

Research Paper

Gene detection and toxin production evaluation of hemolysin BL of *Bacillus cereus* isolated from milk and dairy products marketed in Brazil

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Abstract

Bacillus cereus is an ubiquitous, spore-forming bacterium that can survive pasteurization and the majority of the heating processes used in the dairy industry. Besides, it is a pathogen responsible for different types of food poisoning. One type of foodborne disease caused by *B. cereus* is the diarrheal syndrome, which is caused by the ingestion of vegetative cells producing toxins in the small intestine. One virulence factor for the diarrheal syndrome is the toxin hemolysin BL (HBL), a three-component protein formed by the L₁, L₂ and B components. In order to evaluate the presence of diarrheal strains isolated from milk and dairy products, 63 *B. cereus* isolates were obtained from 260 samples of UHT milk, pasteurized milk and powdered milk, sold in commercial establishments and from different brands. The isolates were subjected to the Polymerase Chain Reaction (PCR) for the detection of the encoding genes for the L₁, L₂ and B components and the toxin production capacity was evaluated with an immunoassay. A total of 23 [36.5%] isolates were identified carrying simultaneously the three tested genes, from which, 20 [86.9%] showed toxigenic capacity. 26 [41.3%] isolates did not carry any of the genes tested and the other 14 [22.2%] were positive for one or two of them. The results showed a high toxigenic capacity among the *B. cereus* isolates able to produce the HBL, indicating a potential risk for consumers.

Key words: milk, dairy products, *Bacillus cereus*, pathogenicity, detection.

Introduction

Bacillus cereus is a widely distributed bacterium that can be found in the soil and in the water. Different kinds of food and food products can be contaminated with the microorganism, including milk and dairy products, meat, rice, pasta, herbs and condiments (Kramer and Gilbert, 1989; Arnesen *et al.*, 2008). *B. cereus* is able to produce a wide range of potentially pathogenic substances including hemolysins, phospholipase C, metalloproteases, collagenases, beta-lactamases and enterotoxins. The complete virulence importance of these substances has not yet been completely elucidated (Martinez-Blanch *et al.*, 2009). Two different foodborne diseases are attributed to *B. cereus*:

emetic syndrome and diarrheal syndrome. Both diseases have mild manifestation and, in most cases, are self-limited, although severe cases resulting in the death of the patient have been reported (Mahler *et al.*, 1997).

The diarrheal syndrome is caused by the ingestion of vegetative cells of *B. cereus* present in food, and once the microorganism reaches the small intestine it starts the colonization and then the toxin production (Arnesen *et al.*, 2008). The production of the diarrheal toxins occurs, in its most, at the exponential growth phase of *B. cereus* (Kotiranta *et al.*, 2000). The mechanism of action of such toxins is not completely known, but it is believed that the diarrhea is caused by the formation of pores in the cellular membrane, what induces the loss of Na⁺ and Cl⁻ ions and water, resulting in

an electrolyte imbalance (Bhunia, 2008). The symptoms are abdominal pain, cramps and watery diarrhea that start in 8 to 16 hours after the ingestion of contaminated food lasting for 12 to 24 hours. The foods most associated to the diarrheal syndrome are milk and dairy products, vegetables and beef (Kotiranta *et al.*, 2000).

The toxins that are considered the main virulence factors of the diarrheal syndrome are the hemolysin BL (HBL), nonhemolytic enterotoxin (NHE) and cytotoxin K (CytK). In addition enterotoxin FM (EntFM), enterotoxin T (BceT) and hemolysin II (Hly II) were also described as potential diarrheal toxins, although there are no data showing that they can cause food poisoning (Kotiranta *et al.*, 2000; Hendriksen *et al.*, 2006). HBL is a three-component toxin consisting of two lytic proteins, L₁ and L₂, that are encoded by *hblD* and *hblC* genes respectively, and a binding component B encoded by *hblA* gene. The presence of all three components is necessary for the toxin activity (Lindback and Granum, 2006).

The distribution of HBL genes within the *B. cereus* species is quite diverse and strains show different capability of producing diarrheal toxins. Several factors can group such strains based on growth temperature, food matrix and nutritional availability to name a few (Carlin *et al.*, 2010).

The aim of this study was to evaluate the presence of the HBL encoding genes and the toxin production of *B. cereus* cultures isolated from milk and dairy products commercialized in the Brazil.

Materials and Methods

B. cereus isolation and identification

Two hundred and sixty samples of dairy products were analyzed (pasteurized milk, UHT milk and powdered milk). The samples were cultured in selective agar plates and then confirmed as *B. cereus* by biochemistry tests according to previously described methods (Silva *et al.*, 1997). Strain NVH 1230/88 (provided by Dr. Per Einar Granum) is known to contain the HBL genes and was used as positive control.

DNA isolation

A total of sixty three *B. cereus* isolates were cultured in nutrient agar and then extraction of genomic DNA were made according to the protocol adapted from Moreira *et al.* (2010). The concentration of the obtained DNA were determined using a spectrophotometer (Nanodrop 2000, Thermo Scientific, Wilmington-MA, USA) and dilutions were made, when necessary, to set the concentration at 100 µg/mL.

PCR amplification

A PCR mixture (25 µL) were prepared according to Aragon-Alegro *et al.* (2008) and consisted of 100 ng of DNA template, 0.2 mM dNTP mix, 2.5 mM MgCl₂, 500 nM of each primer, 0.75 U of Taq polymerase and Taq buffer (750 mM Tris-HCl [pH 8.8 at 25 °C], 200 mM (NH₄)₂SO₄, and 0.1% Tween 20). All reagents were purchased from Fermentas (Burlington, Ontario, Canada). The primers and annealing temperatures used in this study, found in Table 1, were those from Guinebretière *et al.* (2002). Amplifications were carried out in a PCR Maxygene (Axygen, Union City-CA, USA) thermocycler with the following run: a starting cycle of 2 min at 94 °C, followed by 35 cycles of 1 min at 94 °C, 1 min at the annealing temperature, and 2 min at 72 °C, and a final extension of 5 min at 72 °C (Guinebretière *et al.*, 2002).

Toxin production analysis

HBL production was evaluated by the isolates carrying simultaneously *hblACD* genes using the BCET-RAPLA kit (Oxoid Ltd., Basingtoke, England) following the manufacturer's instruction. Shortly, the isolates were cultured in brain heart infusion broth (Merck, Whitehouse Station-NJ, USA) at 37 °C for 24 h. Then 2 mL of each culture were centrifuged at 4 °C at 900 g for 20 min and applied to the test devices. A sample was considered positive when showed distinct agglutination pattern.

Results and Discussion

From the 63 isolates of *B. cereus* obtained (36 from pasteurized milk, 15 from powdered milk and 12 from UHT milk), for the HBL encoding genes, were found 23 [35.6%]

Table 1 - Characteristics of primers used in this study.

Primer	Gene	Annealing temp (°C)	Product size (bp)	Sequence (5-3)
HA F	<i>hblA</i>	56	1.154	AAGCAATGGAATACAATGGG
HA R				AGAATCTAAATCATGCCACTGC
HC F	<i>hblC</i>	58	740	GATAC(T,C)AATGTGGCAACTGC
HC F				TTGAGACTGCTCG(T,C)TAGTTG
HD R	<i>hblD</i>	58	829	ACCGGTAACACTATTCATGC
HD F				GAGTCCATATGCTTAGATGC

Source: Guinebretière *et al.*, 2002.

isolates carrying simultaneously the *hblACD* genes, 37 [58.7%] isolates were positive for at least one of them and 26 [41.3%] isolates didn't harbor any of the tested genes. Individually, *hblA* gene was detected in 26 [41.3%] isolates, 34 [54%] isolates were positive for *hblC* gene and the same result was observed for *hblD* gene (Figure 1).

Many studies have already been done to evaluate the prevalence of pathogenic genes of *B. cereus*, but very few of them are aimed in milk and/or dairy products. One of the most recent ones were developed by Rather *et al.* (2011) in India addressing raw and pasteurized milk and the detection of HBL genes. In that study the percentages of detection for *hblA*, *hblC* and *hblD* were around 70% for the tested samples. Another study made in Thailand by Chitov *et al.* (2008) using milk showed detection percentages of *hblA*, *hblC* and *hblD* genes around 60%. In Brazil, Aragon-Alegro *et al.* (2008) analyzed different types of food, including dairy products, for the presence of *hblA*, *hblC* and *hblD* genes the genetic detection was lower than 40% for *hblA* and *hblC* and *hblD* was detected in 70.6% of the isolates tested. In all of the mentioned studies, including this present one, the gene with the higher detection rate was *hblD*, which could indicate that, in milk and dairy products, *hblD* is the most widely distributed HBL gene of *B. cereus*.

After the gene detection, the 23 isolates carrying the *hblACD* genes simultaneously (14 from pasteurized milk and 09 from powdered milk) were tested with the BCET-RAPLA kit (Oxoid) for the expression of HBL. From that total, 20 [86.9%] isolates were positive for the test, statistical analysis (Table 2) detected no difference between the PCR and the immunoassay positivity, showing a good cor-

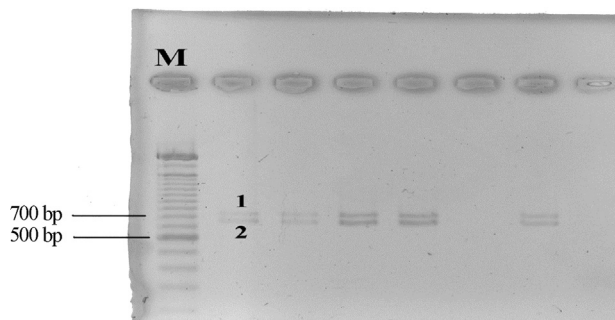


Figure 1 - 1.5% agarose gel of duplex PCR amplification of *hblC* and *hblD* genes (M: 1500 bp marker; 1: amplified *hblC* fragment; 2: amplified *hblD* fragment).

Table 2 - Detection of *hblACD* complex and HBL expression.

Product	Presence of <i>hblACD</i> complex	Expression of HBL
Pasteurized milk	14/36 ^a	13/36 ^a
Powdered milk	9/15 ^a	8/15 ^a
UHT milk	0/12 ^b	0/12 ^b
Total	23/63	21/63

relation between the methods. Among the different dairy products 13 [92.9%] isolates from pasteurized milk were positive for the immunoassay and 08 [88.9%] of the powdered milk cultures were positive for the test. There was also no statistical difference between the positivity observed in the pasteurized milk and the powdered milk. None of the *B. cereus* cultures isolated from UHT milk showed the *hblACD* genes, this could show that there is a higher susceptibility of pathogenic *B. cereus* strains to UHT treatment. Though the immunoassay is able to detect a very small concentration of HBL (2 ng/mL) negative results could represent expression of the toxin at levels below the sensitivity of the kit (Svensson *et al.*, 2006) in the same way that eventual mutation on the pathogenic gene could interfere with the primer specificity (Granum, 2005; Didelot *et al.*, 2009; Oh *et al.*, 2011).

Several circumstances entails the presence of *B. cereus* in milk and dairy products. Since its natural habitat is the soil it is virtually impossible to eliminate the microorganism from the environment where the cows walk around in the dairy farms. However, hygiene procedures such as adequate equipment sanitization, sanitary milking, proper hygiene of the employees during the milking of the cows among other best practices showed to be very effective in the quality control of raw milk in the same way, the negligence of these steps reflects significantly in the increase of the microorganism (Te Giffel *et al.*, 1997; Magnusson *et al.*, 2007; Bartoszewicz *et al.*, 2008; Shaheen *et al.*, 2010).

Concerning the industry the same principles are equivalents since there is a considerable contamination rate of milk post pasteurization. The best practice procedures include a broad scope of critical points of control that goes to attention to the temperatures used in the pasteurization process until the right products used for the cleaning of the equipments, all of this steps work together to ensure the elimination of *B. cereus* (Reys *et al.*, 2007; Salustiano *et al.*, 2009).

In conclusion, the data described in this study shows a high expression of the diarrheal toxin HBL of *Bacillus cereus* isolated in pasteurized milk and powdered milk showing that the pathogenic strains of the microorganism are well adapted for toxins production on these products.

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References

- Aragon-Alegro LC, Palcich C, Lopes GV, Ribeiro VB, Landgraf M, Destro MT (2008) Enterotoxigenic and Genetic Profiles of *Bacillus cereus* Strains of Food Origin in Brazil. *J Food Prot* 71:2115-2118.

- Arnesen LPS, Fagerlund A, Granum PE (2008) From soil to gut: *Bacillus cereus* and its food poisoning toxins. *Microbiol Rev* 32:579-606.
- Bartoszewicz M, Hansen BM, Swiecicka I (2008) The members of the *Bacillus cereus* group are commonly present contaminants of fresh and heat-treated milk. *Food Microbiol* 25:588-596.
- Bhunia AK (2008) *Bacillus cereus* and *Bacillus anthracis*. In: Bhunia, A.K. *Foodborne Microbial Pathogens: Mechanisms and Pathogenesis*. West Lafayette, Springer. p.135-147.
- Carlin F, Brillard J, Broussole V, Clavel T, Dupont C, Jobin M, Guinebretière MH, Auger S, Sorokine A, Nguyen-The C (2010) Adaptation of *Bacillus cereus*, an ubiquitous worldwide-distributed foodborne pathogen, to a changing environment. *Food Res Int* 43:1885-1894.
- Chitov T, Dispan R, Kasinrerker W (2008) Incidence and diarrhegenic potential of *Bacillus cereus* in pasteurized milk and cereal products in Thailand. *J Food Saf* 28:467-481.
- Didelot X, Barker M, Falush D, Priest FG (2009) Evolution of pathogenicity in the *Bacillus cereus* group. *Syst Appl Microbiol* 32:81-90.
- Granum PE (2005) *Bacillus cereus*. In: Fratamico, P.M.; Bhunia, A.K.; Smith, J.L. *Foodborne Pathogens - Microbiology and Molecular Biology*. Norfolk: Caister Academic Press. p. 409-419.
- Guinebretière MH, Broussole V, Nguyen-The C (2002) Enterotoxigenic profiles of food-poisoning and food borne *Bacillus cereus* strains. *J Clin Microbiol* 40:3053-3056.
- Hendriksen NB, Hansen BM, Johansen JE (2006) Occurrence and pathogenic potential of *Bacillus cereus* group bacteria in a sandy loam. *Antonie van Leeuwenhoek*. 89:239 -249.
- Kotiranta A, Lounatmaa K, Haapasalo M (2000) Epidemiology and pathogenesis of *Bacillus cereus* infections. *Microbes Infect* 2:189-198.
- Kramer JM, Gilbert RJ (1989) *Bacillus cereus* and other *Bacillus* species. In: Doyle, M.P. (ed). *Foodborne Bacterial Pathogens*. Marcel Dekker, New York, USA, p.21-70.
- Lindback T, Granum PE (2006) Detection and Purification of *Bacillus cereus* Enterotoxins. In: Adley, C.C. *Food-Borne Pathogens: Methods and Protocols*. Totawa, Humana Press. p. 15-24.
- Magnusson M, Bertilsson J, Svensson B (2007) *Bacillus cereus* spores during housing of dairy cows: factors affecting contamination of raw milk. *J Dairy Sci* 90:2745-2754.
- Mahler H, Pasi A, Kramer JM, Schulte P, Scoging AC, Bär W, Krähenbul S (1997) Fulminant liver failure in association with the emetic toxin of *Bacillus cereus*. *N Engl J Med* 336:1142-1148.
- Martínez-Blanch JF, Sánchez G, Aznar R (2009) Development of a real-time PCR assay for detection and quantification of enterotoxigenic members of *Bacillus cereus* group in food samples. *Int J Food Microbiol* 135:15-21.
- Moreira M, Noschgang J, Neiva IF, Carvalho Y, Higuti IH, Vicente VA (2010) Methodological variations in the isolation of genomic from *Streptococcus* bacteria. *Braz Arch Biol Technol* 53:845-849.
- Oh MH, Ham JS, Cox JM (2011) Diversity and toxigenicity among members of the *Bacillus cereus* group. *Int J Food Microbiol* 152:1-8.
- Rather MA, Aulakh RS, Gill JPS, Verma R, Rao TS (2011) Enterotoxigenic profile of *Bacillus cereus* strains isolated from raw and pasteurized milk. *Indian J Anim Sci* 81:448-452.
- Reys JE, Bastías JM, Gutiérrez MR, Rodríguez MO (2007) Prevalence of *Bacillus cereus* in dried milk products used by Chilean School Feeding Program. *Food Microbiol* 24:1-6.
- Salustiano VC, Andrade NJ, Soares NFF, Lima JC, Bernardes PC, Luiz LMP, Fernandes PE (2009) Contamination of milk with *Bacillus cereus* by post-pasteurization surface exposure as evaluated by automated ribotyping. *Food Control* 20:439-442.
- Shaheen R, Svensson B, Andersson MA, Anders C, Christiansson A, Salkinoja-Salonen M (2010) Persistence strategies of *Bacillus cereus* spores isolated from dairy silo tanks. *Food Microbiol* 27:347-355.
- Silva N, Junqueira VCA, Silveira NFA (1997) Manual de métodos de análise microbiológica de alimentos. In: *Contagem de Bacillus cereus*. 1ª ed. Livraria Varela, São Paulo-SP. p. 59-64.
- Svensson B, Monthán A, Shaheen R, Andersson MA, Salkinoja-Salonen M, Christiansson A (2006) Occurrence of emetic toxin producing *Bacillus cereus* in the dairy production chain. *Int Dairy J* 16:740-749.
- Te Giffel MC, Beumer RR, Granum PE, Rombouts FM (1997) Isolation and characterization of *Bacillus cereus* from pasteurized milk in household refrigerators in the Netherlands. *Int J Food Microbiol* 34:307-318.