Differentiation by Molecular Typing of *Bacillus Cereus* Isolates from Food in Morocco: PFGE-Eric PCR

S. Merzougui[1,2], M. Lkhider[2], N. Grosset[3], M. Gautier[3], N. Cohen[1,*]

[1]Laboratoire de Microbiologie et d'Hygiène des aliments et des eaux, Institut Pasteur du Maroc
[2]Laboratoire de Biotechnologie, Biochimie et Nutrition, Faculté des sciences d'El Jadida, Université Chouaib doukkali, Maroc
[3]Laboratoire de Microbiologie et Hygiène Alimentaire, Agrocampus ouest, Rennes, France

Abstract   *Bacillus cereus* is widespread pathogen. It is widely distributed in several environments such as soil and plants and is commonly isolated from food and additives. In this study we analyzed 18 foodborne B. *cereus* strains isolated in Morocco from food samples (milk and dairy products, rice salade and spices), in order to investigate the genetic diversity (assessed by PFGE and ERIC-PCR). The food samples were collected from hotels (n=38), restaurants (n=32) and private companies (n=26) in several cities in Morocco. The results obtained in this study confirmed the diversity of B. *cereus* strains. The results showed that PFGE analysis has good discriminatory power but it is long to implement, which promotes the use of ERIC-PCR as a complementary tool for molecular typing. However, additional studies involving cytotoxicity tests would be useful to better evaluate this potential and could provide a more accurate indication of the risk.

Keywords   *Bacillus cereus*, ERIC-PCR, Foodborne, Genetic Diversity, Morocco, PFGE

1. Introduction

Bacillus cereus is gram-positive andendospore-forming opportunistic human pathogen, and commonly found in wide range of environments including foods[1], it represents one of the major pathogens because its elimination is not guaranteed by pasteurization and sanitation procedures[2]. Although the virulence of *B. cereus* depends on multifactorial characteristics[3], upon ingestion of contaminated food, it may lead to two types of food poisoning syndromes: emetic and diarrheal[4-5]. Diarrheal syndrome is caused by the production of enterotoxins including hemolysin BL (HBL) and non-hemolytic enterotoxin (NHE), single protein enterotoxins cytotoxin K (CytK), and enterotoxin FM (EntFM)[6]. The symptoms of diarrheal type was abdominal pain and diarrhea, occur 8-16 h after ingestion of contaminated food[7].

The emetic food poisoning is caused by cereulide, a small cyclic heat-stable dodecadepsipeptide toxin[8], the symptoms are characterized by vomiting and nausea, and occur within 1-5 h after ingestion of contaminated food[9].

Different studies demonstrate that biochemical identification of *B. cereus* is not enough for characterisation of *B. cereus* isolates[10], hence the need of molecular methods for characterisation of *B. cereus* group.

Several factors require consideration when selecting a molecular typing strategy. These include discriminatory power, reproducibility and typeability, as well as the biological basis for grouping similar strains, cost, and logistics[11]. Rapid and inexpensive PCR-based typing techniques, such as enterobacterial repetitive intergenic consensus-PCR (ERIC-PCR), can be used to screen for genetic relatedness, while the potentially more discriminatory techniques of pulsed-field gel electrophoresis (PFGE) help confirm results. Several molecular methods have been used to reveal the genetic relationship of the *B. cereus* group species[12-13-14-15-16]. Rapid and reliable molecular subtyping method to epidemiologically trace *B. cereus* is important in monitoring potential bacterial pathogens in foodstuffs[17]. Nevertheless, few studies have been conducted on the genotypic characterization of *B. cereus* isolates from Morocco in terms of foodborne poisoning concerns.

The objectives of this study were to present the genetic diversity of *B. cereus* strains and to compare the effectiveness of PFGE and ERIC-PCR for differentiating the diversity of the *B. cereus* collection.

2. Materials and Methods

2.1. Bacterial Strains and Culture Conditions

A total 18 *B. cereus* strains related to food samples were used for this study. The strains were isolated from milk and dairy products, rice salads and spices. The food samples

were collected from hotels (n=38), restaurants (n=32), and private companies (n=26) in several cities in Morocco (mainly from Casablanca, Tanger, Rabat and Marrakech).

A portion of (10 g) of each sample were homogenized for 1 min with 90 ml of sterile 0.1% (w/v) buffered peptone water (BPW), Bio-Rad (Marne-la-Coquette- France), in a stomacher. Three series of three tubes containing 9 ml of modified PMYP (polymyxin-mannitol-egg yolk-phenol red) selective enrichment broth without mannitol, egg yolk and indicator were prepared[18]. Each series was inoculated with 1 ml per tube from the respective dilution. The tubes were incubated at 30°C for 24 h. The transfer of the positive tube-cultures to selective solid medium PMYPA (Malt, Yeast, Peptone, Agar) from Scharlau Chemie made possible to differentiate characteristics of the *B. cereus* colonies[10]. The cultures were incubated at 30°C for 24 h. Colonies presenting a pink or purple colour with an irregular edge surrounded by a white area were considered as positives and enumerated as described by Valero et al.[10]. The API 50CH galleries were filled, incubated and interpreted according to the instructions specified for each test. The isolates were subjected to a PCR test in order to make sure of their belonging to the *Bacillus cereus* group and kept frozen in a 50 % (v/v) glycerol solution at -20°C.

For this study, all strains were plated on brain heart infusion agar (BHI) and grown at 30°C overnight. In general, cells from one single colony were inoculated in 10 ml BHI-broth.

2.2. PFGE Genotyping

The protocol for the characterization of strains by PFGE is adapted and optimized from previous work[19].

Nine milliliter of cell suspension was centrifuged for 10 min at 7000×g at 4°C. The supernatants were removed and the pellets were resuspended in 1 ml of TE buffer (pH 8; 10 mM Tris, 1.0 mg lysozyme, 1 M NaCl and 50 mM EDTA), partially embedded in 500 µL agarose (Eurobio, les Ulis, France) and then digested with 2.0 mg of lysozyme (Sigma-Aldrich, Saint Louis, MO, USA) and 25 U of lysostaphin (Sigma). The plugs were transferred into a solution containing 0.1 % sodium lauryl sarcosine in 0.5 M EDTA (pH 8), and 200 mg of proteinase K, and the mixture was incubated overnight at 44°C with gentle shaking. DNA was digested with the enzyme *SmaI* (20 U) following migration performed with the CHFE DRII Biorad system (BioRad) in a 1.5 % agarose gel (Ultra-Pure Gibco BRL, Paisley, Ecosse) in 0.5 x TBE buffer at 14°C with a linear ramping time of 2 to 20 s over a period of 18 h, and a gradient of 200 V. After migration the gels were stained with 1 µg/ml ethidium bromide (BET) and photographed under UV light. (Vilber Loumat, Marne-La-Vallée, France).

2.3. ERIC-PCR Genotyping

The primer ERIC1R: 5′ATGTAAGCTCCTGGGGATTCAC3′ was synthesized to amplify the ERIC-PCR fingerprints of *B. cereus*. The PCR (25µl) mixture was described previously by Versalovic et al[20], with a modification that consisted of 200 µM of each dNTP, 1.8 U taq polymerase (Biolabs, USA), 3 mM MgCl2, , 10 mM Tris–HCl (pH=9.0), 1.0 µM primer for ERIC1R, 50 ng DNA templates, and an additional distilled sterile water to 50 µl. The amplification reaction was performed as follows: denatured for 5.0 min at 95°C, followed by 40 cycles of 5.0 min at 94°C, 1.0 min at 55°C, and 2.0 min at 72°C; and a final extension step at 72°C for 10 min. PCR results are visualized with an UV transilluminator on agarose gel 1% (Ultrapure DNA grade agarose, Biorad) containing TBE and 1 µg/ml ethidium bromide after migration to 80 V for 2h to record results[21]. Reproducibility was monitored when warranted by comparing the results of PFGE carried out with the same strains.

3. Results

3.1. PFGE Analysis

After optimization on the protocol of Gautier et al[19], the protocol adopted has the advantage of being easily and feasible in short time (4 days). The *SmaI* macrorestriction profiles of DNA from all isolates tested are shown in Fig. 1. *B. cereus* genomic DNA digested with *SmaI* yielded fragments of approximately 48-388 kb. Eighteen PFGE profiles were founded. The lanes 1 to 18 represent the *B. cereus* strains isolated from different food. These isolates were designated as S1-S18, (isolates S3, S5, S7, S8, S9, S10, S12, S14, S15, S16, S17 and S18 were from milk and dairy products, S2, S4, S11 from spices and S1, S6 and S13 from rice salad). The profiles obtained showed a remarkable polymorphism existing among all strains.
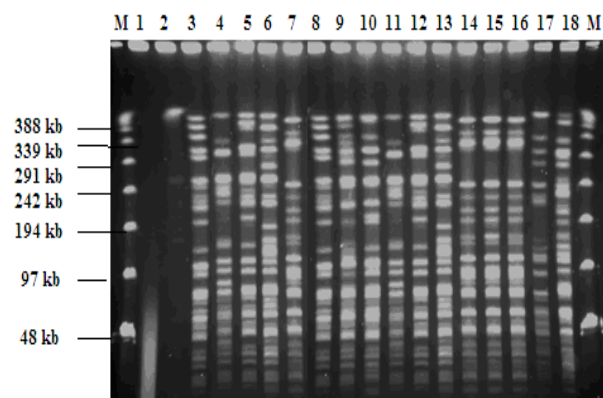
Figure 1.   PFGE separation of *SmaI* digested macrorestriction fragments of genomic DNA of *B. cereus*. M: lambda ladder marker for PFGE (Amersham Biosciences, Piscataway, USA); lanes: 1 to 18 represent the *B. cereus* strains isolated from food: milk and dairy products (lanes 3, 5, 7, 8, 9, 10, 12, 14, 15, 16, 17 and 18), spices (lanes 2, 4, and 11) and rice salad (lanes 1, 6 and 13)

S6 and and S13 had identical *SmaI* PFGE profiles; also, S4 and S11 had identical profiles, while the *SmaI* profiles of *B. cereus* 7, 14, 15 and 16 could not be distinguished from each other, but nevertheless differed from those of the previous group. All the other *SmaI* PFGE profiles of the remaining *B.*

cereus strains were different except S3 with S8 and S5 with S12.

The results obtained showed that there was visible correlation between PFGE types and sources of isolates.

3.2. ERIC-PCR Analysis

The primers based on the repetitive element sequences were tested at different annealing cycle to select suitable primer. For all *B. cereus* strains, the ERIC-PCR patterns obtained with the parameters described in Materials and Methods were constantly reproducible. The eighteen strains typing by PFGE were also analyzed by ERIC-PCR.

Analysis of major band in ERIC-PCR showed a multiple DNA fragments with various intensities. Those bands were generated for all investigated *B. cereus* strains (Fig. 2).
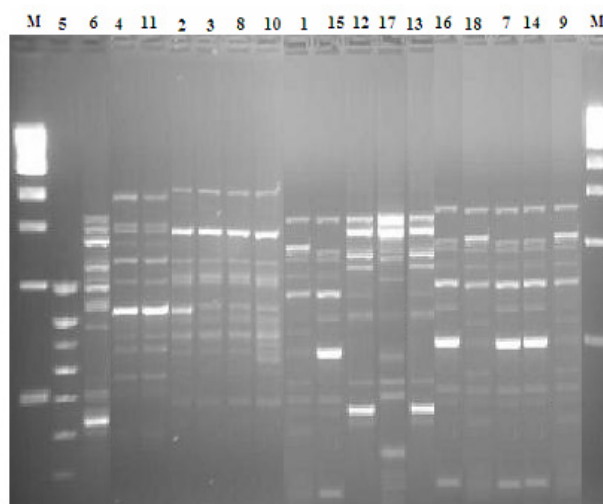
Figure 2. ERIC-PCR profiles of *B. cereus* strains representing the same isolates typing by PFGE. M: Molecular weight marker, 100-bp DNA ladder (BioLabs, Beverli, England)

3.3. Relationship between PFGE and ERIC-PCR

Comparison of the typing results generated by the two techniques revealed few discrepancies (Fig. 1 and Fig. 2). The majority of strains had concordant typing results in PFGE and in ERIC-PCR (Example: S7-S14- S16; S12-S17), However, some strains had diverging results (Example: S9 -S18; S8-S10).

Overall, the results obtained showed that all strains with identical PFGE profiles exhibited a profile similar in ERIC-PCR. However, some strains with identical ERIC-PCR profiles presented different PFGE profiles

4. Discussion

PFGE and ERIC-PCR provide measures of genetic diversity, but they are not equivalent. PFGE reveals restriction site polymorphisms arising from the genomic locations of *SmaI* sites; and ERIC-PCR reveals a profile of DNA fragments of different sizes based, in principle, on the genomic locations of specific repetitive sequences.

The protocol of PFGE adopted in this study is reliable and has the advantage of being feasible in 4 days, which is very short compared with the protocol used for typing strains of group by Harrell et al.[12]. The latter, which was reused by Dubouix et al. indeed takes place on eight days[22].

The use of PFGE and ERIC-PCR for typing strains of group *B. cereus* allows us to conclude that the high discriminatory power of PFGE makes it the "gold standard" for DNA fingerprinting techniques but it has some problems related to the implementation time and limited number of samples to be analyzed simultaneously. Chon JW et al. reported that PFGE is a gold standard method for various foodborne pathogens but its laborious[23].

The ERIC-PCR is an inexpensive, sensitive, and fast tool for molecular typing which requires no prior knowledge of the genome[24-25]. Guimaraes AS et al. reported that the greatest discriminatory power and typeability observed to the ERIC-PCR could be explained by large number of isolates typed[26].

At our knowledge, this study is the first that provides a comparison of the PFGE (using the enzyme *SmaI*) with ERIC-PCR for typing isolates of *B. cereus* group. Otherwise, a same comparison has been the subject of some recent studies, including a study of *Klebsiella pneumoniae*[27] and another on *Mycobacterium fortuitum*[28]. These authors conclude that it was a very similar level of discrimination between the two methods. For Sampaio et al.[28], PFGE has a very powerful method for strain typing but it's laborious, with only a limited number of samples to be analyzed at the same time.

It is often desirable to combine several typing methods. The application of bacterial typing tools requires understanding of both the strengths and limitations of the chosen bacterial[23]. For any bacterial strain assumed to be involved in food poisoning of humans, it is extremely important to possess a wide range of powerful molecular, identification and monitoring tools able to adequately characterize all these strains. Our suggestion for save the time is to use the ERIC-PCR to make a first screening of isolates. Only isolates that had identical profiles can be analyzed by PFGE, which will reduce the number of analyzes with this method more discriminating but also more expensive. In the case of finding a source of contamination in the food industry, ERIC-PCR may be sufficient to trace the bacteria, the high level of discrimination provided by PFGE was not helpful (detection, for example, spontaneous mutation affecting a restriction site). On the contrary, in the case of food poisoning, PFGE, which is the reference method should be used to remove any ambiguity on the differentiation of closely related strains.

During this study we confirmed the ubiquitous nature of these bacteria. Our results indicate that some strains belonging to the same type of foods give the same profile in PFGE and in ERIC-PCR, knowing that different profiles are also obtained for strains of the same type of food, we can assume the existence of other influencing factors such as the environment.

5. Conclusions

In conclusion, our study indicate that all isolates were successfully differentiated, indicating the diversity of *B. cereus* strains present in Morocco samples. The results showed that PFGE is a method that has a high discriminatory power but it is laborious, which promotes the use of ERIC-PCR to be used in priority and previously to PFGE as a typing strategy.

ACKNOWLEDGEMENTS

REFERENCES

[1] Bartoszewicz M, Hansen, BM, Swiecicka I. The members of the *Bacillus cereus* group are commonly presentcontaminant s of fresh and heat-treated milk. Food Microbiology 25:588–596, 2008.

[2] Ehling-Schulz M, Fricker M, Scherer S. Identification of emetic toxin producing *Bacillus cereus* strains by a novel molecular assay. FEMS Microbiol. Lett 232:189-195, 2004.

[3] Minnaard J, Delfederico L, Vasseur V, Hollmann A, Rolny I, Semorile L, Pérez, PF. Virulence of *Bacillus cereus*: a multivariate analysis. International Journal of Food Microbiology 116:197-206, 2007.

[4] Granum PE, Lund T. *Bacillus cereus* and its food poisoning toxins. FEMS Microbiology Letters 157:223–228, 1997.

[5] Vilas-Bôas GT, Peruca APS, Arantes OMN. Biology and taxonomy *of Bacillus cereus, Bacillus anthracis* and *Bacillus thuringiensis*. Canadian Journal of Microbiology 53:673–687, 2007.

[6] Schoeni JL, Wong AC. *Bacillus cereus* food poisoning and its toxins. Journal of Food Protection 68:636-648, 2005.

[7] Koneman E, Allen SD, Janda WM, Schreckenberger PC. Winn Jr., W.C., The aerobic Gram-positive bacilli, Color atlas and textbook of diagnostic microbiology, 5th Ed. Lippincott, New York 651–708, 1997.

[8] Agata N, Ohta M, Mori M. Production of an emetic toxin, cereulide, is associated with a specific class of *Bacillus cereus*. Current Microbiology 33:67-69, 1996.

[9] Ceuppens S, Rajkovic A, Heyndrickx M, Tsilia V, Van De Wiele T, Boon N, Uyttendaele M. Regulation of toxin production by *Bacillus cereus* and its food safety implications. Critical Reviews in Microbiology 1–26, 2011.

[10] Valero M, Herna ndez-Herrero LA, Fernandez PS, Salmeron MC. Characterization of *Bacillus cereus* isolates from fresh vegetables and refrigerated minimally processed foods by biochemical and physiological tests. Food Microbiol 19: 491–499, 2002.

[11] Tenover F C, RD Arbeit, and RV Goering. How to select and interpret molecular strain typing methods for epidemiological studies of bacterial infections: a review for healthcare epidemiologists. Molecular Typing Working Group of the Society for Healthcare Epidemiology of America. Infect. Control Hosp. Epidemiol 18:426–439, 1997.

[12] Harrell, L. J., Andersen, G. L., Wilson, K. H., Genetic variability of *Bacillus anthracis* and related species. J. Clin. Microbiol. 33: 1847–1850, 1995.

[13] Helgason E, Caugant DA, Lecadet MM., Chen Y, Mahillon,J, Lovgren A, Hegna I, Kvaloy K, Kolsto AB. Genetic diversity of *Bacillus cereus/B. thuringiensis* isolates from natural sources. Curr Microbiol 37:80–87, 1998.

[14] Hill KK, Ticknor LO, Okinaka RT, Asay M, Blair H, Bliss KA, Laker M, Pardington PE, Richardson AP, Tonks M, Beecher DJ, Kemp JD, Kolsto AB,Wong AC, Keim P, Jackson PJ. Fluorescent amplified fragment length polymorphism analysis of *Bacillus anthracis, Bacillus cereus*, and *Bacillus thuringiensis* isolates. Appl. Environ. Microbiol 70:1068–1080, 2004.

[15] Cherif A, Borin S, Rizzi A, Ouzari H, Boudabous A, Daffonchio D. *Bacillus anthracis* diverges from related clades of the *Bacillus cereus* group in 16S–23S ribosomal DNA intergenic transcribed spacers containing tRNA genes. Appl. Environ. Microbiol 69:33–40, 2003.

[16] Valjevac S, Hilaire V, Lisanti O, Ramisse F, Hernandez E, Cavallo JD, Pourcel C, Vergnaud G. Comparison of minisatellite polymorphisms in the *Bacillus cereus* complex: a simple assay for large-scale screening and identification of strains most closely related to *Bacillus anthracis*. Appl. Environ. Microbiol. 71: 6613–6623, 2005.

[17] Hyeon JY, Chon JW, Hwang IG, Kwak HS, Kim MS, Kim SK, Choi IS, Song CS, Park C, Seo KH. Prevalence, antibiotic resistance, and molecular characterization of Salmonella serovars in retail meat products. Journal of Food Protection 74:161-166, 2011.

[18] Guinebretiere M.H, Girardin H, Dargaignaratz F, Carlin F. The, C., Contamination flows of *Bacillus cereus* and sporeforming aerobic bacteria in a cooked, pasteurized and chilled zucchini pure processing line. Int. J. Food Microbiol 82:223–232, 2003.

[19] Gautier M, de Carvalho A, and Rouault, A DNA fingerprinti ng of dairy propionibacteria Strains by Pulsed-Field Gel Electrophoresis. Current Microbioly. 32:17-24, 1996.

[20] Versalovic J, Koeuth T, Lupskil JR. Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. Nucleic Acids Res. 19: 6823–6831, 1991.

[21] From C, Pukall R, Schumann P and Granum PE. Toxin-producing ability among *bacillus* spp. Outside the *bacillus cereus* group. Applied and Envirinmental Microbioly 71: 1178-1183, 2005.

[22] Dubouix A. Bonnet E, Alvarez M, Bensafi H, Archambaud M. *Bacillus cereus* infections in traumatology-orthopaedics department: Retrospective investigation and improvement of healthcare pratices. The Journal of Infection; the Journal on Infection 50: 22-30, 2005.

[23] Chon JW, Kim JH, Lee SJ, Hyeon JY, Seo KH. Journal of food Microbiology. 32:217-222, 2012.

[24] Foxman B, Zhang L, Koopman JS, Mannig SD, Marrs CF.

Choosing an appropriate bacterial typing technique for epidemiologic studies. Epidemiol. Perspect Innov 2-10, 2005.

[25] Li W, Raoult D, Fournier PE. Bacterial strain typing in genomic era. FEMS. Microbiol. Rev. 33:892–916, 2009.

[26] Guimaraes A, Seles Dorneles EM, Andrade G, Pereira Lage A, Miyoshi A, Azevedo V, Guimaraes Gouveia AM, Heineman M. B. Molecular characterization of Corynebacter ium pseudotuberculosis isolates using ERIC-PCR. Journal of Veterinary Microbiology. 153:299-306, 2011.

[27] Ben-Hamouda T, Foulon T, Ben-Cheikh-Masmoudi A, Fendri C , Belhadj O, Ben-Mahrez K. Molecular epidemiolo gy of an outbreak of multiresistant Klebsiella pneumoniae in a Tunisian neonatal ward. Journal of Medical Microbiology 52:427-433, 2003.

[28] Sampaio J, Junior D, de Freitas D, Hofling-Lima A, Miyashiro K, Alberto F, Cardoso Lea S. An Outbreak of Keratitis Caused by *Mycobacterium immunogenum*. Journal of Clinical Microbiology 3201-3207, 2006.