Letter to the Editor

Optimization of Pulsed-field Gel Electrophoresis Procedure for *Bacillus cereus*[*]

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In order to develop a rapid and reliable method for *B. cereus* genotyping, factors influencing PFGE results, including preparation of bacterial cells embedded in agarose, lysis of embedded cells, enzymatic digestion of intact genomic DNA, and electrophoresis parameters allowing for reproducible and meaningful DNA fragment separation, were controlled. Optimal cellular growth (Luria-Bertani agar plates for 12-18 h) and lysis conditions (4 h incubation with 500 μg/mL lysozyme) produced sharp bands on the gel. Restriction enzyme *Not*I was chosen as the most suitable. Twenty-two isolates were analyzed by *Not*I digestion, using three electrophoretic parameters (EPs). The EP-a was optimal for distinguishing between isolates. The optimized protocol could be completed within 40 h which is a significant improvement over the previous methods.

Bacillus cereus is an aerobic, spore-forming, Gram-positive bacillus with close phenotypic and genetic relationships to several other *Bacillus* species, especially *B. anthracis*[1]. *B. cereus* is widely distributed in nature, largely because of the resistance of the bacterial endospore to various environmental stresses, which contribute to their long-term survival under unfavorable conditions. The natural environmental reservoirs of *B. cereus* include decaying organic matter, fresh and marine waters, vegetables, and fomites. *B. cereus* is recognized as an important etiological agent in food-borne infectious disease outbreaks in Europe and China[2]. Outbreaks are often associated with boiled rice being left overnight at room temperature, rather than in the refrigerator. Two main types of *B. cereus* food poisoning have been described: emetic and diarrhea. What's more, it is reported that this pathogen can also infect both immunologically compromised and immunocompetent individuals and cause systemic and local infections.

Because of its ubiquitous nature, genetic differentiation among isolates of *B. cereus* is necessary to identify, and also rule-out potential sources of contamination, and to determine routes of transmission. Development of a reliable and efficient molecular typing strategy would allow rapid detection of outbreaks and prevent the spread of *B. cereus* infections. An international molecular typing network for foodborne disease surveillance, PulseNet, has been established, and has successfully detected numerous multistate (USA) and international food-borne infectious disease outbreaks . The China CDC became a member of PulseNet International in 2004, and PFGE along with other molecular sub-typing methods have been widely used to detect outbreaks and trace pathogen sources in China[3].

A standardized PFGE protocol that could be universally implemented would assist with local, national, and international outbreak investigations by allowing increased confidence in source tracking of contamination and determination of routes of transmission. An optimal PFGE protocol produces a suitable number of large-molecular-weight restriction fragments that, when separated, provide distinct patterns for genetically unique strains

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following agarose separation. Several PFGE protocols related to *B. cereus* have been described previously[4-8]. *Not*I and *Sma*I are the most frequently used enzymes in these PFGE protocols. However, most laboratories using PFGE to subtype *B. cereus* cannot compare their results because the protocols used vary at critical parameters, including the restriction enzymes used and the electrophoresis conditions. As a result, there is currently no universally accepted protocol and thus no international database of *B. cereus* PFGE patterns.

In this study, key factors influencing band number, pattern, and quality were addressed in the development of a reliable and robust PFGE method for *B. cereus*. Variations in culture conditions, lysis time, restriction enzymes, and electrophoresis parameters were investigated to establish the most robust method for subtyping *B. cereus* isolates.

In total, 22 *B. cereus* isolates were examined in this study, including 14 isolates recovered from foodstuffs and 8 isolates from soil from various locations in China. All isolates were cultivated on Luria-Bertani (LB) agar medium. Three isolates of the strains were used to optimize the culture medium for bacterial growth, lysis of the bacterial cell wall, and enzyme selection. *Salmonella enterica* serotype *Braenderup* H9812 was used as a DNA size marker, as recommended by PulseNet.

Bacteria were grown in LB broth medium or streaked onto LB agar plates for 12-18 h at 37 °C. The fresh culture was then suspended in 2 mL of TE buffer (10 mmol/L Tris, 1 mmol/L EDTA, pH 8.0) and adjusted to an optical density of 5.0-5.5 using a Densimat (bioMérieux, France). Lysozyme (2 mg/mL) (Amresco Inc., Solon, OH, USA) was added to the cell suspension and incubated for 10-20 min at 37 °C. Following incubation, proteinase K (1 mg/mL)(Merck, Darmstadt, Germany) was added, and 300 µL of the mixture were immediately added to 300 µL of Seakem Gold Agarose (Lonza, Rockland, ME, USA) and pipetted into plug molds (Bio-Rad, Hercules, CA, USA). Plugs were incubated for 2-16 h in 5 mL of TE buffer containing lysozyme (0.5 mg/mL) at 37 °C, and then for a further 2 h at 54 °C in 5 mL of CLB buffer (10 mmol/L Tris, 1% sarcosyl, 50 mmol/L EDTA, pH 8.0) containing proteinase K (75 µg/mL). Each agarose plug was subsequently washed four times at 50 °C with gentle shaking, followed by a single wash for 10 min in ultrapure water, and three washes (each 15 min) in TE buffer. A slice of each plug (2.5 mm) was cut and then incubated for 3 h with 20 U of each restriction endonuclease, using buffers and reaction conditions recommended by the manufacturer. The restriction endonucleases examined in this study were *Xba*I, *Nhe*I, *Mlu*I, *Sma*I, *Apa*I, *Bln*I, *Spe*I, and *Not*I (TaKaRa Bio, Dalian, China). Electrophoresis was performed using a CHEF-DRIII system (Bio-Rad). Images were captured on a Gel Doc 2000 system (Bio-Rad) and converted to TIFF files for computer analysis. Plugs of *S. Braenderup* strain H9812 were prepared and digested along with the test isolates, using 40 U/slice *Xba*I. All electrophoresis steps were conducted with a voltage gradient of 6 V/cm, an included angle of 120°, and a linear ramp.

Solid and liquid media were compared to find the most appropriate medium for producing clear DNA fragments. Bacteria were streaked onto LB agar plates and typical colonies were picked and subcultured on LB agar plates or in LB liquid medium, respectively. Bacteria were incubated for 12-18 h at 37 °C, and subsequently 5 µL of an overnight liquid culture were inoculated in 5 mL of LB liquid medium and incubated at 37 °C for another 3 h.

For lysis, bacterial suspensions including lysozyme (final concentration: 2 mg/mL) were incubated for 10-20 min at 37 °C prior to adding agarose and being dispensed into a plug mold. Resulting agarose plugs containing bacteria cells were placed in a mixture of TE buffer and lysozyme and incubated for 2 h, 4 h, or 16 h under gentle shaking.

Restriction enzymes were selected using DNASTAR 5.01 software (DNASTAR, Inc., Madison, USA), with analyses based on available *B. cereus* whole genome sequences (GenBank accession numbers NC003909, NC004722, NC006274, and NC011773). A pilot test using three isolates was conducted using PFGE, and the optimal enzyme was selected based on the distribution of the bands. The candidate enzyme was then further evaluated by PFGE using all 22 *B. cereus* isolates.

Twenty-two isolates were analyzed following *Not*I digestion using three different EPs, named EP-a, EP-b, and EP-c. EP-a is carried out for 19 h with pulse from 5 to 80 s as described in previous literature[4]. EP-b is last for 19 h with pulse from 2.4 to 60.8 s. EP-c is last for 20 h with pulse from 2.4 to 60.8 s for 16 h and followed from 1 to 25 s for 4 h. The Simpson diversity index (*D*-value)[9] and similarity coefficients were used to compare the discriminatory powers of the three methods. The *D*-value was calculated by the equation $D=1-(\sum[n_j(n_j-1)])/[N(N-1)]$, where N is the total