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 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 patógeno 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 enterotoxigenicos, mientras que en el almidón los consorcios enterotoxigenicos 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 patogênico nos seres humanos ocasionando síndromes de vômitos e diarreia. Neste estudo foi avaliada a presença de *B. cereus* toxigênicos por PCR multiplex diretamente em suplementos 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 enterotoxigenicos, não obstante no amido os consórcios enterotoxigenicos 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 cereulein or emetic toxin (2), which is encoded by the ces gene 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.

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**, **hneA**, **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 MgCl₂, 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.

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Primer</th>
<th>Primer sequence (5’→ 3’)</th>
<th>Product size (bp)</th>
<th>Primer position*</th>
<th>GenBank accession number</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>hblC</strong></td>
<td><strong>hblCF</strong> 1318</td>
<td>CGAAAAATTAGGTCGCCGAAT</td>
<td>411</td>
<td><strong>hblCR</strong> 1728</td>
<td>U63928</td>
<td>(10)</td>
</tr>
<tr>
<td></td>
<td><strong>hblCR</strong> 1728</td>
<td>TAATATGCCCTGGCCGATTTG</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>nheA</strong></td>
<td><strong>nheAF</strong> 430</td>
<td>ACGAATGTAATTTGAGTGCCTC</td>
<td>755</td>
<td><strong>nheAR</strong> 1185</td>
<td>Y19005</td>
<td>(11)</td>
</tr>
<tr>
<td></td>
<td><strong>nheAR</strong> 1185</td>
<td>TGCGTAAGGGGGAAGGGG</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>cesB</strong></td>
<td><strong>cesF</strong> 21816</td>
<td>GGTGACACATTATCATATAAGGTG</td>
<td>1271</td>
<td><strong>cesR</strong> 23087</td>
<td>DQ360825</td>
<td>(12)</td>
</tr>
<tr>
<td></td>
<td><strong>cesR</strong> 23087</td>
<td>CGTAGCCAGGCTCGTGTTGAAACA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>cytK</strong></td>
<td><strong>cytKF2</strong> 286</td>
<td>CGAGCTACAGAATGGTGAACA</td>
<td>565</td>
<td><strong>cytKR2</strong> 850</td>
<td>AJ318876.2</td>
<td>(13)</td>
</tr>
<tr>
<td></td>
<td><strong>cytKR2</strong> 850</td>
<td>CGTGTGTAAATACCCCAGTT</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>16S rDNA</strong></td>
<td><strong>16S rDNAF</strong></td>
<td>AGATTGTAGATCTGGCTCA</td>
<td>1514</td>
<td><strong>16S rDNAR</strong> 10713-10696</td>
<td>CP001407.1</td>
<td>(14)</td>
</tr>
</tbody>
</table>

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

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* ATCC 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 discussion

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.**

<table>
<thead>
<tr>
<th>Toxin gene consortia</th>
<th>Positive samples n (%)</th>
<th>Genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>5 (9.8)</td>
<td>nheA, hblC, cytK</td>
</tr>
<tr>
<td>II</td>
<td>1 (2)</td>
<td>nheA, hblC</td>
</tr>
<tr>
<td>III</td>
<td>22 (41.2)</td>
<td>hblC, cytK</td>
</tr>
<tr>
<td>IV</td>
<td>25 (47)</td>
<td>hblC</td>
</tr>
</tbody>
</table>
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 nheABC 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 (consortium II) in a cassava starch sample suggest that frequencies of ces containing strains may be low in Medellín, Colombia, and possibly this is the case for other Latinamerican countries. However, more studies are needed to prove this hypothesis.

### Table 3. Toxigenic consortia in cassava starch samples.

<table>
<thead>
<tr>
<th>Toxin gene consortia</th>
<th>Positive samples n (%)</th>
<th>Genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>16 (48.5)</td>
<td>nheA, hblC, cytK</td>
</tr>
<tr>
<td>II</td>
<td>1 (3)</td>
<td>nheA, hblC, cytK, cesB</td>
</tr>
<tr>
<td>III</td>
<td>10 (30.3)</td>
<td>hblC, cytK</td>
</tr>
<tr>
<td>IV</td>
<td>6 (18.2)</td>
<td>hblC</td>
</tr>
</tbody>
</table>

### 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.

### Acknowledgements

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### References


16. Altayar, M.; Sutherland, A. Bacillus cereus is common in the environment but emetic toxin producing isolates are rare. *J Appl Microbiol.* 2006. 100(1): 7-14.


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