Identification of Bacillus cereus Group Species Associated with Food Poisoning Outbreaks in British Columbia, Canada

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The genus Bacillus currently consists of at least 148 validly named species (List of Prokaryotic Names with Standing in Nomenclature—Genus Bacillus [http://www.bacterio.cict.fr/bbacillus.html]). The Bacillus cereus group contains five species, B. cereus sensu stricto, B. thuringiensis, B. anthracis, B. mycoides, and B. weihenstephanensis. They are difficult to discern using standard biochemical schemes, chemotaxonomic methods, or phylogenetically relevant target genes (1, 2), and many distinguishing pathogenicity markers in this group can be attributed to mobile plasmids (18, 21, 22, 23). The differentiation of B. cereus group members using molecular techniques is not routine in food-poisoning diagnostic methods and may cause underreporting of species such as B. thuringiensis (1, 8). In 2005, Bacillus was identified in a food-poisoning event implicating imported strawberries. The isolate was initially identified phenotypically as B. cereus and then later by PCR as B. thuringiensis. To assess the proportion of food poisonings caused by different B. cereus group species, 155 B. cereus group-like isolates collected from food or clinical specimens in food-borne outbreaks between 1991 and 2005 were characterized using molecular and phenotypic typing methods.

Frozen isolates were retrieved onto blood agar plates and incubated at 35°C for 24 h. Phenotypic characterization was conducted following established procedures (12). No B. cereus group-like bacterium reviewed here was consistent with B. weihenstephanensis or B. anthracis (data not shown).

DNA was extracted by lysing pure culture in a heating block at 102°C for 10 min. Microcentrifuged supernatant was frozen at −80°C until required. Pathogenicity genes for emetic cereulide toxin (nonribosomal peptide synthetase [NRPS]) and ICP (cry1 or cry2) were detected in multiplex PCR assays (7, 10) shown in Fig. 1. Each master mix contained 0.8 μM of each primer, hot start master mix, diethyl pyrocarbonate water (20 μl), and 5 μl of DNA. The PCR products were loaded onto 2% agarose gels made with 0.5× Tris-borate-EDTA buffer and ethidium bromide (1 μg ml−1). The gels were electrophoresed at 120 V for 30 min and then visualized on a Bio-Rad Gel Doc 2000.

Strains positive for NRPS were designated as B. cereus, those positive for ICP (by microscopy or PCR) as B. thuringiensis, those with rhizoidal growth on nutrient agar as B. mycoides, and all other strains with the typical B. cereus phenotype as B. cereus NRPS+ICP+. PCR-negative isolates were further examined for ICP crystals using transmission electron microscopy (TEM) since B. thuringiensis strains may carry one to six cry genes and there is no universal method available to detect all cry genes (there are currently more than 150 cry toxins) (17; Bacillus thuringiensis toxin nomenclature [http://www.lifesci.sussex.ac.uk/Home/Neil_Crickmore/Bt/]). Samples of B. cereus group bacteria were prepared for electron microscopy by fixation with 2% glutaraldehyde and 1% para-formaldehyde, followed by post-fixation with 1% osmium tetroxide in 0.1 M sodium cacodylate buffer and dehydration in acetone. Subsequently, they were embedded in Epok resin and sectioned using a Leman ultramicrotome. Sections were placed on grids, stained with uranyl acetate and lead citrate, and observed with a Jeol 1200 EX Transmission Electron Microscope at 80 kV. Dark areas on the sections were then imaged using a digital camera and ImageJ software. Bacterial samples were identified by TEM as positive or negative for ICP crystals.

FIG. 1. Multiplex PCR of NRPS and cry1 to distinguish between B. cereus and B. thuringiensis. Lanes 1 and 11, 100-bp ladder; lane 2, B. cereus NRPS+ (emesis isolate 8-91-71); lane 3, B. cereus (fried rice isolate 28-240-5); lane 4, B. cereus (chow mein isolate 28-251-1); lane 5, B. cereus (stool isolate 36-254-1); lane 6, B. thuringiensis (stool isolate 36-254-2); lane 7, B. thuringiensis (strawberry isolate 43-05-36-1); lane 8, B. thuringiensis (mixed-salad isolate 41-04-259-1); lane 9, positive control; lane 10, negative control.
cies were significantly different (Pearson’s coefficient, 0.0001). There was a significant correlation found between the Bacillus species, as indicated by a mixed model analysis.

B. thuringiensis isolates were identified in 23 of the 39 outbreaks (median, 14 h). In summary (Table 1), B. cereus were identified as a significant pathogen in 11 of 39 outbreaks (28.2%). A small number (2.6%) of isolates were positive for either cry1 or ICP, or both (in one case, B. thuringiensis). A large number (26.5%) of isolates were identified as B. mycoides based on rhizoid growth demonstrated on nutrient agar. In summary (Table 1), B. cereus isolates were identified in 23 of the 39 outbreaks (B. cereus NRPS in 5 [12.8%] and B. cereus NRPS- ICP in 18 [46.1%]). B. thuringiensis was identified in 4 (10.3%), B. mycoides in 1 (2.6%), and mixed species of Bacillus in 11 (28.2%).

Records of food-poisoning investigations were reviewed, and epidemiological information included the number of people that ate the implicated meal (at risk), the number ill, and the onset and duration of the illness and symptoms. Data were available for 108 individuals, collected in 37 of the 39 investigations. The attack rate was 32%, and the mean age was 37 years (standard error, 1.9; median, 37 years; range, 2 to 75 years). The average time to symptom onset was 6 h, with the duration ranging from 1 to 144 h (median, 16 h). The predominant symptoms are shown in Table 2. When symptom data from ill individuals were separated by Bacillus species identified (excluding outbreaks involving mixed Bacillus species), the symptom profiles between species were significantly different (Pearson’s coefficient, P < 0.0001). There was a significant correlation found between the symptom of vomiting and the identification of B. cereus NRPS+ isolates (Fisher’s exact test [right-tailed], P = 0.038). This was not true when NRPS gene presence was compared to any other symptom. Twenty-five (64%) investigations were traced back to the consumption of Asian foods, with 65% associated with restaurant foods, 17% with foods obtained at retail stores, and 7.5% with foods prepared at home. Raw foods accounted for 11.5% of the food poisonings and included fruit, green salads, and raw oysters.

Multiple Bacillus species were detected in more than 25% of the outbreaks. On average, four Bacillus-positive isolates were collected in each investigation (median, two isolates). If additional isolates for each sample had been collected and cryopreserved, more heterogeneous Bacillus populations in foods and clinical samples could potentially have been detected. In this study, 23 B. thuringiensis isolates were identified as the only Bacillus spp. associated with four food-poisoning outbreaks. The initial incorrect isolate identification may have occurred from a failure to test for ICP or from the failure of the culture to produce recognizable ICP under light microscopy. Standard methods that either do not differentiate B. cereus from B. thuringiensis (8) or specify that a 3- to 7-day culture followed by staining and microscopic examination for ICP be performed (12) are not as sensitive or rapid as detecting cry genes by PCR (unpublished data) to discriminate between these Bacillus species. Although B. thuringiensis is not considered a food-borne pathogen (11, 24), and is rarely found linked to food-borne (15) or other (4, 8, 20) human illness this pesticide is currently under review by the European Union (9). This study suggests that there is an association between this bacterium and previously recognized food-borne gastrointestinal illnesses.

The rapid identification of Bacillus species implicated in food poisonings can be facilitated by PCR. We recommend the use of PCR in tandem with phenotypic tests to assist in the

### Table 1. Bacillus food-borne illness outbreaks and isolate summary relating to Bacillus species retrieved from cryopreservation in this study

<table>
<thead>
<tr>
<th>Species</th>
<th>No. of outbreaks</th>
<th>No. of people at risk</th>
<th>No. of ill people</th>
<th>No. of Bacillus-positive food specimens</th>
<th>No. of Bacillus-positive clinical specimens</th>
<th>No. of isolates tested</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. cereus NRPS+</td>
<td>5</td>
<td>14</td>
<td>12</td>
<td>9</td>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td>B. cereus NRPS- ICP-</td>
<td>18</td>
<td>518</td>
<td>115</td>
<td>36</td>
<td>4</td>
<td>55</td>
</tr>
<tr>
<td>B. thuringiensis</td>
<td>4</td>
<td>22</td>
<td>14</td>
<td>5</td>
<td>0</td>
<td>20</td>
</tr>
<tr>
<td>B. mycoides</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>3</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Mixed Bacillus spp.</td>
<td>11</td>
<td>78</td>
<td>57</td>
<td>38</td>
<td>3</td>
<td>71b</td>
</tr>
<tr>
<td>Total Bacillus species</td>
<td>39</td>
<td>634</td>
<td>200 (5, 2)</td>
<td>91 (2, 2)</td>
<td>7 (0, 2, 0)</td>
<td>155 (4, 2)</td>
</tr>
</tbody>
</table>

*Values in parentheses are the average and median, respectively.
*b Bacillus species identified: B. cereus NRPS+, n = 31; B. cereus NRPS- ICP-, n = 35; B. thuringiensis, n = 3; and B. mycoides, n = 2.

### Table 2. Symptom profiles for ill individuals associated with Bacillus species identified in food-borne illness outbreaks

<table>
<thead>
<tr>
<th>Species</th>
<th>% of individuals with indicated symptom</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nausea</td>
</tr>
<tr>
<td>All Bacillus species (n = 108)</td>
<td>61</td>
</tr>
<tr>
<td>B. cereus NRPS+ (n = 12)</td>
<td>50</td>
</tr>
<tr>
<td>B. cereus NRPS- ICP- (n = 41)</td>
<td>54</td>
</tr>
<tr>
<td>B. mycoides (n = 2)</td>
<td>0</td>
</tr>
<tr>
<td>B. thuringiensis (n = 13)</td>
<td>62</td>
</tr>
</tbody>
</table>
identification of all B. cereus group species implicated in food-borne illnesses.

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REFERENCES