Food Microbiology 46 (2015) 195–199

Contents lists available at ScienceDirect

Food Microbiology

journal homepage: www.elsevier.com/locate/fm

Short communication

A case of intoxication due to a highly cytotoxic *Bacillus cereus* strain isolated from cooked chicken

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ARTICLE INFO

Article history:
Received 31 March 2014
Received in revised form
11 August 2014
Accepted 14 August 2014
Available online 27 August 2014

Keywords:
Bacillus cereus
Epithelial cells
Virulence
Genes
Detachment
Necrosis
Food poisoning

ABSTRACT

Outbreaks of *Bacillus cereus* infection/intoxication are not commonly reported because symptoms are often mild, and the disease is self-limiting. However, hypervirulent strains increase health risks. We report a case, which occurred in Argentina, of severe food poisoning illness on a healthy adult woman associated to *B. cereus* strain MVL2011. The studied strain was highly cytotoxic, showed high ability to detach Caco-2 cells and was positive for the hblA, hblB, and hblC genes of the hbl complex, bceT, entS and ces. As it is considered that *B. cereus* emetic cluster evolved from a panmictic population of diarrheal strains, *B. cereus* MVL2011 could constitute an intermediate strain between diarrheal and emetic strains.

1. Introduction

Bacillus cereus is a spore-forming rod-shaped bacterium, commonly present in food. It is an opportunistic microorganism widely recognized as the etiological agent of food-borne outbreaks (emetic and diarrheic syndromes) as well as non-intestinal pathologies (Kramer and Gilbert, 1992; Stenfors Arnesen et al., 2008). Emesis is caused by cereulide (Ehling-Schulz et al., 2005a), whereas diarrhea probably involves diverse extracellular factors (Stenfors Arnesen et al., 2008). The virulence of *B. cereus*, whether intestinal or non-intestinal, is intimately associated with the production of tissue destructive/reactive proteins (Bottone, 2010). Two of these virulence factors are protein complexes, i.e. hemolysin BL (HBL) and the non-hemolytic enterotoxin NHE (Guinebretiere et al., 2002). Other virulence factors are single proteins, i. e. entFM (enterotoxin FM), cytK (cytolysin K) and bceT (*B. cereus* enterotoxin) (Fagerlund et al., 2004; Kramer and Gilbert, 1992; Stenfors Arnesen et al., 2008), phosphatidylinositol specific phospholipase (PI-PLC), enterotoxin S (EntS), sphingomyelinase (SMase), cereolysin O (Clo), InhA1, NprA and HlyII (Cadot et al., 2010; Kramer and Gilbert, 1992; Stenfors Arnesen et al., 2008). Moreover, other factors such as adhesion to and invasion of epithelial cells also play a role in the biological effects of *B. cereus* strains (Minnaard et al., 2004, 2007, 2013).

B. cereus symptoms are usually mild (Kramer and Gilbert, 1992) and the pathology is not commonly reported. However more severe cases including fatal outcomes have increased in the last few years (Al-Abri et al., 2011; Bottone, 2010; Dierick et al., 2005; Mahler et al., 1997; Naranjo et al., 2011; Saito et al., 2010; Shiota et al., 2010). In Argentina, diagnostic testing for *B. cereus* is not routinely performed for patients with gastrointestinal diseases. Nevertheless, statistical data provided by the Health Ministry (2008–2013) showed 5783 cases of food-borne disease, of which 26 were positive for *B. cereus* and 9 occurred in 2011 (year of highest incidence).

In this context the aim of the present work was the isolation, identification and characterization of the etiologic agent of a food poisoning episode associated to consumption of chicken by a young healthy woman.

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http://dx.doi.org/10.1016/j.fm.2014.08.005
0740-0020/

2. Materials and methods

2.1. Case presentation

In Buenos Aires, Argentina, a 39-year-old healthy woman was hospitalized due to dehydration. She had purchased chicken stuffed with carrots, eggs, pepper and cheese which had been refrigerated immediately and the following day (for lunch) warmed in the microwave oven. One hour after eating, she left home for sport activities (5 km running). Ten hours after eating, she started vomiting and had 5 episodes of watery diarrhea. After 3 h from onset, the symptoms had not subsided, and the doctor ordered oral rehydration and rest. However, diarrhea and vomiting continued for further 5 h, which resulted in severe dehydration and required 7 h hospitalization for reposition of water and electrolytes. Analyses performed on faeces and vomitus did not detect the presence of *Escherichia coli*, *Shigella* spp, *Staphylococcus aureus* or *Salmonella* spp.

The rapid onset of symptoms, in conjunction with the results of the microbiological analysis, suggested *B. cereus* as the most likely etiological agent for this case.

2.2. Isolation of bacteria

Two chicken samples (10 g) were diluted 1:10 in sterile distilled water and homogenized. Ten microliters were spread on polymyxin–pyruvate–egg-yolk–mannitol agar (PEMBA) plates (Holbrook and Andersson, 1980), which were incubated at 32 °C for 48–96 h until bacterial growth was detected (Lancette and Harmon, 1980). Gram staining and determination of catalase activities were performed. Colony appearance regarding shape, color, type of growth and presence of opaque halos due to lecithinase activity were also evaluated.

2.3. Bacterial identification

The identity of the bacterial isolates was confirmed by using API 20E and API 50CH strips plus API 50CHB medium (Biomerieux, France) and data base Apiweb (Biomerieux, www.biomerieux.com). In addition, starch hydrolysis, hemolytic activity and production of a discontinuous hemolytic pattern on blood agar plates according to standard protocols (Beecher and Wong, 1994) were tested.

2.4. Detection of enterotoxin genes

Total genomic DNA was isolated from 24 h-cultures grown on TSA using the procedure previously described by López and Alippi (2007). Presence of sequence associated to virulence genes were assessed as previously reported (Minnaard et al., 2007). Genes encoding for enterotoxin-T (bceT) (Guinebretiere et al., 2002), cytotoxin K (cytk) (Ehling-Schulz et al., 2006), sphingomyelinase (sph), enterotoxin FM (entFM), enterotoxin S (entS), phosphatidylinositol, phospholipase C (piplC) (Ghelardi et al., 2002), the components of HBL (hemolysin BL: hblA, hblB, hblC, hblD) and NHE (non-hemolytic enterotoxin: nheA, nheB, nheC) complexes (Guinebretiere et al., 2002) were studied. In addition, the isolated was assessed for the *ces* gene related to the production of cereulide (Ehling-Schulz et al., 2005a). DNA amplifications were performed in a thermal cycler (Mastercycler personal; Eppendorf Hamburg, Germany). Amplicons were analyzed by 1.6% (W/V) agarose gel, in TBE buffer, stained with Gel Red® (Biotium, U.S.A) for 2 h at 80 V.

2.5. Culture of epithelial cells

Caco-2 cells (Fogh and Orfeo, 1977) were routinely grown in Dulbecco's modified Eagle's minimum essential medium (DMEM) (25 mM glucose) (Life Technologies, Cergy, France), supplemented with 15% (v/v) heat-inactivated (30 min, 56 °C) fetal calf serum (FCS) (PAA Laboratories GmbH, Pasching, Austria) and 1% (v/v) non-essential amino acids (Life Technologies, Cergy, France). Monolayers were prepared in 24-tissue culture plates (Greiner Bio One, Frickenhausen, Germany) by seeding 2.5×10^4 cells per well. Experiments and cell maintenance were carried out at 37 °C in a 5% CO_2/95% air atmosphere. Cells at late post confluence (15 days in culture) were used.

Hep-2 cells were routinely grown in Dulbecco's modified Eagle's medium (DMEM, 25 mM glucose, Life Technologies, Carlsbad, CA, USA), supplemented with 10% (v/v) heat-inactivated (56 °C, 30 min) fetal calf serum (FCS, PAA Laboratories, Pasching, Austria), 12 IU/ml penicillin-12 μg/ml streptomycin (Life Technologies) and 1% (v/v) non-essential amino acids (Life Technologies). Cells were seeded at 6.2×10^4 cells per well in 48-well tissue culture plates (Greiner Bio One, Frickenhausen, Germany) and incubated for 48 h at 37 °C in 5% CO_2.

2.6. Detachment of Caco-2 cells

Detachment of enterocyte-like cells was performed as previously reported (Minnaard et al., 2001). Briefly, differentiated Caco-2 monolayers were incubated at 37 °C for 1 h with 0.5 ml of serial dilutions of culture filtrate supernatants (CFS, pH 6.8) from 16 h-old bacteria cultures at 32 °C in BHIG (BHI broth (BIOKAR Diagnostics) supplemented with 0.1% (w/v) glucose). Cells were washed twice with phosphate buffered saline (PBS) (pH 7.2), fixed at room temperature for 1 min with 2% (v/v) formaldehyde in PBS and washed again with PBS. Afterward, cells were stained by incubating for 20 min at room temperature with 500 μl of a crystal violet solution (0.13% (w/v) crystal violet, 5% (v/v) ethanol and 2% (v/v) formaldehyde in PBS). After washing to remove stain excess, samples were treated with freshly prepared 50% (v/v) ethanol at room temperature for 1 h. Absorbance was measured in a microplate reader at 620 nm (Biotek Instruments, Winooski, USA). Percentage of cell detachment was calculated as follows:

$$\text{Cell detachment } \% = 100 \times (\text{Ac} - \text{As})/\text{Ac},$$

where Ac : A_{620} of control cells and As : A_{620} of sample cells.

2.7. Necrosis

Assessment of necrosis was performed as reported previously (López et al., 2013). Briefly, Caco-2 monolayers were incubated with serial dilutions of CFS as describe above. After 1 h of incubation, wells with monolayer not detached were washed twice with binding buffer containing 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 125 mM NaCl, 2.5 mM $CaCl_2$, (pH 7.2), and 0.2% (p/v) gelatin. Afterward, 1 μg of propidium iodide was added in 100 μl of binding buffer per well, and cells were incubated on ice for 15 min. Then, samples were mounted in 50% (v/v) glycerol in PBS and analyzed by conventional fluorescence microscopy using a Leica DMLB microscope coupled to a Leica DC 100 camera (Leica Microscopy Systems, Heerbrugg, Switzerland).

2.8. Mitochondrial dehydrogenase activity

Activity of mitochondrial dehydrogenases was determined by assessing the reduction of 3-(4, 5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium (MTT) by means of modification of a previously reported protocol (Finlay et al., 1999; Minnaard et al., 2007).

Briefly, autoclaved (15 min at 121 °C) or non-heated CFS diluted in DMEM with 2% methanol were added to each well of Hep-2 cells and incubated 24 h at 37 °C in 5% CO_2. CFS (autoclaved or non-