Diversity of Toxigenic Bacillus cereus in Powder Milk

Diversidad de Bacillus cereus Toxigénicos en Leche en Polvo

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Abstract. Bacillus cereus *is a bacterium commonly isolated from food and produces multiple toxins such as hemolysin BL (HBL), non-hemolytic enterotoxin (NHE), cytotoxin K (CytK) and emetic toxin or cereulide that cause human food poisoning. The detection of* ***B. cereus*** *by traditional plating does not allow detecting the toxic potential and toxigenic diversity of the strains in food. Therefore, in this work, a multiplex PCR to direct detection of toxigenic B. cereus from powder milk samples collected in Medellín, Colombia, was applied. According to the pattern of toxic genes found in the samples, was possible to establish five different toxigenic consortia: I (nheA, hblC), II (I (nheA, hblC, cytK), III (hblC), IV (hblC, cytK) and V (nheA, cesB). These results suggest a wide diversity of toxigenic B. cereus in the evaluated samples.*

Key words: *Enterotoxins, emetic toxin, multiplex PCR, foodborne pathogen.*

Resumen. Bacillus cereus *es una bacteria comúnmente aislada de alimentos, la cual produce toxinas como la hemolisina BL (HBL), enterotoxina no hemolítica (NHE), citotoxina K y toxina emética o cereúlida, que causan intoxicaciones alimentarias al hombre. La detección de B. cereus mediante pruebas tradicionales de cultivo en placa no permite determinar el potencial tóxico y diversidad toxigénica de las cepas; por lo cual, en este trabajo se aplicó una PCR múltiple que permitió la detección directa de* ***B. cereus toxigénicos*** *en muestras de leche en polvo colectadas en Medellín, Colombia. De acuerdo a los patrones de genes tóxicos encontrados en las muestras, fue posible establecer cinco consorcios toxigénicos diferentes: I (nheA, hblC), II (I (nheA, hblC, cytK), III (hblC), IV (hblC, cytK) y V (nheA, cesB). Estos resultados sugieren una amplia diversidad de B. cereus toxigénicos en las muestras evaluadas.*

Palabras clave: *Enterotoxinas, toxina emética, PCR múltiple, patógeno de alimentos.*

INTRODUCTION

Bacillus cereus is a Gram-positive, rod-shaped, spore-forming, motile bacterium that is commonly isolated from dried foods such as milk powder and farinaceous products contaminated with spores that germinate when in contact with water during food preparation, leading to spoilage or food poisoning (Logan, 2012). *B. cereus* may produce multiple toxins; diarrheal enterotoxins such as hemolysin BL (HBL), non-hemolytic enterotoxin (NHE), and cytotoxin K (CytK) are produced after colonization of the human small intestine by this microorganism (Bhunia, 2008). The emetic or cereulide toxin (ces) is produced in the foods before consumption (Granum, 2005).

The detection of *B. cereus* is performed by traditional plating and biochemical assays that are time-consuming and do not allow detecting the toxic potential and toxigenic diversity of the strains (Bhunia, 2008) in food. Molecular approaches currently available, for example multiplex PCR, are inexpensive and easy to perform alternatives that could be used to assess the diversity of toxigenic *B cereus* in food (Ehling-Schulz *et al*., 2004). Therefore, the aim of this study was to assess the diversity of toxigenic *B. cereus* by multiplex PCR from powder milk samples collected in Medellín, Colombia.

MATERIALS AND METHODS

Bacterial strains. The reference strains ATCC 14579 *(hbl, nhe, cytK),* NVH 1230/88 *(hbl, nhe, cytK)*, NVH 1257 *(ces)* and F4810/72 *(ces*) were used for the standardization of molecular techniques.

Sample preparation. 25 g of powder milk were dissolved in sterile distilled water and filtered through Whatman N°1 filter. The resulting supernatant, which contained the spores and cells of *B. cereus* was centrifuged at 6000 *g* for 30 min and the pellet used for DNA extraction.

DNA extraction. DNA was extracted according to a methodology previously described (D'Alessandro, 2007).

Primers. The primer pairs used in this work were selected after previous laboratory screening of several reported primers (Table 1).

Table 1. Characteristics of PCR primers used for multiplex detection of *B. cereus* toxin genes

Target gene	Primer	Primer sequence (5' —> 3')	Product size (bp)	Primer position	GenBank accession number	Reference
hblC	hblCF 1318 hblCR 1728	CGAAAATTAGGTGCGCAATC TAATATGCCTTGCGCAGTTG	411	1318- 1337 1709-1728	U63928	(Moravek *et al.*, 2004)
nheA	nheAF 430 nheAR 1185	ACGAATGTAATTTGAGTCGC TACGCTAAGGAGGGGCA	755	430-449 1166-1185	Y19005	(Guinebretiere *et al.*, 2002)
cesB	cesF 21816 cesR 23087	GGTGACACATTATCATATAAGGTG GTAAGCGAACCTGTCTGTAACAACA	1271	21816-21839 23063-23087	DQ360825	(Ehling-Schulz *et al.*, 2005b)
CytK	cytK F2 cytK R2	CGACGTCACAAGTTGTAACA CGTGTGTAAATACCCCAGTT	565	286-305 850-831	AJ318876.2	(Ngamwongsatit *et al.*, 2008)
16S rDNA	ITSF 8 ITSR 1511	AGAGTTTGATCCTGGCTCA CGGCTACCTTGTTACGAC	1514	9200-9218 10713-10696	CP001407.1	(Ehling-Schulz *et al.*, 2005a)

Multiplex PCR. The final reaction mixture (16 μL) consisted of 0.6 mM dNTPs mix, 4 mM MgCl2, 0.2 μM forward and reverse primers for amplification of hblC, nheA, cesB and cytK genes (0.1 μM for ITS1), 1.3 U of Taq platinum polymerase (Invitrogen), 1.6 μL 10$\times$ reaction buffer and 100 ng of DNA template extracted directly from the samples. Amplification was performed on a G-Storm GS482 thermocycler with an initial denaturation step at 94 °C for 5 min, followed by 40 cycles of 1 min denaturation at 94 °C, 40 annealing at 49 °C and 2 min elongation at 72 °C, and final incubation at 72 °C for 10 min.

Test to evaluate specificity. The specificity of the multiplex PCR was evaluated by amplifying the DNA of various bacterial strains that are considered important in food safety, such as *Salmonella* spp., *Shigella* spp., *Escherichia coli, Staphylococcus aureus* and *Listeria monocytogenes.*

RESULTS AND DISSCUSION

Amplified fragments for reference strains corresponding to *hblC, nheA, cesB, cytK* and the internal control ITS1 were obtained and confirmed by sequencing. High homology values were obtained (95–100%) when these sequences were compared with sequences in GenBank, indicating that the amplified products were those expected. The multiplex PCR showed high specificity since the toxigenic genes were amplified only in the *B. cereus* reference strains.

Sixty four samples (85.3%) of 75 powder milk samples evaluated in the multiplex PCR were contaminated with toxigenic *B. cereus*. The most predominant toxin gene was *hblC* (81.3%), followed by *nheA* (46.7%), *cytK* (17.3%) and *cesB* (4%). These results are in agreement with those previously reported for *B. cereus* strains isolated from powder milk in Brazil and Kenya for *hblC, nheA* and *cytK t*oxin genes (Chaves *et al.*, 2011; Ombui *et al.*, 2008). Remarkably, the *cesB* toxin gene has not been previously reported. In addition, with the results obtained was possible to establish five different toxigenic consortia, according to the pattern of toxic genes found in the samples (Table 2).

Table 2. Toxin gene consortiums in powder milk samples.

Toxin gene consortiums	Positive samples n (%)	Genes
I	20 (31.25)	nheA, hblC
II	12 (18.75)	nheA, hblC, cytK
III	28 (43.75)	hblC
IV	1 (1.56)	hblC, cytK
V	3 (4.69)	nheA, cesB

CONCLUSIONS

A broad diversity of toxigenic *B. cereus* in samples of powder milk collected in Medellín, Colombia was found by the application of a molecular test such as the multiplex PCR. The above could not be assessed by traditional plating and biochemical assays.

ACKNOWLEDGMENTS

This work was funded by the Administrative Department of Science, Technology and Innovation (COLCIENCIAS) and the University of Antioquia, Colombia.

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Calidad de las Harinas de Corozo Producidas por Deshidratación

Quality of the Corozo Flours Produced Trough the Drying

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Resumen. *El objetivo del trabajo fue evaluar la calidad de la harina de la pulpa de corozo* ***(Acrocomia aculeata (jacq.) lodd****) de frutos procedentes de tres diferentes localidades brasileñas. Fueron evaluados los constituyentes nutricionales, el pH, la actividad de agua y flora microbiana presente. El rendimiento del proceso de secado también fue evaluado. Las harinas de los frutos de corozo presentaron baja humedad, baja actividad de agua y alto contenido de minerales. Todas las harinas fueron consideradas como productos de baja acidez. Los valores encontrados para coliformes fecales,* ***Staphylococcus aureus****,* ***Bacillus cereus****,* ***Salmonella sp****., mohos y levaduras estaban dentro de los padrones establecidos por la legislación brasileña, lo que permite que el producto sea considerado apropiado para el consumo. Los frutos de dos localidades proporcionaron rendimiento de pulpa con potencial de aplicación industrial.*

Palabras clave: *Acrocomia aculeata, deshidratación, seguridad microbiológica.*

Abstract. *The aim of this work was to evaluate the quality of corozo* ***(Acrocomia aculeata (jacq.) lodd****) pulp flour originated from three different brazilian localities. The nutritional constituents, pH, water activity and microbial flora were tested. The drying performance was also evaluated. Corozo flours of the fruits presented low humidity, low water activity and high mineral content. All meals were considered*