Evidence for a further enterotoxin complex produced by Bacillus cereus

Per Einar Granum*, Annika Andersson, Claire Gayther, Meike te Giffel, Helle Larsen, Terje Lund, Kristin O'Sullivan

Department of Pharmacology, Microbiology and Food Hygiene, Norwegian College of Veterinary Medicine, PO Box 8146 Dep., N-0033 Oslo, Norway

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Abstract

Out of 321 strains of Bacillus cereus from several sources and isolated in four different countries, 239 (74%) produced cytotoxins. Only 127 (53%) of the cytotoxic strains were positive for the B-component gene of the haemolysin BL (enterotoxin) by polymerase chain reaction (PCR). Western blots using antiserum produced against enterotoxin(s) gave positive results for 199 (83%) of the cytotoxic Bacillus cereus strains. On closer examination of seven of the strains, involved in food poisoning, we found that two strains completely lacked the L2- and B-components (of the haemolysin BL), and two strains were negative for the B-component gene by PCR, but were positive for the L2-component. From our experiments we concluded that there is at least one enterotoxin complex in addition to the haemolysin BL enterotoxin and enterotoxin T.

Keywords: Bacillus cereus; Enterotoxin; Haemolysin; Food poisoning

1. Introduction

Bacillus cereus is responsible for two different types of food poisoning: the diarrhoeal type first recognised after a hospital outbreak in Oslo, Norway, in 1948 and the emetic type described about 20 years later after several outbreaks in London involving rice (for reviews see [1,2]). The diarrhoeal type of food poisoning is caused by enterotoxin(s), produced during vegetative growth of B. cereus in the small intestine [3], while the emetic toxin is produced by growing cells in the food [1]. Both types of food poisoning are usually caused by heat-treated foods, where surviving spores are the source of the food poisoning. There is still confusion regarding how many different enterotoxins are produced by B. cereus. At least two different enterotoxins have been characterised [2,4,5] so far. One is a three-component enterotoxin with haemolytic and necrotising activity [4,6,7], while the other is a single component enterotoxin [5]. Whether these two types of enterotoxin can explain all the different outbreaks of diarrhoeal B. cereus food poisoning is unclear. During 1994 and 1995 several strains of B. cereus from four different European countries were investigated for the ability to produce enterotoxin in our laboratory. We compared three different techniques: in vitro cell

* Corresponding author. Tel.: +47 22 96 48 45; Fax: +47 22 96 48 50; E-mail: per.e.granum@veths.no
assays, Western blots and PCR for detection of enterotoxin(s). Both the B-component of the hemolytic enterotoxin and the enterotoxin T have been cloned and sequenced [4,5], and these sequences were used to design primers for the PCR. Correlation between the results using these three techniques was investigated in an attempt to define, and thus understand, the role of enterotoxin production by B. cereus in food poisoning.

2. Material and methods

2.1. Strains

From four different European countries (Denmark, Norway, The Netherlands and UK) 321 strains of B. cereus were obtained for this study. Of these strains, 207 were from dairy products, 28 were from food-borne outbreaks (18 diarrhoeal and 10 emetic) and 86 were from other sources. All isolates were identified as B. cereus using lecithinase (positive) and mannitol (negative) reactions on selective medium (Oxoid, UK) and additional tests for motility, aerobic and anaerobic growth, and haemolysis on blood agar.

2.2. Growth conditions and crude enterotoxin preparation

The B. cereus strains were cultured in Brain Heart Infusion broth (Oxoid with an additional 10 g/l glucose), in an incubator with shaking (200 cycles/min) at 32°C for 6 h. The cells were harvested by centrifugation (5000×g for 20 min) and the supernatant was used in the cell assay and for immunoblotting. The strains that did not show cytotoxicity using the unconcentrated supernatant fluids were then 20-fold concentrated by precipitation, using 70% saturated ammonium sulfate solution. The precipitated protein was dissolved in 20 mM phosphate buffer, pH 6.8, and dialysed against the same buffer for 16 h at 4°C.

2.3. Electrophoresis and immunoblotting

Western immunoblots were carried out as described previously [8], using the Mini-Protean II Dual Slab Cell (Bio-Rad, USA). Antiserum against the purified enterotoxin was provided by John M. Kramer, PHLS Food Hygiene Laboratory, London, UK [9]. SDS polyacrylamide gels were stained using the Bio-Rad Silver Stain Kit (Bio-Rad, CA, USA). The molecular mass of the proteins was estimated on SDS gels using the Bio-Rad SDS-PAGE Low Molecular Weight Standards.

2.4. Kit for detection of enterotoxin

Antiserum and enterotoxin from the Oxoid B. cereus enterotoxin detection kit, BCET-RPLA, was used according to the instruction manual.

2.5. Vero cell assay

Toxicity was determined using a Vero cell assay [10]. The anti-enterotoxin neutralised the cytotoxic activity of the enterotoxin extracts. All experiments were performed in duplicate and repeated twice. Cell extracts (20 times concentrated) that gave at least 40% inhibition of protein synthesis of the Vero cells were recorded as cytotoxic. It should be noted that assays with Vero cells and human intestinal epithelial cells (Caco-2 cells) gave the same results. As Caco-2 cells took much longer to grow, they were not used for routine assays.

2.6. Polymerase chain reaction

PCR reactions were carried out in a Perkin Elmer Cetus DNA Thermal Cycler or an MJ Research MiniCycler using the following program: 30 cycles

| Table 1
| The PCR primers used in this study |
| Name of primer | Position of primer | Primer sequence (5’ to 3’) |
| B 1F | 169–188 | AGAACAATTGGAGATACGGC |
| B 2R | 790–769 | TGTTAGACCCAAAATAGCACC |
| B 7R | 1186–1126 | ATTTTGTGGAGTACAGTTTCTAC |
| B’ 3F | 1810–1830 | ATACAATATGGAAAAATACA |
| B’ 4R | 2041–2019 | CTCCCTGTAATCTGTAATCCCT |
| ET F | 188–208 | TTACATTACGAGACCTGCTT |
| ET R | 615–595 | TTGTGTGATTAATCCAG |

*a B, B-component gene of the haemolysin BL; B’, B’-component gene following 3’ to the B-component (from reference [12]); ET, enterotoxin T (from reference [5]); F, forward; R, reverse.
3. Results and discussion

Table 2 shows an overview of our results. These show that 239 (74%) of the strains were positive in the Vero cell assay, while only 127 of these strains (53% of the cytotoxic) were positive by PCR for the B-component gene of the haemolysin BL. Examples of the results from the PCR experiments are given in Fig. 1. A 1018 bp fragment is observed as expected for the positive strains. Of the cytotoxic strains, 199 (83%) showed a band on immunoblotting where the B-component (and/or the L1-component) was expected. Seven of the cytotoxin-negative strains were positive for the B-component gene by PCR and Western blots. The reason for this was most probably the lack of the L2- and/or L1-component, as judged from the Western blots. Both proteins are necessary for cytotoxic activity of the haemolysin BL [4]. During our studies, Agata et al. [5] described a further single protein enterotoxin (enterotoxin T). We tested for the presence of this gene ($bceT$) in 95 of our strains (Table 2). This protein has not been shown to be related to food poisoning and it is interesting to note that the gene is present only in 40% of the 95 strains and in 52% of the cytotoxic strains tested (Table 2). However, $bceT$ is not present in five of the seven food-poisoning strains investigated further (Table 3). It is also interesting to note that while this gene was found in 40% of the strains we tested, Agata et al. [5] found the gene in all 10 strains they tested.

It is clear from our results that cytotoxicity (and most probably enterotoxicity) cannot be explained by the production of haemolysin BL alone. Neither is addition of enterotoxin T to the list of potential

Table 2

| Summary of enterotoxin properties of 321 B. cereus strains from the four countries$^a$ |
|-----------------------------------------------|-----------------|-----------------|
| Cytotoxin positive strains                   | 239/321         | 127/239         |
| Cytotoxin negative strains                   | 82/321          | 7/82            |
| Total number of positive strains             | 134/321         | 206/321         |

$^a$The results are given as number of positives number of strains tested in each group.
$^b$Primers: B 1F-B 2R or B 1F-B 7R.
$^c$Tested on 95 strains.
enterotoxins sufficient to explain the results. Therefore, we decided to further investigate several properties of seven strains that have been involved in food poisoning (Table 3). As expected, all of these strains were positive on cell tests (unconcentrated supernatant). These seven strains could be divided into three main groups (Table 3). Strains 1230-88, 3 and 9 all have the haemolysin BL, organised as in B. cereus strain F837/76 (the strain used for cloning of the B-component), with both the B- and B’-component gene present. Two strains, 0075-95 and svm, both involved in large outbreaks of food poisoning in 1995, lack at least both the B- and L2-components (Oxoid BCET-RPLA TD950 kit), of the haemolysin BL, in addition to the enterotoxin T. Thus, another explanation for enterotoxicity must be found (Table 3) for these two strains. Finally, two strains from The Netherlands (21 and 23) appear to have the intact haemolysin BL complex, judged from the immunoblotting results, although at least the B-component must be missing (Table 3), as the gene coding for this protein does not seem to be present. It is, however, possible that the gene is quite different in the areas where the primers are expected to bind. We have indications from using five different sets of primers for the B-component gene that there is heterogeneity for some few strains (results not shown). However, it is possible that the activity is due to yet another protein of similar molecular mass to the B-component that may substitute for it and react with Kramer’s antiserum. From the immunoblotting results (Fig. 2), it appears that there may be at least two different types of ‘enterotoxins’. Three strains (0075-95, svm and 44) belong to one group while the three remaining strains (1230-88, SNT3 and 101A) do not belong to this group based on the patterns on the Western blots (Fig. 2).

It is also interesting to note that some strains have a protein of molecular mass approximately 105 kDa, which reacts with the antibodies (Fig. 2, Table 3). Both strains without the haemolysin BL complex (0075-95 and svm) produce this protein, in addition to other proteins, that react with the antiserum (Fig. 2). It has indeed been shown that strain 0075-95 contains another enterotoxin complex, consisting of two proteins of about 40 kDa and including the 105 kDa protein [13]. Some strains of B. cereus seem to

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**Table 3**

Comparison of enterotoxin properties of seven B. cereus strains involved in food poisoning

<table>
<thead>
<tr>
<th>Strain</th>
<th>Country of origin*</th>
<th>Cytotoxic to Vero cells</th>
<th>Toxin components detected by immunoblotting</th>
<th>Toxin genes detected by PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>L2-comp.</td>
<td>B-comp.</td>
</tr>
<tr>
<td>0075-95</td>
<td>N</td>
<td>+</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>1230-88</td>
<td>N</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>svm</td>
<td>DK</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>3</td>
<td>NL</td>
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<td>9</td>
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<td>21</td>
<td>NL</td>
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<td>+</td>
</tr>
<tr>
<td>23</td>
<td>NL</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

*+, positive reaction; —, negative reaction.

*N*, Norway; *DK*, Denmark; *NL*, Netherlands.

*Negative with the Oxoid BCET-RPLA TD950 kit (although a band is present close to the position of L2 in Fig. 2).
contain only one of the enterotoxin complexes, while other strains may contain both (Table 3, Fig. 2). The enterotoxin complexes appears to be heterogeneous, and this can only be resolved through protein purification, followed by cloning, sequencing and mutation studies.

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