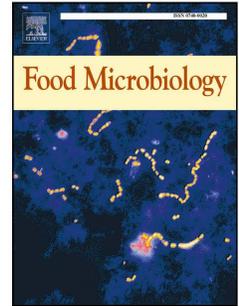# Accepted Manuscript

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

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2 Short Communication

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4 **A case of intoxication due to a highly cytotoxic *Bacillus cereus* strain**

5 **isolated from cooked chicken**

6

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26

27 **Abstract**

28

29 Outbreaks of *Bacillus cereus* infection/intoxication are not commonly reported because

30 symptoms are often mild, and the disease is self-limiting. However, hypervirulent strains

31 increase health risks. We report a case, which occurred in Argentina, of severe food

32 poisoning illness on a healthy adult woman associated to *Bacillus cereus* strain MVL2011.

33 The studied strain was highly cytotoxic, showed high ability to detach Caco-2 cells and was

34 positive for the hblA, hblB, and hblC genes of the hbl complex, bceT, entS and ces. As it is

35 considered that *B. cereus* emetic cluster evolved from a panmictic population of diarrhoeal

36 strains, *B. cereus* MVL2011 could constitute an intermediate strain between diarrhoeal and

37 emetic strains.

38

39 *Keywords: Bacillus cereus,* ephitelial cells, virulence, genes, detachment, necrosis, food

40 poisoning

41

42 **Introduction**

43

44   *Bacillus cereus* is a spore-forming rod-shaped bacterium, commonly present in

45 food. It is an opportunistic microorganism widely recognized as the etiological agent of

46 food-borne outbreaks (emetic and diarrheic syndromes) as well as non-intestinal

47 pathologies (Kramer and Gilbert, 1992; Stenfors Arnesen et al., 2008). Emesis is caused by

48 cereulide (Ehling-Schulz et al, 2005a), whereas diarrhoea probably involves diverse

49 extracellular factors (Stenfors Arnesen et al., 2008). The virulence of *B. cereus*, whether

50 intestinal or non-intestinal, is intimately associated with the production of tissue

2

51 destructive/reactive proteins (Bottone, 2010). Two of these virulence factors are protein

52 complexes, i.e. hemolysin BL (HBL) and the non-haemolytic enterotoxin NHE

53 (Guinebretiere et al., 2002). Other virulence factors are single proteins, i. e. entFM

54 (enterotoxin FM), cytK (cytolysin K) and bceT (*B. cereus* enterotoxin) (Fagerlund et al,

55 2004, Kramer and Gilbert, 1992; Stenfors Arnesen et al., 2008), phosphatidylinositol

56 specific phospholipase (PI-PLC), enterotoxin S (EntS), sphingomyelinase (SMase),

57 cereolysin O (Clo), InhA1, NprA and HlyII (Cadot et al., 2010, Kramer and Gilbert, 1992;

58 Stenfors Arnesen et al., 2008). Moreover, other factors such as adhesion to and invasion of

59 epithelial cells also play a role in the biological effects of *B. cereus* strains (Minnaard et al.,

60 2004, 2007, 2013).

61   *B. cereus* symptoms are usually mild (Kramer and Gilbert 1992) and the pathology

62 is not commonly reported. However more severe cases including fatal outcomes have

63 increased in the last few years (Al-Abri et al., 2011; Bottone 2010; Dierick et al., 2005;

64 Mahler et al., 1997; Naranjo et al., 2011; Saito et al., 2010; Shiota et al., 2010). In

65 Argentina, diagnostic testing for *B. cereus* is not routinely performed for patients with

66 gastrointestinal diseases. Nevertheless, statistical data provided by the Health Ministry

67 (2008-2013) showed 5783 cases of food-borne disease, of which 26 were positive for *B.*

68 *cereus* and 9 occurred in 2011 (year of highest incidence).

69   In this context the aim of the present work was the isolation, identification and

70 characterization of the etiologic agent of a food poisoning episode associated to

71 consumption of chicken by a young healthy woman.

72

73 **1. Materials and Methods**

74

75   *2.1. Case presentation*

76

77   In Buenos Aires, Argentina, a 39-year-old healthy woman was hospitalized due to

78   dehydration. She had purchased chicken stuffed with carrots, eggs, pepper and cheese

79   which had been refrigerated immediately and the following day (for lunch) warmed in the

80   microwave oven. One hour after eating, she left home for sport activities (5 km running).

81   Ten hours after eating, she started vomiting and had 5 episodes of watery diarrhoea. After 3

82   hours from onset, the symptoms had not subsided, and the doctor ordered oral rehydration

83   and rest. However, diarrhoea and vomiting continued for further 5 h, which resulted in

84   severe dehydration and required 7 h hospitalization for reposition of water and electrolytes.

85   Analyses performed on faeces and vomitus did not detect the presence of *Escherichia coli*,

86   *Shigella* spp, *Staphylococcus aureus* or *Salmonella* spp.

87   The rapid onset of symptoms, in conjunction with the results of the microbiological

88   analysis, suggested *Bacillus cereus* as the most likely etiological agent for this case.

89

90   *2.2. Isolation of bacteria*

91

92   Two chicken samples (10 g) were diluted 1:10 in sterile distilled water and

93   homogenized. Ten microliters were spread on polymyxin–pyruvate–egg-yolk–mannitol

94   agar (PEMBA) plates (Holbrook and Andersson, 1980), which were incubated at 32 °C for

95   48 to 96 h until bacterial growth was detected (Lancette and Harmon, 1980). Gram staining

96   and determination of catalase activities were performed. Colony appearance regarding

97   shape, color, type of growth and presence of opaque halos due to lecithinase activity were

98   also evaluated.

99

100   *2.3. Bacterial identification*

101

102   The identity of the bacterial isolates was confirmed by using API 20E and API

103   50CH strips plus API 50CHB medium (Biomerieux, France) and data base Apiweb

104   (Biomerieux, www.biomerieux.com). In addition, starch hydrolysis, haemolytic activity

105   and production of a discontinuous haemolytic pattern on blood agar plates according to

106   standard protocols (Beecher and Wong 1994) were tested.

107

108   *2.4. Detection of enterotoxin genes*

109

110   Total genomic DNA was isolated from 24h-cultures grown on TSA using the

111   procedure previously described by López and Alippi (2007). Presence of sequence

112   associated to virulence genes were assessed as previously reported (Minnard et al., 2007).

113   Genes encoding for enterotoxin-T (bceT) (Guinebretiere et al., 2002), cytotoxin K (cytk)

114   (Ehling-Schulz et al., 2006), sphingomyelinase (sph) , enterotoxin FM (entFM) ,

115   enterotoxin S (entS), phosphatidylinositol, phospholipase C (piplC) (Ghelardi et al., 2002),

116   the components of HBL (haemolysin BL: hblA, hblB, hblC, hblD) and NHE (non-

117   haemolytic enterotoxin: nheA, nheB, nheC) complexes (Guinebretiere et al., 2002) were

118   studied. In addition, the isolated was assessed for the *ces* gene related to the production of

119   cereulide (Ehling-Schulz et al., 2005a). DNA amplifications were performed in a thermal

120   cycler (Mastercycler personal; Eppendorf Hamburg, Germany). Amplicons were analyzed

121   by 1.6% (W/V) agarose gel, in TBE buffer, stained with Gel Red ® (Biotium, U.S.A) for 2

122   h at 80 V.

123 *2.5 Culture of epithelial cells*

124

125 Caco-2 cells (Fogh and Orfeo, 1977) were routinely grown in Dulbecco's modified

126 Eagle's minimum essential medium (DMEM) (25 mM glucose) (Life Technologies, Cergy,

127 France), supplemented with 15% (v/v) heat-inactivated (30 min, 56° C) fetal calf serum

128 (FCS) (PAA Laboratories GmbH, Pasching, Austria) and 1% (v/v) non-essential amino

129 acids (Life Technologies, Cergy, France). Monolayers were prepared in 24-tissue culture

130 plates (Greiner Bio One, Frickenhausen, Germany) by seeding 2.5 x 10^4 cells per well.

131 Experiments and cell maintenance were carried out at 37°C in a 5% CO_2/ 95% air

132 atmosphere. Cells at late post confluence (15 days in culture) were used.

133 Hep-2 cells were routinely grown in Dulbecco's modified Eagle's medium (DMEM,

134 25 mM glucose, Life Technologies, Carlsbad, CA, USA), supplemented with 10 % (v/v)

135 heat-inactivated (56 °C, 30 min) fetal calf serum (FCS, PAA Laboratories, Pasching,

136 Austria), 12 IU/ml penicillin-12 µg/ml streptomycin (Life Technologies) and 1 % (v/v)

137 non-essential amino acids (Life Technologies). Cells were seeded at 6.2×10^4 cells per well

138 in 48-well tissue culture plates (Greiner Bio One, Frickenhausen, Germany) and incubated

139 for 48 h at 37 °C in 5% CO_2.

140

141 *2.6 Detachment of Caco-2 cells*

142

143 Detachment of enterocyte-like cells was performed as previously reported (Minnaard

144 et al., 2001). Briefly, differentiated Caco-2 monolayers were incubated at 37 °C for 1 h

145 with 0.5 ml of serial dilutions of culture filtrate supernatants (CFS, pH 6.8) from 16 h-old

146   bacteria cultures at 32 °C in BHIG (BHI broth (BIOKAR Diagnostics) supplemented with

147   0.1% (w/v) glucose). Cells were washed twice with phosphate buffered saline (PBS) (pH

148   7.2), fixed at room temperature for 1 min with 2 % (v/v) formaldehyde in PBS and washed

149   again with PBS. Afterwards, cells were stained by incubating for 20 min at room

150   temperature with 500 µl of a crystal violet solution (0.13 % (w/v) crystal violet, 5 % (v/v)

151   ethanol and 2 % (v/v) formaldehyde in PBS). After washing to remove stain excess,

152   samples were treated with freshly prepared 50 % (v/v) ethanol at room temperature for 1 h.

153   Absorbance was measured in a microplate reader at 620 nm (Biotek Instruments, Winooski,

154   USA). Percentage of cell detachment was calculated as follows:

155   Cell detachment % = 100 X (Ac – As)/Ac, where Ac: A_{620} of control cells and As:

156   A_{620} of sample cells.

157

158   *2.7. Necrosis.*

159

160   Assessment of necrosis was performed as reported previously (López et al., 2013).

161   Briefly, Caco-2 monolayers were incubated with serial dilutions of CFS as describe above.

162   After 1 h of incubation, wells with monolayer not detached were washed twice with

163   binding buffer containing 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

164   (HEPES), 125 mM NaCl, 2.5 mM $CaCl_2$, (pH 7.2), and 0,2 % (p/v) gelatin. Afterwards, 1

165   µg of propidium iodide was added in 100 µl of binding buffer per well, and cells were

166   incubated on ice for 15 min. Then, samples were mounted in 50 % (v/v) glycerol in PBS

167   and analyzed by conventional fluorescence microscopy using a Leica DMLB microscope

168   coupled to a Leica DC 100 camera (Leica Microscopy Systems, Heerbrugg, Switzerland).

169

170   *2.8. Mitochondrial dehydrogenase activity*

171

172   Activity of mitochondrial dehydrogenases was determined by assessing the

173   reduction of 3-(4, 5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium (MTT) by means of

174   modification of a previously reported protocol (Finlay et al., 1999; Minnaard et al, 2007).

175   Briefly, autoclaved (15 min at 121°C) or non-heated CFS diluted in DMEM with

176   2% methanol were added to each well of Hep-2 cells and incubated 24 h at 37°C in 5%

177   CO_2. CFS (autoclaved or non-heated) from a *ces* (-) *B. cereus* strain (B10502) were also

178   evaluated. The medium was removed and DMEM (without phenol red) containing 0.5

179   mg/ml of MTT was added to each well; plates were incubated at 37 °C for 4 h and then, the

180   medium was removed. Intracellular formazan was solubilized with 0.25 ml of isopropyl

181   alcohol/0.1N HCl and absorbance at 570 nm was measured in a microtiter plate reader

182   (Bioteck Instruments, Winooski, USA).

183

184   *2.9. Cell association assays*

185

186   Bacterial from a 3 h-old cultures (32 °C) in BHIG were centrifuged and pellets were

187   suspended in DMEM containing 100 µg/ml chloramphenicol. As reported (Minnaard et al.,

188   2004), in these conditions bacterial viability is preserved but no bacterial growth occurs.

189   Cell monolayers were washed twice with PBS before the infection assays and bacterial

190   suspensions were added to the monolayers (Multiplicity of infection, MOI = 100 bacteria

191   per cell) and incubated for 2 h at 37°C in a 5% CO_2/ 95% air atmosphere. To evaluate

192   association (adhering plus invading bacteria), monolayers were exhaustively washed with

193   PBS and incubated with 1 ml distilled water per well to lyse eukaryotic cells. Serial

194   dilutions of the samples were plated onto nutrient agar (BIOKAR Diagnostics, Beauvais,

195   France) and incubated at 37 °C for 16 h. Invasion was assessed by the aminoglycoside

196   protection assay as previously reported (Minnaard et al., 2004). All the infection assays

197   were performed in DMEM FCS-free.

198

199   **2.   Results**

200

201   After 48 h of incubation, typical mannitol negative and lecithinase positive colonies

202   were observed in PEMBA plates. Gram staining showed Gram-positive rods with

203   subterminal spores and no significant swelling of the sporangia. The isolate was named

204   MVL2011. Using the API 20E and API 50 CH strips and data base Apiweb, MVL2011

205   matched as *Bacillus cereus*. In addition, the strain hydrolyzed starch and lead to a

206   discontinuous haemolytic pattern.

207   The presence of sequences related to genes associated to the virulence of *Bacillus*

208   *cereus* was determined. As compared with 4 well characterized *B. cereus* strains, the strain

209   under study shows a very different pattern of virulence genes (table 1). Strain MVL2011

210   was positive for the sequence corresponding to the genes *bceT, cytK, sph, entFM, entS,* ces

211   and *piplC* (Table 1); moreover it presented 3 of the sequences of the hemolyisn BL (*hbl A,*

212   *B* and *C*) and one of the sequences of the non hemolytic enterotoxin (*nhe B*) (Table 1). All

213   the genes gave amplification fragments of the expected sizes.

214   Biological activity of the strain MVL2011 was studied on epithelial cells.

215   Coincubation of Caco-2 monolayers with CFS of MVL2011 leads to cell detachment and

216   dose-response behaviour was observed (Fig. 1). Low doses of CFS did not detach cells but

217   led to significant necrosis as assessed by propidium iodide staining (Fig. 2). Interestingly,

9

218   when CFS was heated for 15 min at 121°C, the ability to detach Caco-2 cells was abrogated

219   (data not shown).

220   Hep-2 bioassay has been employed to analyzed cereulide. Results showed that, after

221   thermal treatment, 80 % of the biological activity was retained as compared to non-heated

222   CFS. In contrast, biological activity of CFS from strain B10502 was completely abolished

223   after thermal treatment.

224   Infection of enterocyte-like Caco-2 cells was performed with vegetative culture of

225   MVL2011. Association and invasion values were 1.16 ± 0.03 x 10^6 cfu/ml and 1.15 ± 0.07

226   x 10^2 cfu/ml, respectively. The ratio of associated/initial bacteria was 0.01 ± 0.003 and

227   invading/associated bacteria was 9.95 ± 0.61 x 10^{-5}.

228

229   **3.   Discussion**

230

231   Results presented in this case study provided evidence that *B. cereus* strain

232   MVL2011 isolated from cooked chicken and related to a case of serious food poisoning in

233   a healthy adult woman, was highly cytotoxic and unable to invade Caco-2 cells in spite of

234   adhesion ability similar to other *B. cereus* strains. Along with the clinical symptoms,

235   evidence suggest that the strain is an emetic isolate since it is positive for this sequences in

236   the PCR assay and the biological activity of the extracellular factors on Hep-2 cells is not

237   completely abrogated by thermal treatment (Stark et al., 2013). Even though levels of

238   cereulide lower than 8 µg/Kg body weight can be lowered with proper hydration, these

239   concentrations can be fatal in children (Saito et al., 2010). Some strains of *B. cereus* are

240   psychrotropic since they lead to the highest production of the emetic toxin between 12 °C

241   and 15 °C (Dierick et al., 2005). Fatal outcomes have been reported for these emetic strains

242   (Dierick et al., 2005). Note that the meal had been refrigerated for 24 h after being

243   purchased, and the same food had been eaten 24 h before without symptoms of food-

244   poisoning. Usually *B. cereus* symptoms are mild (Kramer et al., 1992) and the pathology is

245   not commonly reported. However cases involving more serious health risks and fatal

246   outcomes (Al-Abri, et al, 2011; Dierick et al., 2005; Mahler et al., 1997; Naranjo et al.,

247   2011; Saito et al., 2010, Shiota et al., 2010) have increased in the last few years (Bottone

248   2010).

249   In Argentina, diagnostic test for *B. cereus* is not routinely performed for patients

250   with gastrointestinal diseases. As compared with other non-invasive *B. cereus* strains

251   previously studied (Minnaard et al., 2007) stated in Table 1, strain MVL2011 is the sole

252   strain showing high ability to detach Caco-2 cells. In addition, it is positive for the hblA,

253   hblB and hblC genes of the hbl complex, bceT, entS and ces (cereulide synthase).

254   According to our results and the clinical presentation of the case, *B. cereus* MVL2011 is an

255   emetic strain. In early reports on strains positive for the gene *ces* it has been proposed that,

256   on the basis of gene analysis, they belong to a well-defined "emetic cluster" (Ehling-Schulz

257   et al., 2005b). Noteworthy, our strain is positive for 3 out of 4 genes of the hbl complex that

258   is common in non-emetic strains (Ehling-Schulz et al., 2005b). These apparently

259   contradictory findings are in agreement with current knowledge on the virulence of *B.*

260   *cereus* (Castiaux et al., 2014; Messelhäusser et al., 2014). Indeed, it has been demonstrated

261   that the distribution of the *ces* gene is not homogeneus along *B. cereus* populations and

262   strains positive both for *ces* and *hbl* complex have been isolated (Kim et al., 2010; Rahmati,

263   T. and Labbe, R., 2008).

264

265   **4.   Conclusion**

11

266

267   Results presented in this case study provided evidence that *B. cereus* strain

268 MVL2011 was involved in serious food poisoning based on symptoms, microbiological

269 analysis of patient´s samples and the presence of *B. cereus* in the implicated food. It has

270 been proposed that *B. cereus* emetic cluster evolved from a panmictic population of

271 diarrhoeal strains. In this context and according to the results of the present study and the

272 current knowledge on the distribution of virulence traits of *B. cereus*, strain MVL2011

273 belongs to the sub-population of strains positive for several virulence genes. These

274 characteristics could correlate with the high biological activity of the strain under study.

275

276 **Acknowledgments**

277

282

283 **Figure Captions**

284

285 **Fig. 1**: Detachment of enterocyte-like cells (Caco-2) after incubation with different

286 concentrations of culture filtrate supernatants of *Bacillus cereus* strain MVL2011. Values

287 represent averages from 3 determinations $\pm$ standard deviation

288

289 **Fig. 2** Fluorescence microscopy of Caco-2 cells co-incubated with culture filtered

290 supernatants of *Bacillus cereus* MVL2001 (A) and control co-incubated with BHIG

291 medium (B). Cells were labeled with propidium iodide indicating red nuclei in necrotic

292 cells.

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294

295

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Table 1

Screening by PCR of genes encoding for components of HBL (haemolysin BL: *hblA, hblB, hblC, hblD*) and NHE (non-haemolytic enterotoxin: *nheA, nheB, nheC*) complex; enterotoxin-T (*bceT*); cytotoxin K (*cytk*); sphingomyelinase (*sph*); enterotoxin FM (*entFM*); enterotoxin S (*entS*); cereulide*(ces)* and phosphatidylinositol phospholipase C(*piplC*) [a]

Strain	Origin	*hbl* complex				*nhe* complex			*bce*T	*cty*K	*sph*	*entFM*	*entS*	*ces*	*piplC*	Reference
		*hbl*A	*hbl*B	*hbl*C	*hbl*D	*nhe*A	*nhe*B	*nhe*C								
MVL2011	Chicken (food-borne illness)	+	+	+	-	-	+	-	+	+	+	+	+	+	+	This study
B10502	Food-borne illness	-	-	-	-	+	-	-	-	+	+	+	-	-	+	Minnaard et al, 2004 and 2007
T1	Unknown	+	+	+	+	+	+	+	+	+	+	+	+	-	+	Minnaard et al, 2007
2	Skim milk powder	-	-	-	+	-	+	+	+	+	+	-	-	-	-	Minnaard et al,
M2	Skim milk powder	-	-	-	-	-	+	-	-	-	+	+	-	-	+	2001 and 2007

[a] Toxin gene presence was assessed by PCR analysis. The "+" and "-" symbols refer to the presence and absence of the gene, respectively.
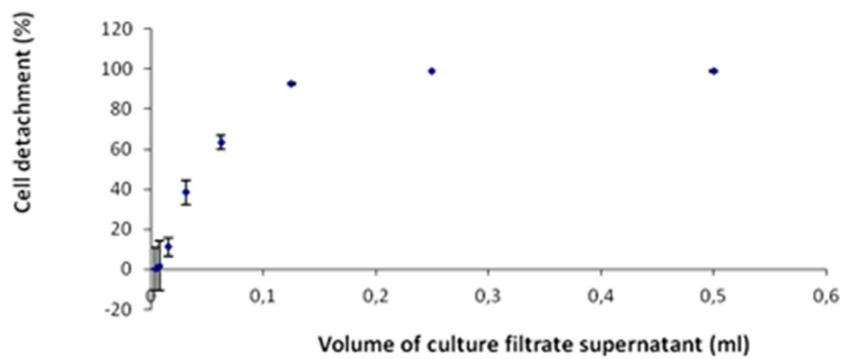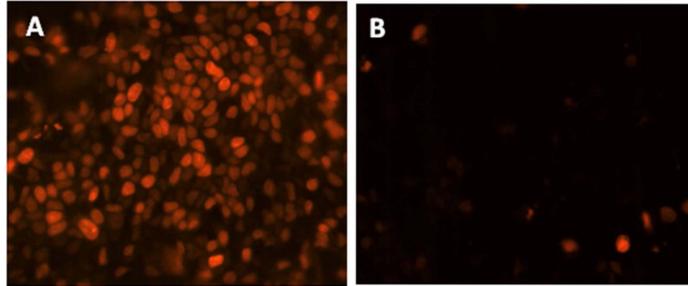
Highlights

A *Bacillus cereus* strain (MVL2011) isolated from cooked chicken caused severe poisoning in a healthy woman

The woman was hospitalized due to severe dehydration

B. cereus MVL2011 was highly cytotoxic and unable to invade Caco-2 cells in spite of adhesion ability

Strain MVL2011 could belong to the "emetic cluster"