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Original Article

Application of pulsed-field gel electrophoresis (PFGE) in Bacillus cereus typing

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Abstract: Bacillus cereus (B. cereus) is an opportunistic pathogen. B. cereus food poisoning can cause diarrhea and even meningitis. B. cereus can be isolated from infant food, effective typing of B. cereus is helpful in tracking of pollution sources. In this study, PFGE was used for typing B. cereus isolated from infant milk powder and rice flour from different regions and different time points. The PFGE typing and homology analysis of 52 B. cereus were analyzed to provide scientific basis for disease control. 52 B. cereus were isolated and lysed to extract DNA. Genomic DNA was digested with Not I restriction enzyme, followed by pulsed-field gel electrophoresis. Cluster analysis is performed using unweighted pair group method with arithmetic mean (Bionumerics 5.10). Different concentrations of lysozyme and treatment time were used to explore the optimum process conditions. Not I restriction enzyme digestion resulted 8-155-500 kb bands. Using 100% similarity criteria, 52 strains can be divided into 47 different PFGE types, no dominant PFGE type was found. There are five strains from different manufacturers showed the same PFGE type. The PFGE typing methods established in this study showed high repeatability and high sensitivity, providing an effective technique for tracking of pollution sources of B. cereus-induced foodborne diseases and hospital infections.

Keywords: Bacillus cereus, PFGE typing, epidemiology

Introduction

B. cereus is an opportunistic pathogen. It is generally believed that the B. cereus will cause light vomiting and diarrhea. No enough attention has been given to B. cereus worldwide. But in recent years, food poisoning caused by the B. cereus increases year by year with main symptoms of nausea, headache, vomiting, abdominal pain, watery diarrhea, and tenesmus. Diarrhea caused B. cereus accounts for 3.5% [1]. B. cereus can also cause more serious diseases, such as meningitis [2-5], and even death [4, 5].

Pulsed-field gel electrophoresis (PFGE) can be used to analyze large DNA fragments of the bacterial chromosome with high resolution, high repeatability, and good comparability, and is recognized as the gold standard for bacterial typing [6, 7]. Analyzing the strains isolated from patients, food, or environment using PFGE can help us to conduct epidemiological traceability, pinpoint the source of disease outbreaks, take effective control measures, and provide a scientific basis for disease control.

Therefore, PFGE methods for typing B. cereus is need to analyze B. cereus isolated from different regions and different food to track and trace its origin and provide scientific basis for controlling the disease.

Material

Strains

The 52 B. cereus stains isolated from infant food were kept by our laboratory. Salmonella standard H9812 was purchased from Chinese

Center for Disease Control and Prevention. Bacillus thuringiensis was purchased from Fujian Agriculture and Forestry University.

Instruments

Pulsed field gel electrophoresis system (CHEF MAPPERTM) and gel imaging system (BIO RAD Gel DocTMXR +) were purchased from Bio-Rad. Turbidity meter (WGZ-1M12100) was purchased from Shanghai Precision. Refrigerated centrifuge was purchased from Eppendorf.

Reagents

Restrictive enzyme was purchased from TaKaRa. Lysozyme was purchased from Sigma. Proteinase K was purchased from Meck. Gold Agorose was purchased from Seakem Gold Agorose.

Methods

Banerjee's method with optimized experimental conditions was used in this study [1]. NotI restriction enzyme was used to digest experimental strains and XbaI restriction enzyme was used to digest standard strain H9812.

Preparation of gel block

Preparation of the bacterial suspension: Lessons 2 mL TE buffer in a tube, scraped with A sterile cotton swab was used to scrape bacterial cultures on the plate and 2 mL TE buffer was used to suspend the bacterial cultures to a concentration of 5.0 measured by Maxwell turbidity meteor.

Lysozyme lysis: 300 μL of bacterial suspension was removed to a 1.5 mL centrifuge tube, mixed with 6 μl of lysozyme, and incubated in 37°C water bath for 10~20 min.

Protease K lysis: After removal tubes out of water bath, 15 μL of proteinase K was added to each tube, mix gently, then immediately cast gel.

Preparation of gel: 300 μL of dissolved 1% SeaKem gold agarose was mixed with 300 μL lysed bacterial suspension, then quickly injected into the gel plug and allowed to stand at room temperature for 15 min until agarose solidified.

Cell lysis in gel

Preparation of lysozyme solution: 25 μl of lysozyme was added to 5 ml bacteria suspension and mixed by inversion, each dispensing tube 5 ml in 50 ml centrifuge tubes.

lysozyme lysis: Carefully transfer gel blocks to tubes with lysozyme solution, incubated at 37°C water bath with shaking speed of 150 rpm for 4~6 h.

Preparation of protease K solution: 5 μl of CLB and 30 μl of protease K were added to each bacteria suspension and mixed by inversion.

Protease K lysis: The lysozyme-lysed gel blocks were moved to tubes containing protease K and incubated at 54°C water bath overnight with shaking speed of 150 rpm.

Wash of gel blocks

15 ml of preheated water was added to each tube and incubated in 50°C water bath shaker for 10 min. Discard water, 15 ml of preheated TE was added to each tube and incubated in 50°C water bath shaker for 10 min. Discard TE, 5 ml of TE was added to each tube and stored at 4°C.

DNA digestion in gel

2 mm wide gel pieces containing DNA were cut with a razor blade, placed into 1.5 ml centrifuge tubes, and mixed with 200 μl TE. Tubes containing XbaI and NotI were incubated at 37°C water bath. Tubes containing SmaI were incubated at 30°C water bath for 10~15 min. After incubation, 200 μl endonuclease mixture was added to each tube. Tubes with XbaI and NotI were incubated at 37°C; rubes with SmaI were incubated at 30°C for 3 h.

Electrophoresis setting

The initial conversion time was 5 s, terminal switching time was 80 s, voltage drop was 6 V, field angle was 120°, and electrophoresis time were 19 hrs.

image acquisition and data analysis

Gels were removed, placed in trays with 500 ml of Gelred solution, and stained for 20~30 min. Then de-stained with 500ml water for 60~90

Table 1. Optimal dosage and duration of action for cell lysis and digestion

Enzyme	Concentration	Volume (uL)	Time (h)
lysozyme	0.1 mg/ul	25	6
Proteinase K	0.02 mg/ul	20	18
XbaI	20 U/ul	3.0	3
SmaI	10 U/μl	5.0	3
NotI	10 U/μl	2.0	3

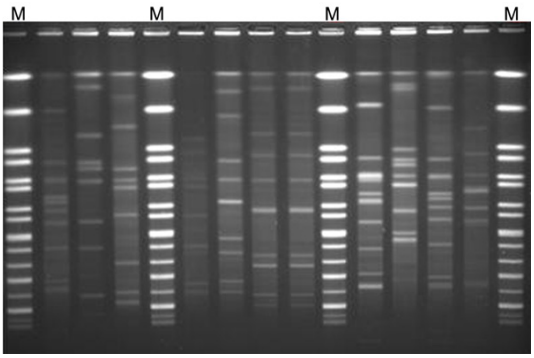

Figure 1. PFGE images of 11 B. cereus strains digested with Not I.

min with changing of water every 20~30 min. Capture images with BIO RAD Gel DocTMXR +.

Data analysis: Bionumerics 5.10 was used to process PFGE images. Images of Salmonella H9812 were used as a marker to calibrate images' position, manual correction if necessary. Cluster analysis is performed using unweighted pair group method with arithmetic mean. The tolerance of band positional difference was 1.0%. The similarity of different electrophoretic bands of different strains was represented by Dice coefficient.

Results

Different concentrations of lysozyme and different treatment time were used to lyse B. cereus. Results showed that 25 μl (0.1 mg/μl) of lysozyme and a reaction time of 6h gave the best results (**Table 1**).

After 18~19 hours' NotI digestion and electrophoresis, B. cereus showed 8 to 15 DNA fragments with molecular weight of 5~50 Kb (**Figure 1**). Cluster analysis of 52 B. cereus strains was shown in **Figure 2**.

A total of 52 multiple B. cereus strains were PFGE typed at different time points, the results test results showed high repeatability. Cluster analysis of 52 B. cereus strains showed that there are 47 different PFGE types. The similarity of #21, #23, #44, #45, and #46 was 100%. The similarity of #7 and #12 was 100%. The PFGE typing of other strains showed certain differences, indicating that there is a high degree of genetic diversity in B. cereus isolated from food.

Discussion

Cluster analysis of 52 B. cereus strains showed that there was a high degree of genetic diversity. B. cereus strains isolated from food of different area showed no aggregation. The similarity of #21, #23, #44, #45, and #46 was 100%. The similarity of #7 and #12 was 100%. Epidemiological analysis showed that #21 and #46 were isolated from different batches of infant milk powder made by the same food factory in Fujian Province. There were 1 year difference between sampling date and 7 months difference between production date (sampling date: March 22, 2011 for #21 and April 24, 2012 for #46; production date: October 25, 2010 for #21 and May 10, 2011 for #46). #23 and #44 were isolated from different batches of infant milk powder made by the same food factory in Inner Mongolia. There were 9 months difference between sampling date and 10 months difference between production dates. This suggested that a certain step of the process was contaminated by B. cereus and no effective disinfection measures were taken, leading to the long-term survival of B. cereus in the equipment. (sampling date: March 22, 2011 and December 20, 2011; production date: November 17, 2010 and September 14, 2011). #45 was isolated from infant milk powder made by a food factory in Shandong. The similarity of PFGE typing of #21, #23, #44, #45, and #46 was 100%. #7 and #12 were isolated from rice flour. #7 was isolated from rice flour made by a food factory in Zhejiang. #12 was isolated from rice flour made by a food factory in Guodong. Whether the two food factories used same raw materials needed for further investigation. All the data suggested that PFGE typing of different strains of B. cereus can be used to trace the sources of pollution [8, 9].

To control B. cereus contamination in food processing and prevent B. cereus proliferation and toxin production in the harvest, transportation,

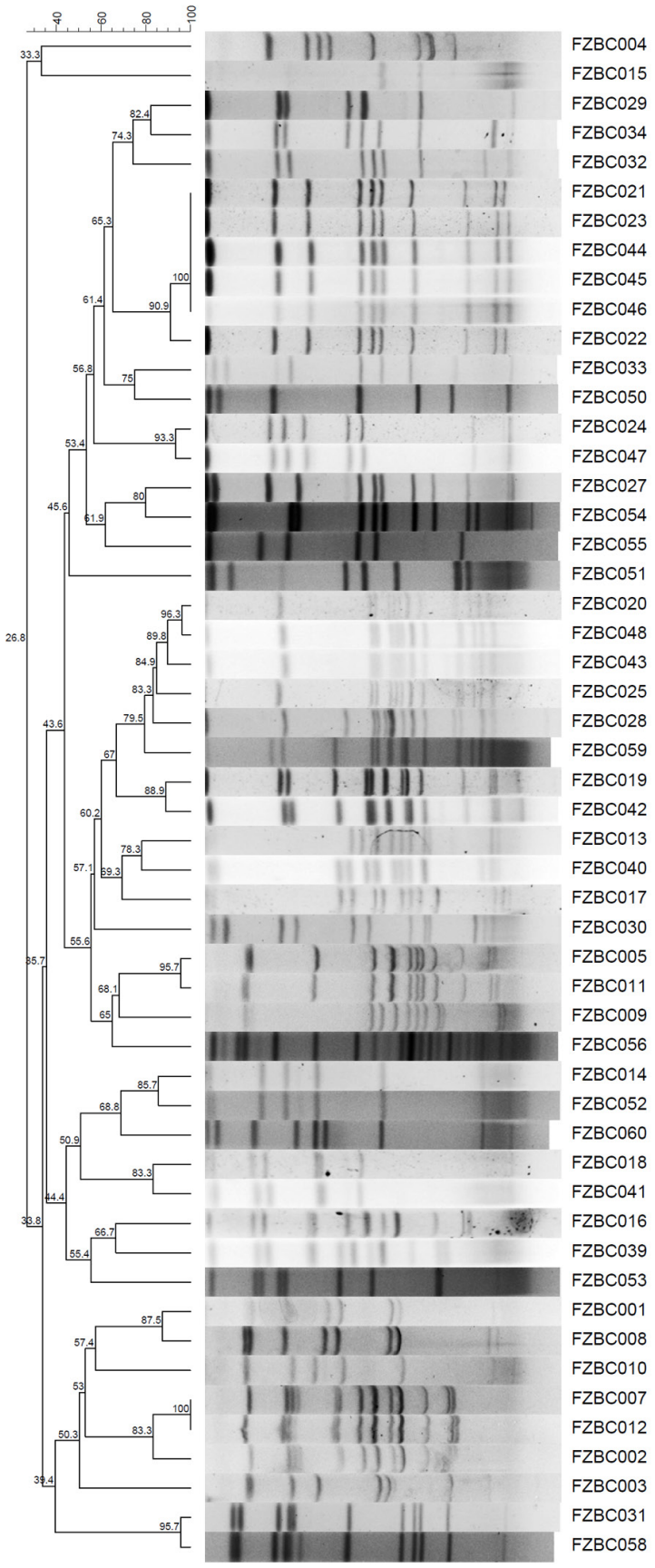

Figure 2. Cluster analysis of 52 B. cereus strains digested with Not I.

and storage process of food, food companies need to strengthen the supervision and guidance, make sure the companies strictly follows good agricultural practices (GAPs), good manufacturing practices (GMPs), and hazard analysis and critical control point (HACCP). In addition, the health condition of raw materials, the use of additives, and sterilization of indoor air and equipment are also crucial to reduce the number of microorganisms in baby food.

At present, there are few reports on PFGE typing of B. cereus in China. The reason is that the condition is B. cereus is an opportunistic pathogen with cell wall which is difficult to be lysed. Some scholars inoculated B. cereus into LB broth and incubated for 3 hours [10], such that only a small number of spores formed and the lysis of B. cereus was relatively easy. But this method needs long time incubation with lysozyme, lysostaphin, and proteinase K, and needs long time to clean gel block. All these inhibited the practical application of this approach.

In this study, PFGE typing of B. cereus was established. The concentration and duration time of lysozyme, proteinase K, and Not I were optimized through several tests. The results showed that the incubation time of restriction endonucleases is critical. Compared with Salmonella, B. cereus needs longer digestion time [11].

The concentration and uniformity of the bacterial suspension will affect the quality of the gel block. So when making bacterial suspension, good quality cotton swab should be used to mix the bacteria culture with liquid before measuring the turbidity. Too much bacteria can result in uncompleted lysis, too little bacteria can cause faint DNA bands after electrophoresis [11-15].

Incubation of B. cereus with different endonuclease results in different DNA fragments. XbaI incubation results in 13-15 DNA fragments with relatively larger molecular weights, which will be concentration the in the first half of the gel block and unfavorable for typing. Sma I incubation results in 23-15 DNA fragments with similar molecular weights, which cannot be separated very well after electrophoresis and is unfavorable for typing. Not I enzyme digestion results in 8-15 DNA fragments with different molecular weights which can be clearly separate very well after electrophoresis [16-20].

Serotyping and biochemical methods are commonly used typing methods for microbe. These phenotyping methods have many limitations and can be influenced by many factors. The use of serum or reagents from different manufacturers may give inconsistent results. Typing results can be affected by different personnel or pathogens' physiology [6]. PFGE typing recognizes large molecular weight DNA fragments with high resolution, high repeatability, and good comparability. It is recognized as the gold standard for bacterial typing [7]. Analyzing the strains isolated from patients, food, or environment using PFGE typing can help us to conduct epidemiological traceability and provide a scientific basis for disease control.

Conclusion

PFGE can be used to effectively analyze the type of B. cereus and is of significance in epidemiology and disease control.

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Disclosure of conflict of interest

None.

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