemination of this species in hospitals had been reported. Most of these episodes were pseudo-outbreaks and were usually secondary to equipment or environmental contamination. We report here on the use of pulsed-field gel electrophoresis (PFGE) to analyze a pseudo-outbreak of *B. cereus* in a pediatric unit. Different restriction endonucleases had been tested, and *Sma*I was found to give the best result for PFGE. Among the 26 clinical isolates of *B. cereus* and the type strain of the species, 15 distinct PFGE patterns were distinguished. PFGE after DNA macrorestriction with *Sma*I could clearly differentiate between the epidemiologically related isolates and the unrelated isolates. Because the same epidemic strain of *B. cereus* was isolated from the settle plates which were exposed near the outlet of the ventilation system, the source of this pseudo-outbreak was suspected to be the unit's air filtration system. This is one of the first reports of the application of PFGE to the study of *B. cereus*, and this method is useful for epidemiological investigation.

Bacillus cereus, a gram-positive, rod-shaped, spore-forming aerobe is widely distributed in nature and is frequently considered a saprophyte or contaminant when recovered from cultures of different sources. Only in relatively recent times has the potential of *B. cereus* as a systemic pathogen come to be more widely recognized and of concern (5, 16). The most common infection caused by this species is food poisoning (1, 4). The rare nongastrointestinal infections seen are associated with wound injuries and burns, hemodialysis, immunocompromised hosts, parenteral drug abuse, blood transfusion, and spinal anesthesia (5). Dissemination of this species in maternity units (18) and intensive care units (2) has been reported previously. Most of these episodes were pseudo-outbreaks and were usually secondary to equipment or environmental contamination (6, 8, 11, 13, 17). A pseudo-outbreak is a situation in which an organism is recovered in culture at a rate that is greater than expected and that cannot be correlated clinically with the supposed infection implied by the culture results (9). Recognizing and tracking the source of such pseudo-outbreaks can be a difficult task (9, 11). This situation may result from systematic extrinsic contamination during specimen collection or processing or intrinsic contamination at the time that the culture medium is manufactured or prepared (12). For tracing the source of contamination, molecular analysis, such as pulsed-field gel electrophoresis (PFGE), has been applied successfully to investigations of pseudo-outbreaks (3, 10, 12). PFGE had been tried by Harrell et al. (7) to differentiate *Bacillus anthracis* from related species and among strains of *B. anthracis*. They found that PFGE may be useful in separating *B. anthracis* from closely related species, but more sensitive methods are needed for identification of strains of this species.

We report here on the use of PFGE as an adjunct in a clinical epidemiological investigation of a pseudo-outbreak due to *B. cereus*.

MATERIALS AND METHODS

Background and pseudo-outbreak investigation. In early August 1996, the infection control committee in a 450-bed tertiary care hospital became aware that *B. cereus* strains were being isolated at an unusual frequency from the pediatric unit. Between August and October, the proportion of positive blood cultures that were identified as *B. cereus* was 50% and was higher than that identified in the previous 6 months (11%). Accordingly, an investigation was begun. Epidemiological and clinical data on culture-positive patients was gathered as soon as an increase in the number of positive specimens was noted, and the clinical isolates of *B. cereus* were stored in the laboratory. Examination of each patient's clinical status through consultations with their attending physicians suggested contamination rather than true infection with this organism. Because the unusual frequency of isolation of *B. cereus* occurred only in the pediatric unit, it was speculated that the contamination might occur within the pediatric unit, not the microbiology laboratory. In view of the widespread dispersal of the organism, extensive environmental sampling (including patients' skin, bedding, disinfectants, and the tops of blood culture bottles) was performed in the pediatric unit. Settle plates were exposed for 4 h in different areas of the pediatric unit, especially near the outlet of the ventilation system. They were also placed in the microbiology laboratory and randomly selected general wards in this hospital.

Bacterial strains. Eleven clinical isolates of *B. cereus* isolated from blood, pus, and sputum of 11 pediatric patients during the pseudo-outbreak period which occurred between August 1996 and October 1996 were selected for this study. Representative *B. cereus* isolates collected from environmental investigations were also included. Seven isolates collected from different wards of this hospital and five isolates collected from another hospital were selected as epidemiologically unrelated controls. The type strain of the species ATCC 11778 was also included in this study for comparison. Suspected *B. cereus* isolates were subcultured onto phenol red-mannitol-egg yolk medium (Oxoid Ltd., Basingstoke, United Kingdom). Strains found to be lecithinase positive and mannitol negative after overnight incubation at 37°C were confirmed to be *B. cereus* by conventional biochemical tests (15).

PFGE. Genomic DNA was prepared by the technique of Harrell et al. (7), with some modifications. A 5-ml early-log-phase culture was grown in Trypticase soy broth (Difco Laboratories, Detroit, Mich.) (shaking at 37°C for 3 h). Fifteen milliliters of SE buffer (75 mM NaCl, 25 mM EDTA [pH 7.5]) was added, and the tubes were centrifuged at 3,000 × g to pellet the cells. The cell pellet was resuspended in 0.5 ml of SE buffer containing 1 mg of lysozyme (Sigma Chemical Co., St. Louis, Mo.) and 10 U of lysostaphin (Sigma). This bacterial suspension was then mixed with 0.5 ml of 2% Low-Melting agarose (Bio-Rad Laboratories, Richmond, Calif.), dispensed in a plug mold (Bio-Rad Laboratories), and allowed to solidify. For lysis, the resulting plugs were then placed in a mixture of 10 mM Tris-HCl (pH 7.6), 100 mM EDTA, 1 mM NaCl, 0.2% sodium deoxycholate, 0.5% sodium lauryl sarcosine, 0.5 mg of lysozyme per ml, and 5 U of lysostaphin per ml. Following overnight incubation at 37°C, the plugs were transferred to a solution which contained 1% sodium lauryl sarcosine, 0.5 M EDTA (pH 9.5), and 500 µg of proteinase K per ml, and the mixture was incubated for 2 days at 56°C under gentle shaking. The plugs were washed once for 1 h at room temperature in TE buffer (10 mM Tris-HCl [pH 7.5], 10 mM

*Corresponding author. Mailing address: Number 30, Ching-Cheng 20th St., Taichung, Taiwan. Phone and Fax: 886-4-2595425. E-mail: liu712@tc.globalnet.com.tw.

TABLE 1. Phenotypic and genotypic characteristics of epidemiologically related and unrelated isolates of *B. cereus*

Isolate group and no.	Date of isolation (mo/day/yr)	Source	PFGE genotype ^a
Epidemiologically related isolates from hospital A (pediatric unit)			
S1	8/25/1996	Blood	A
S2	9/1/1996	Blood	B
S3	9/19/1996	Blood	C
S4	9/23/1996	Blood	A
S5	9/23/1996	Blood	A
S6	9/24/1996	Blood	A
S7	9/28/1996	Blood	C
S8	9/30/1996	Blood	A
S9	10/26/1996	Pus	A
S10	10/28/1996	Blood	A
S11	10/31/1996	Sputum	A
S12	10/15/1996	Settle plate 1	A
S13	10/15/1996	Settle plate 2	A
S14	10/15/1996	Settle plate 3	A
Epidemiologically unrelated isolates from hospital A			
S15	8/22/1996	Pus	D
S16	8/25/1996	Spinal fluid	A
S17	9/4/1996	Pus	E
S18	9/11/1996	Blood	F
S19	9/24/1996	Pus	G
S20	10/3/1996	Pus	H
S21	10/4/1996	Pus	I
Epidemiologically unrelated isolates from hospital B			
S22	9/15/1995	Eye aspirate	J
S23	3/7/1996	Pleural fluid	K
S24	5/1/1996	Blood	L
S25	9/25/1996	Blood	M
S26	1/8/1996	Blood	N
Type strain, ATCC 11778			O

^a PFGE after digestion of DNA with *Sma*I.

EDTA), once for 1 h at 37°C in TE buffer containing 1 mM phenylmethylsulfonyl fluoride (Sigma), and twice for 1 h at 4°C in TE buffer. A slice of each plug (2.5 mm) was cut out and was incubated overnight with 20 U of each restriction endonuclease (each from GIBCO-BRL Laboratories, Grand Island, N.Y.) with the buffers and the reaction conditions recommended by the manufacturer. The restriction endonucleases tried in this study included *Xba*I, *Dra*I, *Ssp*I, *Sma*I, *Apa*I, *Sfi*I, and *Not*I. They were then loaded into the wells of 1.2% SeaKem GTG agarose (FMC Bioproducts, Rockland, Maine) in 0.5× TBE buffer (Tris, borate, EDTA). Electrophoresis was done in a Bio-Rad contour-clamped homogeneous electric field (CHEF-DRII) apparatus for 18 h at 14°C, with an electric field of 6 V/cm, and the pulse time was increased from 1 to 25 s. A bacteriophage lambda ladder (Bio-Rad Laboratories) was used as the molecular weight marker. The reproducibility of the PFGE fingerprinting was examined by three different tests. PFGE chromosomal fingerprints were compared by use of the criteria of Tenover et al. (14).

RESULTS AND DISCUSSION

In this study, we found that *Sma*I is the most suitable restriction endonuclease for digestion of *B. cereus* DNA in a PFGE typing system. Minimal cutting of *B. cereus* DNA by *Dra*I, *Ssp*I, *Apa*I, and *Spe*I and overactive cutting of *B. cereus* DNA by *Xba*I were found. Macrorestriction of *B. cereus* DNA by either *Not*I or *Sfi*I produced fewer than seven bands by PFGE. The discriminatory power of PFGE provided by these two enzymes thus was as poor as expected (data not shown).

Table 1 lists the epidemiological data and the PFGE pat-

FIG. 1. PFGE of *Sma*I-digested genomic DNA from epidemiologically related *B. cereus* isolates. Lane M, bacteriophage lambda ladder (Bio-Rad), which served as a molecular size marker; lanes 1 to 14, DNA digests of isolates S1 to S14, respectively (see Table 1 for the origins of the isolates).

terns for the isolates of *B. cereus* included in this study. Colonies of *B. cereus* were only found on the settle plates that had been exposed in the pediatric unit. Isolates from three of these plates, which had been put near the outlet of the ventilation system in the pediatric unit, were selected for the molecular analysis. No *B. cereus* was isolated from settle plates that had been put in other hospital units or the microbiology laboratory. *B. cereus* was also isolated from the skin of most patients hospitalized in the pediatric unit. Some of the bedding was also contaminated with this microorganism, but the disinfectants and the tops of the blood culture bottles were not.

The fingerprints generated by macrorestriction with *Sma*I comprised approximately 15 to 25 bands of approximately 5 to 500 kb (Fig. 1 and 2). According to the interpretive criteria of Tenover et al. (14), 15 distinct PFGE patterns were distinguished among these 26 clinical isolates of *B. cereus* and the type strain of the species. PFGE after macrorestriction of the DNA with *Sma*I could clearly differentiate between the epidemiologically related isolates and the unrelated isolates. Hence, the discriminatory power of PFGE typing for *B. cereus* is satisfactory. Minor band differences (fewer than three bands) were noted among the epidemic strains (isolates S1, S4 to S6, and S8 to 14). However, according to the criteria of Tenover et al. (14), they were considered to be epidemiologically closely related. The PFGE patterns of each isolate on 3 different days were consistent. Hence, the reproducibility of PFGE analysis of *B. cereus* is good. The problem of preparation of chromosomal DNA for macrorestriction is the sporulation of *B. cereus*.

FIG. 2. PFGE of *Sma*I-digested genomic DNA from epidemiologically unrelated *B. cereus* isolates. Lane M, bacteriophage lambda ladder (Bio-Rad), which served as a molecular size marker; lane 1, DNA digest of type strain ATCC 11778; lanes 2 to 13, DNA digests of isolates S15 to S26, respectively (see Table 1 for the origins of the isolates).

However, this can be prevented by using a fresh early-log-phase culture for DNA extraction. Harrell et al. (7) mentioned the use of ampicillin-sulbactam to lyse the cell walls of *Bacillus* species during extraction of chromosomal DNA. However, we found that this step is not really essential.

The predominant PFGE genotype was type A in the pediatric unit. This epidemic strain was also isolated from the three settle plates which had been exposed to the outlet of the ventilation system in the pediatric unit. It was possible that the epidemic strain of *B. cereus* had contaminated the unit's air filtration system. The spores of this strain might then disseminate in the air of the unit and contaminate the skin of the patients. Youngs et al. (18) reported a similar *B. cereus* outbreak in a maternity unit. The primary source of the organism, although suspected to be contamination of the unit's air filtration system, could not positively be established (18). We tried to control the dissemination of this epidemic strain by cleaning the ventilation system of the pediatric unit. This was difficult to achieve because the system in this unit was old, and the filters did not work very well and could not be changed. However, the isolation of *B. cereus* decreased after November when we encouraged hand washing and the use of aseptic procedures in this pediatric unit.

In conclusion, the PFGE technique with *Sma*I for DNA macrorestriction was found by this study to be a highly discriminatory and reproducible method for the epidemiological investigation of *B. cereus* infection. To our knowledge, this is one of the first reports of the use of PFGE for typing this species. This typing method can facilitate the reliable evaluation of the clonal relationship of *B. cereus* isolates and the identification of the common sources of outbreaks or pseudo-outbreaks. However, since the results of this study are based on an analysis of only a small number of isolates, further studies involving much larger numbers of epidemiologically unrelated strains will be required in order to validate the results presented above.

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