Vol. 12(30), pp. 730-735, 14 August, 2018
DOI: 10.5897/AJMR2018.8921
Article Number: 27AEE7B58211
ISSN: 1996-0808
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African Journal of Microbiology Research

Full Length Research Paper

Prevalence and genetic diversity of the strains of *Bacillus cereus* groups in food for infants and young children in México

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Received 23 June, 2018; Accepted 2 August, 2018

The aim of this study was to determine the prevalence and genetic diversity of the strains of *Bacillus cereus* groups isolated in México from foods for infants and young children. A total of 94 foods from a single commercial brand were analyzed to find *B. cereus* through a pre-enrichment method of colonial morphology in agar mannitol yolk polymyxin. Specific colonies were selected to be analyzed by polymerase chain reaction (PCR) determining the amplification of the 16S *rRNA* gene from *B.* cereus and the cytotoxin K gene. Eight strains were selected to determine genetic diversity (and relation) between the isolates from a PCR of repeated elements (rep-PCR), technique of molecular phylogeny that uses the primer (GTG) 5. The genetic similarity was determined by the Dice coefficient and from this a dendrogram was carried out. Isolates corresponding to strains from *B. cereus* group were detected in 9.2% (8/92) of the samples analyzed, 87.5% of the eight isolates showed the *cytK* gene. Groups of isolated strains were detected in meats and fruits and there was only one isolated strain from vegetables. The study shows the presence and propagation of strains from *B. cereus* group in foods for infants and young children commercialized in México.

Key words: *Bacillus cereus*, México, genetic diversity, enterotoxin, infant food.

INTRODUCTION

Foods for infants and children are mainly used during the normal weaning period and during the gradual adaptation of infants and children to normal feeding. They are prepared to be directly administered or dehydrated to be reconstructed through water dilution. The microbiological criteria for these foods state that they must be free from pathogenic microorganisms or self-produced substances in amounts that could cause disease (CAC, 1979; FAO/WHO, 1981).

The *Bacillus cereus* group is made up of six species of bacteria closely related: *B. cereus* sensu stricto, *Bacillus antrhacis*, *Bacillus mycoides*, *Bacillus pseudomycoides*,

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Bacillus thuringiensis and *Bacillus weihentephanensis* (Vilas-Bôas et al., 2007). This group of bacteria is spore-forming Gram-positive bacilli with a wide spread in the environment, decomposing organic matter, fresh or salty water bodies, fomites, and naturally in the gastrointestinal tract of invertebrate organisms. Foods may be contaminated by these reservoirs and colonize the human intestine upon ingestion (Samapundo et al., 2011).

Some species from the *B. cereus* group may cause food poisoning due to the production of different toxins: cerulide is a thermostable toxin resistant to acid pH and proteases, composed of four amino acids and/or oxyacids [D-O-Leu-D-Ala-L-O-Val-L-Val] that form a cyclic complex (dodecadepsipeptide) (Stenfors-Arnesen et al., 2008). This toxin causes the afferent vagus nerve stimulation through the attachment to 5-HT3 receptor of serotonin, causing the emetic syndrome characterized by nausea and vomit. The main related foods are rice, pasta and dairy products. The presence of the preformed toxin in the foods is enough to cause the disease (Delbrassine et al., 2012; Dommel et al., 2010).

In contrast, diarrhea toxins like hemolysin BL (Hbl), non-hemolytic enterotoxin (Nhe) and cytotoxin K (CytK) are produced during the vegetative growth of the bacteria in the small intestine (Naranjo et al., 2011). The Hbl and Nhe enterotoxins are made up of three subunits; L2, L1B, and NheA, NheB, ans NheC, respectively, whereas the CytK is made up of a single protein from the family of barrels β (Bottone, 2010). The three toxins have lytic activity against enterocytes, the mechanism is not precisely known, however, it suggests the formation of pores in the lipid membranes of cells, which leads to osmotic lysis (Stenfors-Arnesen et al., 2008; Tsilia et al., 2012).

A high frequency of isolated toxigenic strains from group *B. cereus* has been recently reported in different food groups, including foods rich in starch (rise and potatoes), raw or partially cooked vegetables, dairy and meat products, and ready-to-eat foods (Chon et al., 2012; Lee et al., 2012; Samapundo et al., 2011).

Diseases related to foods contaminated by species from group *B. cereus* generally occur when toxigenic strains multiply and reach around 10^4 to 10^6 CFU, however, due to the high variability of infectious doses, as well as toxin production and spore formation, it is not possible to rule out the risk of smaller size inoculates (Logan, 2012).

In 2015, in the United States of America, two food poisoning cases caused by *B. cereus* were reported with 25 people without going to hospital (Centers for Disease Control and Prevention (CDC), 2017). Meanwhile, in countries like Norway, Finland, and Hungary, *B. cereus* has been held accountable for gastrointestinal diseases (diarrhea syndrome) in contrast with countries like China, Japan, and Belgium, where it is linked to the emetic syndrome (Granum and Lund, 1997; Logan, 2012). The existence of *B. cereus* as a contaminant of food for babies has been reported in China (Li et al., 2014; Zhang et al., 2017) and Iran (Rahimi et al., 2013), and currently, in Mexico there are no data about this microorganism. In Mexico, until March 2018, over 600 food poisoning cases and more than 90,000 gastrointestinal diseases have been reported without identifying the causal agent (Dirección General de Epidemiología, 2018). The aim of this study was to analyze and compare the *B. cereus* frequency in a wide array of foods for infants and small children, the presence of cytotoxin K gene, and possible strain cloning.

MATERIALS AND METHODS

Sampling and microbiological analysis

The products were chosen per the general description of foods for infants made by the Codex Alimentarius described earlier, considering in this study a total of 94 foods of a single commercial brand, which were purchased in supermarkets in Chilpancingo, a southwestern city from Mexico. Remarking that even when they were purchased in this city, they are widely distributed and purchased throughout the country. The samples were collected from January to July, 2017. The 94 samples comprised varieties fruit (35 samples), vegetables (32 samples) and meat (27 samples).

The microbiological analysis was limited to the count of mesophyll microorganisms in plaque and a pre-enrichment method for the research of *B. cereus*. For the mesophilic count, 25 g of the sample were used, which were taken aseptically and mixed in 225 mL of peptone saline solution. After 10 min, aliquots of 1 mL were taken and from these, decimal dilutions were made in sterile saline. The dilutions were inoculated by the dispersion method placing a volume of 0.1 mL on nutritive agar plates. The plates were incubated for 24 h at 30°C. The colonies were counted to determine the total count of aerobic microorganisms in plaque. From the remaining mixture in peptone saline solution, a 1 mL aliquot was transferred to a brain heart infusion broth supplemented with 10 U/mL of polymyxin and after incubation at 30°C, aliquots of 0.1 mL were inoculated in mannitol agar polymyxin egg yolk (MYP). The pink colonies with an opaque halo were considered as suspicious colonies of *B. cereus* and were confirmed by beta hemolysis in trypticase soy agar supplemented with sheep blood.

Molecular confirmation of strains of the *B. cereus* group and presence of cytotoxin K

From bacterial cultures of the bacterial strains, a thermal shock was performed to obtain the chromosomal DNA. In brief, cells from one colony were suspended in sterile water, heated at 95°C for 3 min and then placed on ice. After centrifugation, the supernatant was used as template for the amplification of the 16S *rRNA* gene of *Bacillus*. The primer for the 16S *rRNA* gene amplification was derived from the data record GenBank: AE016877 for the reference strain *B. cereus* ATCC 14579. In order to amplify the 16S *rRNA* gene, the reaction mixes (25 µL) contained the following: 25 µl of REDTaq Ready Mix DNA polymerase (Sigma- Aldrich, USA), 11 µL of sterile MiliQ water, 0.5 µL of the genomic DNA template (concentration about 10 to 20 ng/µL) and 1 µL of each primer (FRNA, 5-AGA GTT TGA TCC TGG CTC-3; RRNA, 5-CGG CTA CCT TGT TAC GAC-3). The reactions were carried out with an initial denaturation at 94°C for 1 min, followed by 35 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 3 min and the final extension at 72°C for 3 min. For the detection of the *cytK* gene, polymerase chain reaction (PCR) reaction was set up. The reaction mixes

Table 1. Aerobic plaque count and presence of *B. cereus* in different varieties of food for infants.

APC count (Log CFU/g)	Variety		
	Fruits [n (%) N=35]	Vegetable [n (%) N=32]	Meat [n (%) N=27]
1	24 (68.6)	23 (71.8)	9 (33.3)
2	6 (17.1)	9 (28.2)	11 (40.7)
3	3 (8.6)	0	4 (14.8)
4	2 (5.7)	0	3 (11.2)
B. cereus	2 (5.7)	1 (3.1)	5 (18.5)

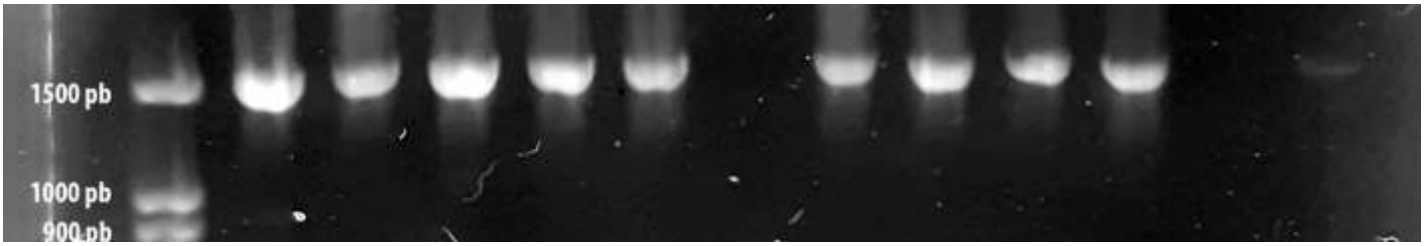

Figure 1. Agarose gel electrophoresis of the PCR products obtained for selected strains from the *B. cereus* group with primers 16S *rRNA* gene. Lane 1, 100 bp molecular marker; Lane 2, *B. cereus* ATCC 14579, Lane 3-13, samples.

contained: 12.5 µL of RedTaq Ready Mix DNA polymerase (Sigma-Aldrich, USA), 11.5 µL of sterile MilliQ water, 0.2 µL of each primer solutions (concentration 100 µM) P1-5′-CAA AAC TCA (T/C) CTA TGC AAT TAT GCA T-3′, P3-5′-ACC AGT TGT ATT AAT AAC GGC AATC-3′), 1 µL of the template DNA with concentrations equaling 10 to 20 ng/µL. The amplification cycle composed of initial denaturation at 94°C for 2 min, followed by 40 cycles at 94°C for 30 s, 55°C for 30 s, 72°C for 1 min and final extension at 72°C for 2 min. Electrophoresis was performed on 2% agarose gels at 80 V for 120 min. The gels were stained with Midori Green (Nippon Genetics, Germany) and visualized with UV light.

Genetic diversity of *B. cereus*

The phylogenetic relationship between the strains of *B. cereus* was carried out using the technique of repetitive palindromic elements PCR (rep-PCR)[13] using primers (GTG) 5 (GTG GTG GTG GTG GTG) with the following reaction conditions; initial denaturation at 95°C for 2 min, 30 cycles of 94°C for 30 s, 40°C for 1 min, 60°C for 1 min and for a final extension, 65°C for 5 min. Electrophoresis was performed on 2% agarose gels at 80 V for 120 min. The gels were stained with Midori Green (Nippon Genetics, Germany) and visualized with UV light.

To establish the genetic distances of the profiles, the coefficient of similarity of Dice was calculated. The genetic distance matrix was analyzed by the UPGMA method (acronym in English of Unweighted Pair Group Method with Arithmetic Mean). A dendrogram was developed with the analyzed data using the BioNumerics 7.1 package (Applied Maths Inc, US).

Statistical analysis

Descriptive statistics are used to describe the basic features of the data in a study. They provide simple summaries about the sample and the measures.

RESULTS

In the present study, numbers above 4.0 log CFU/g of aerobic mesophilic in foods was observed for infants of meat and fruit varieties. However, one of the most important results was the presence of *B. cereus* in all varieties of foods for toddlers (Table 1).

To confirm the proper classification of isolates, the amplification by PCR of the 16S *rRNA* gene was used. PCR amplification products of the nearly complete 16S *rRNA* gene were observed (1500 bp about) in eight strains (Figure 1). The results obtained for their strains confirmed their taxonomic position as belonging to the *B. cereus* group.

PCR was used for the detection of the *cytK* gene in eight strains belonging to the *B. cereus* group. An amplicon, 238 bp long was obtained containing the N-terminal fragment of the structural gene. It was found that among eight isolates, most of them (7 strains, 87.5%) contained the *cytK* gene (Figure 2)

Upon identifying possible cloning of strains, four different groups could be observed; three of them positive for cytotoxin K. In one of them, an isolated vegetable strain was grouped, whereas isolated meat strains were distributed among the remaining groups. In the first group both meat and fruit strains were grouped (Figure 3).

DISCUSSION

In recent years, the role and importance of *B. cereus* as a

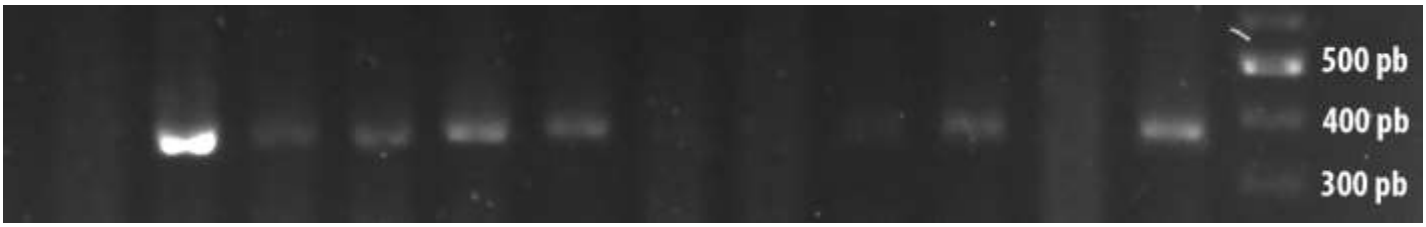

Figure 2. Agarose gel electrophoresis of the PCR products obtained for selected strains from the *B. cereus* group with primers *cytK* gene. Lane 1, Negative control; Lane 2- 12, samples; Lane 13, *B. cereus* ATCC 14579; Lane 14, molecular marker.

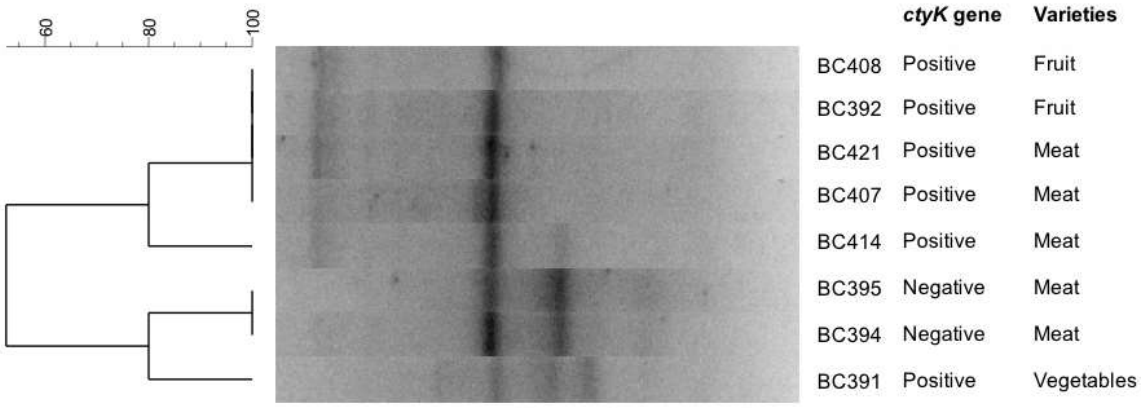

Figure 3. Dendrogram (obtained by GTG5) of cloning of strains of the *B. cereus* group. Eight strains of processed foods for infants positive to 16s *rRNA* were characterized and grouped into four main groups.

pathogen associated with food poisoning has increased (Jeßberger et al., 2015). Even though risk groups have not been described, it has been reported that the infection is not self-limited in all cases and that it can even progress to death (Dierick et al., 2005; Lund et al., 2000).

In children under two years old, in addition to breastfeeding, different sorts of packaged foods are included in the diet, importantly; this group of foods is recognized as non-sterile, establishing internationally the microbiological parameters that these must meet (FAO/WHO, 1981).

At a national level, the Mexican regulations framework considers as a quality parameter the amount of CFU/g of aerobic mesophilic (Secretaria de Salud (SSA), 1995). In this study, five fruit samples and seven meat samples exceed this amount; an important feature in this regulation, is not included the amount of CFU/g or the presence of *B. cereus* as an important microbiological indicator, even when the population to which these products are directed, could be a risk group. In this study, *B. cereus* was isolated in all varieties with a frequency of 9.2%; in studies carried out in China, the prevalence ranges from 1 to 6% (Li et al., 2014; Zhang et al., 2017), while in Iran it is 42% (Rahimi et al., 2013), considering that the main difference is the type of variety analyzed, in these three studies the base was rice and in the present study, it was rice-based foods, fruits and vegetables were included.

These data as a whole denote the capacity of the spores of the *Bacillus* genus to resist thermal treatments (wet and dry heat), radiation (UV and ionizing) and chemical agents (Setlow, 2006), treatments to which these products are subjected to their preparation and packaging, particularly sterilization (variety of meats) and airtight seal (varieties of vegetables and fruits) (SSA, 1995); even when only the presence was determined (not the quantity), it is an important fact because these foods are stored for long periods of time either in shops or at home, which can favor the growth of the microorganism to an infectious dose (Valero et al., 2003), which has been described in vegetable purees, where the aerobic spore increased 7.8±0.1 log CFU/g in the puree after five days at 20 to 25°C and the vegetative cells of *B. cereus* approximately 6.4±0.5 log CFU/g in the same period of time and temperature (Guinebretiere et al., 2003). In addition, minor inoculants should therefore not be ruled out due to the toxigenic potential of each strain (Logan, 2012).

B. cereus is a microorganism with a wide variety of virulence factors, including emetic toxin and the enterotoxins Hbl, NHE and CytK (Bottone, 2010). In this

study, the *cytK* gene was molecularly identified, because the presence of this gene has been determined in strains that caused food poisoning (and that are negative for NHE and Hbl) (Lund et al., 2000). The frequency in this study was 87.5%, which is like that reported in a study in Belgium in ready-to-eat foods (Samapundo et al., 2011).

The intention to identify a possible clone in this type of food was focused on the search for a clone that will be isolated from all varieties of these foods and that may be related to a systematic contamination during its production and processing, due to its persistence in the area and resistance of the spores produced. For example, certain characteristics of spores, such as hydrophobicity or the presence of exosporium favor their ability to adhere to surfaces involved in the food processing (Tauveron et al., 2006). Additionally, it has been observed that this microorganism is capable of producing biofilm, which protects both the spores and vegetative cells from disinfectants (Ryu and Beuchat, 2005); this could be explained with the strains that belong to the first generated group. However, the presence of strains, such as the strain isolated from vegetables, could explain that the contamination of this microorganism may not only be found in the process, but also in the raw material. Related to this, the presence of *B. cereus* in dairy products was shown from early stages of product processing, which include contamination of raw material (Svensson et al., 2004) or even in raw vegetables for the preparation of puree (Choma et al., 2000), which is capable of enduring thermal and pressure processes to which the food is subjected (Guinebretiere et al., 2003) and this could explain the presence of the strain found in vegetables and not in other food groups.

This study proposes the incorporation of this microorganism in the current Mexican legislation, due to the characteristics that allow it to be found in food, as well as virulence factors that could cause major food poisonings in the group to which these foods are directed.

Conclusion

The study shows the presence and propagation of strains from *B. cereus* group in foods for infants and young children commercialized in México

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

The authors would like to express their heartfelt thanks and deep appreciation to University Autonomy of Guerrero. The work was financed by the “Programa de Incorporación de Nuevos Profesores de Tiempo Completo” by the Secretaria de Educación Pública, México

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