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Direct detection of toxigenic *Bacillus cereus* in dietary complement for children and cassava starch

Abstract

Bacillus cereus is a food contaminant and a known human pathogen that can cause emetic and diarrheal syndromes. In this study we evaluated the presence of toxigenic *B. cereus* by multiplex PCR directly in dietary complement for children and cassava starch samples collected on Medellin, Colombia. Of 75 dietary complement for children samples evaluated, 70.7% were contaminated with toxigenic *B. cereus* and four different toxigenic consortia were detected: I: *nheA*, *hblC*, *cytK* (9.8%), II: *nheA*, *hblC* (2%), III: *hblC*, *cytK* (41.2%), IV: *hblC* (47%). Of 75 cassava starch samples, 44% were contaminated with toxigenic *B. cereus* and four different toxigenic consortia were determined: I: *nheA*, *hblC*, *cytK* (48.5%), II: *nheA*, *hblC*, *cytK*, *cesB* (3%), III: *hblC*, *cytK* (30.3%), IV: *hblC* (18.2%). In general, in dietary complement for children only enterotoxigenic consortia were detected while in cassava starch the enterotoxigenic consortia predominated over the emetic. Multiplex PCR was useful to detect toxigenic *B. cereus* contamination allowing direct and simultaneous detection of all toxin genes in foods. This study is the first in Colombia to evaluate toxigenic *B. cereus*, providing information of importance for microbiological risk evaluation in dried foods.

Keywords: *Bacillus cereus*, enterotoxins, emetic toxin, dried foods, Multiplex PCR.

Detección directa de *Bacillus cereus* toxigénicos en complementos dietarios para niños y en almidón de yuca

Resumen

Bacillus cereus es un contaminante de alimentos conocido por ser patogénico para los humanos, causando síndromes de vómito y diarrea. En este estudio se evaluó la presencia de *B. cereus* toxigénicos utilizando PCR múltiple directamente en complementos dietarios para niños y en almidón de yuca colectados en Medellín, Colombia. De 75 muestras de complemento dietario para niños, 70,7% estuvieron contaminadas con *B. cereus* toxigénicos y se detectaron cuatro diferentes consorcios toxigénicos: I: *nheA*, *hblC*, *cytK* (9,8%), II: *nheA*, *hblC* (2%), III: *hblC*, *cytK* (41.2%), IV: *hblC* (47%). De 75 muestras de almidón de yuca, 44% estuvieron contaminadas con *B. cereus* toxigénicos y se determinaron cuatro diferentes consorcios toxigénicos: I: *nheA*, *hblC*, *cytK* (48.5%), II: *nheA*, *hblC*, *cytK*, *cesB* (3%), III: *hblC*, *cytK* (30,3%), IV: *hblC* (18.2%). En general, en los complementos dietarios para niños sólo se detectaron consorcios enterotoxigénicos, mientras que en el almidón los consorcios enterotoxigénicos predominaron sobre el emético. La PCR múltiple fue de utilidad para detectar contaminación con *B. cereus* toxigénicos permitiendo la detección directa y simultánea de todos los genes tóxicos en los alimentos. Este estudio es el primero en Colombia en evaluar *B. cereus* toxigénicos y proporciona información importante para la evaluación de riesgos microbiológicos en los alimentos pulverizados.

Palabras clave: *Bacillus cereus*, enterotoxinas, toxina emética, alimentos pulverizados, PCR múltiple.

Detecção direta de *Bacillus cereus* toxigênicos em suplementos alimentares para crianças e amido de mandioca

Resumo

Bacillus cereus é um contaminante de alimentos e é conhecido por ser patogénico nos seres humanos ocasionando síndromes de vômitos e diarreia. Neste estudo foi avaliada a presença de *B. cereus* toxigênicos por PCR multiplex diretamente em complementos da dieta para crianças e amido de mandioca, em amostras coletadas em Medellín, na Colômbia. De 75 amostras dos complementos da dieta para crianças, 70,7% estiveram contaminadas com *B. cereus* toxigênicos e foram detectados quatro diferentes consórcios: I: *nheA*, *hblC*, *cytK* (9,8%), II: *nheA*, *hblC* (2%), III: *hblC*, *cytK* (41,2%), IV: *hblC* (47%). De 75 amostras de amido de mandioca, 44% estiveram contaminadas com *B. cereus* toxigênicos e quatro consórcios diferentes foram determinados: I: *nheA*, *hblC*, *cytK* (48,5%), II: *nheA*, *hblC*, *cytK*, *cesB* (3%), III: *hblC*, *cytK* (30,3%), IV: *hblC* (18,2%). Em geral, nos complementos da dieta para crianças foram detectados apenas consórcios enterotoxigênicos, não obstante no amido os consórcios enterotoxigênicos predominaram sobre o emético. A PCR multiplex foi útil para detectar contaminação com *B. cereus* toxigênico permitindo a detecção direta e simultânea de todos os genes tóxicos em alimentos. Este estudo é o primeiro na Colômbia em avaliar *B. cereus* toxigênico e providencia informação importante para a avaliação de riscos microbiológicos em alimentos pulverizados.

Palavras-Chave: *Bacillus cereus*, enterotoxinas, toxina emética, alimentos pulverizados, PCR multiplex.

Introduction

Bacillus cereus is a spore-forming, aerobic to facultative, Gram-positive and motile rod bacteria that can be commonly found in many types of environments and is also a known human pathogen that can cause emetic and diarrheal syndromes (1). Emetic syndrome occurs after ingestion of food containing a preformed toxin called cereulide or emetic toxin (2), which is encoded by the *ces* gen cluster, that comprises seven coding DNA sequences, *cesH*, *cesP*, *cesT*, *cesA*, *cesB*, *cesC* and *cesD*, located on a plasmid (3).

Three different enterotoxins responsible for the diarrheal syndrome have been described: hemolysin BL (HBL), encoded by the *hbl* operon that comprises the *hblC*, *hblD* and *hblA* genes; nonhemolytic enterotoxin (NHE), encoded by the *nhe* operon composed by the *nheA*, *nheB* and *nheC* genes; and cytotoxin K (CytK) encoded by the *cytK* gene. These genes are located on the bacterial chromosome (4).

Different foods such as rice, meat, pasta, chicken, fruits, grain, spices, and vegetables may be contaminated by cell or spores of *B. cereus* (5). Moreover dried foods such as powder milk and starch foods are frequently contaminated by *B. cereus*; this is because of the presence of starch degrading amylases and spores that can survive to treatments such as drying and heat, factors that eliminate other competing microorganisms present. Spores can germinate when in contact with water during food preparation, leading to spoilage or food poisoning (6).

The detection of *B. cereus* is traditionally performed by plating and biochemical assays that are time-consuming and do not allow detecting the toxigenic potential and diversity of the strains (2). Molecular approaches currently available, for example multiplex PCR, are inexpensive, easy to perform, and allow the evaluation of the toxigenic potential of the strains (7). In addition, some tests do not require isolation of the microorganism in pure culture (8).

In Colombia, the toxigenic potential of *B. cereus* in foods is unknown, and a rapid detection test for this pathogen in foods is not available, which impede the application of rapid quality control measures to eliminate *B. cereus* from food. Therefore, the objective of this study was to evaluate the presence of toxigenic *B. cereus* by a simple and rapid test, a multiplex PCR, directly in dietary complement for children and cassava starch samples collected in Medellin, Colombia. This study is the first in Colombia to evaluate toxigenic *B. cereus* and can provide an approximation of the type of toxins that strains can cause in food. Also, this study helps to better understand the toxigenic *B. cereus* in dried foods and provides information for microbiological risk evaluation.

Materials and methods

Dried food samples

The following dried foods products were selected for analysis: cassava starch (n=75) and dietary complement for children (n=75), which were collected in public and private educational institutions, bakeries and powdered food companies located in Medellin, Colombia.

Extraction of spores and cells of *B. cereus* from dried foods

Twenty-five grams of the dried food were dissolved in 225 ml sterile distilled water and filtered through Whatman N°1 filter. The resulting liquid portion containing *B. cereus* spores and cells was centrifuged at 6000 g for 30 min and the pellet used for DNA extraction.

DNA extraction

Total DNA from *B. cereus* spores and cells was extracted according to the method described by D'Alessandro (9).

Multiplex PCR

To develop the multiplex PCR assays to test for toxigenic *B. cereus* in dried foods, the selected primer pairs were directed to amplify *hblC*, *nheA*, *cesB* and *cytK* genes. In addition, 16S rDNA sequence was targeted as the amplification internal control (Table 1).

The final reaction mixture (16 µL) consisted of 0.6 mM dNTPs mix, 4 mM $MgCl_2$, 0.2 µM forward and reverse primers for *hblC*, *nheA*, *cesB* and *cytK* genes and 0.1 µM for ITS1, 1.3 U of Taq platinum polymerase

Table 1. Description of primers used for multiplex PCR detection of *B. cereus* 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[1] 1709-1728	U63928	(10)
nheA	nheAF 430 nheAR 1185	ACGAATGTAATTTGAGTCGC TACGCTAAGGAGGGGCA	755	430-449[2] 1166-1185	Y19005	(11)
cesB	cesF 21816 cesR 23087	GGTGACACATTATCATATAAGGTG GTAAGCGAACCTGTCTGTAACAACA	1271	21816-21839[3] 23063-23087	DQ360825	(12)
cytK	cytKF2 cytKR2	CGACGTCACAAGTTGTAACA CGTGTGTAAATACCCCAGTT	565	286-305[4] 850-831	AJ318876.2	(13)
16S rDNA	16S rDNAF 16S rDNAR	AGAGTTTGATCCTGGCTCA CGGCTACCTTGTTACGAC	1514	9200-9218[5] 10713-10696	CP001407.1	(14)

*Primer position in reference strains: [1]*Bacillus cereus* F837/76. [2]*B. cereus* 1230-88. [3]*B. cereus* 4810/72 containing plasmid pBCE4810. [4]*B. cereus* 1230-88 with partial *cytK*-2 gene. [5]*B. cereus* 03BB102.

(Invitrogen, Germany), and 1.6 µL 10X reaction buffer. Amplification was performed on a G-Storm GS482 thermocycler with an initial denaturation step at 94 °C for 5 min, followed by 35 cycles of 1 min denaturation at 94 °C, 40 s annealing at 50 °C and 2 min elongation at 72 °C, and final incubation at 72 °C for 10 min. After electrophoresis on a 2% agarose gel, PCR products were stained with ethidium bromide and visualized under ultraviolet light (UVP Gel Doc).

DNA cloning and sequencing

Multiplex PCR amplification products from *B. cereus* reference strains F4810/72 and 1257 (*ces*, *nhe*), ATCC 10987, NVH 1230-88, 307, and ATCC 14579 (*hbl*, *nhe*, *cytK*), ATCC 21281, ATCC 27348, ATCC 6464, and F4094/73 (*hbl*, *nhe*), and F0075/95 (*nhe*), were cloned using TA cloning kit (Invitrogen, Germany) and the cloned genes were sequenced. The resulting sequences were searched against *B. cereus* genome sequences available in GenBank.

Specificity and sensitivity determination

To assess the specificity of the multiplex PCR developed in this study, a panel of *B. cereus* reference strains were included: *B. cereus* F4810/72 (*ces*, *nhe*), *B. cereus* 1257 (*ces*, *nhe*), *B. cereus* ATCC 10987 (*hbl*, *nhe*, *cytK*), and *B. cereus* ATCC 14579 (*hbl*, *nhe*, *cytK*). Also, various bacterial strains considered important in food safety available in the reference lab collection, such as *Salmonella* spp., *Shigella* spp., *Escherichia coli*, *Staphylococcus aureus* and *Listeria monocytogenes* were evaluated.

To assess the minimum amount of *B. cereus* DNA detectable by the multiplex PCR, 0.1, 0.5, 1, 10, 20, 50, 100, 200 or 500 ng of DNA template from *B. cereus* reference strains was added separately to tubes that contained multiplex PCR mix.

Results and disscusion

Specificity and sensitivity of the multiplex PCR

Cloned PCR products from *B. cereus* reference strains corresponding to *hblC*, *nheA*, *cesB*, *cytK*, and the internal control 16S rDNA were confirmed by sequencing. High identity values were obtained (95-100%) when these sequences were compared with those registered in GenBank, indicating that the amplified products were those expected. The multiplex PCR only amplified toxigenic genes in the *B. cereus* reference strains and no in other bacterial genera evaluated demonstrating the specificity of the PCR. The DNA detection limit of the multiplex PCR was 100 ng; at this concentration strong bands were visualized for all the evaluated genes (Figure 1).

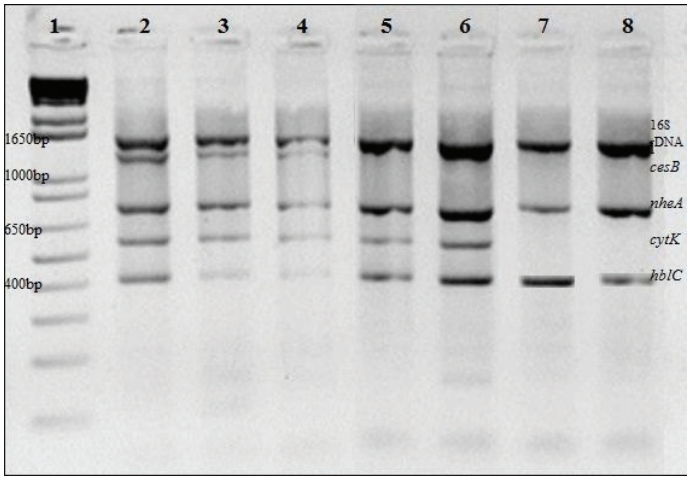

Figure 1. Toxigenic patterns of *B. cereus* strains after multiplex PCR. 2% agarose gel electrophoresis. Gene regions amplified: 16S rDNA, cesB, nheA, cytK and hblC. Lanes: **1.** Molecular weight marker (1Kb plus, Invitrogen), **2.** Positive control (DNA mix of *B. cereus* ATCC 14579 and *B. cereus* 1257 strains), **3** and **4**. DNA of toxigenic *B. cereus* extracted from cassava starch, **5** to **8**: DNA of toxigenic *B. cereus* extracted from dietary complement for children.

Direct detection of toxigenic *B. cereus* from dried foods

Fifty three (70.7%) of 75 dietary complement for children samples evaluated by multiplex PCR were contaminated with toxigenic *B. cereus*. The most predominant toxin gene was *hblC* (65.8%), followed by *cytK* (34.2%), and *nheA* (7.9%). The emetic gene *cesB* was not detected. The standardized multiplex PCR allowed the detection of four toxigenic consortia, differing in the pattern of toxic genes present in the samples (Table 2).

Thirty three (44%) of 75 cassava starch samples were contaminated with toxigenic *B. cereus*. The most predominant toxin gene was *hblC* (44%), followed by *cytK* (36%) and *nheA* (22.7%). Unlike dietary complement for children samples, the emetic gene *cesB* was detected in 1.3% of cassava starch samples. According to the pattern of toxic genes determined in the samples, four different toxigenic consortia were established (Table 3).

The results of toxin gene consortia present in dietary complement for children and cassava starch samples are in agreement with those previously reported for toxigenic *B. cereus* detected directly by multiplex PCR in other foods (8, 15). In India, a study in meat and meat products evaluated the presence of *hblA*, *hblC*, *hblD*, *nheA*, *nheB*, *nheC*, *cytK*, and

Table 2. Toxigenic consortia in dietary complement for children samples.

Toxin gene consortia	Positive samples n (%)	Genes
I	5 (9.8)	*nheA*, *hblC*, *cytK*
II	1 (2)	*nheA*, *hblC*
III	22 (41.2)	*hblC*, *cytK*
IV	25 (47)	hblC

Table 3. Toxigenic consortia in cassava starch samples.

Toxin gene consortia	Positive samples n (%)	Genes
I	16 (48.5)	*nheA*, *hblC*, *cytK*
II	1 (3)	*nheA*, *hblC*, *cytK*, *cesB*
III	10 (30.3)	*hblC*, *cytK*
IV	6 (18.2)	hblC

entFM genes. Six toxigenic consortia were detected: Group I (50.84%), all eight genes were detected; Group II (10.16%) and Group III (8.47%), the foods lacked *hbl* complex (*hblCDA*) and *cytK*, respectively; Group IV (16.94%), foods not containing the *hbl* complex and *cytK*; Group V (6.78%), foods in which none, one, two or all the three genes of the *hbl* complex were present but *cytK*, *entFM* and at least one gene of *nhe* complex (*nheABC*) were present; Group VI, similar to Group V but lacking *cytK* gene (15). In a study conducted in Kenya, the presence of *hblA*, *hblC*, *hblD*, *nheA*, *nheB*, *nheC*, and emetic toxin genes were evaluated by multiplex PCR directly in ready-to-eat foods such as rice and milk. Emetic toxin genes and the *hblC* gene were detected in rice while in milk *nheA*, *hblD*, and *hblC* genes were identified (8).

Only the toxin gene consortium II of cassava starch presented the *cesB* gene, which is in agreement with previous studies that indicated that the detection of the emetic toxin was rare (16). Its production seems to be restricted to a particular lineage of *B. cereus* (4).

Few studies have been carried out using multiplex PCR for direct detection of *B. cereus* in foods, and usually the toxigenic potential of *B. cereus* strains is determined by strain isolation from foods. In a study in Korea, *B. cereus* isolates from cereal presented a high frequency of *nheA* (99%) and *hblDC* (84%) genes, but *cytK* gene was less frequent (55%) (17). In Belgium, various strains of the *B. cereus* group were isolated from marketed food products, none harbored the *ces* gene required for the production of the emetic toxin, but 52.5% strains carried all seven genes required for the production of the diarrhoeal enterotoxins: haemolytic BL, non-haemolytic enterotoxin and cytotoxin K (18).

In United States 47 (56.6%) *B. cereus* isolates from rice contained the *hblA* and *hblD* genes and 74 (89.1%) isolates the *nheA* and *nheB*, but the *ces* gene was not detected in any of the isolates (19). In Argentina, of 132 *B. cereus* isolates from honey, 42% harbored the *hblABCD* genes, 53% the *cytK* gene and 73% the *bceT* (20), the latter gene encodes the BceT enterotoxin and its biological activity is not clear yet, therefore, it was not evaluated in the present study (2). In Brazil, 97 foodborne *B. cereus* sensu stricto strains isolated in the 1980's, 1990's and 2000's were analyzed. The *nhe* genes were detected in 84.5% strains and *hbl* genes in 62.9% strains; all strains were negative for *ces* and the *cytK-2* gene was found in 45.4% strains. The predominant toxigenic pattern included enterotoxin genes positive strains but no *ces* (21).

In countries such as Korea, emetic strains have been isolated from grain, korean rice cake (22) and sunsik (23). It is known that emetic strains have been reported in oriental countries where the emetic syndrome has been associated with the consumption of rice (2). In a study in Argentina only one emetic strain in cooked chicken was reported (24) and this was the first report of emetic strains in the Americas. The finding in our study of a sample containing the *cesB* gene (consortia II) in a cassava starch sample suggest that frequencies of *ces* containing strains may be low in Medellin, Colombia, and possibly this is the case for other Latinamerican countries. However, more studies are needed to prove this hypothesis.

Conclusions

The predominant consortium found in dietary complement for children included *hblC* and *cytK* genes and the predominant consortium found in cassava starch included *nheA*, *hblC* and *cytK* genes. In general, in dietary complement for children only enterotoxigenic consortia were detected while in cassava starch the enterotoxigenic consortia predominated over the emetic.

The standardized multiplex PCR can be used to test contamination of dried foods by toxigenic *B. cereus* by simultaneous detection of all toxin genes directly in foods such as dietary complement for children and cassava starch. This assay will help to optimize the time and resources in the laboratory and serves the bases for carrying out survey or studies directed to better understand the epidemiological risk represented by toxigenic *B. cereus*.

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