Laboratory Investigation of the First Case of Botulism Caused by \textit{Clostridium butyricum} Type E Toxin in the United States

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We report here the laboratory investigation of the first known case of botulism in the United States caused by \textit{Clostridium butyricum} type E. This investigation demonstrates the importance of extensive microbiological examination of specimens, which resulted in the isolation of this organism.

Botulism is a rare but acute paralytic disease caused by botulinum neurotoxin (BoNT). Botulism can occur when contaminated food is ingested (food-borne) or when BoNT-producing clostridia colonize wounds or the intestine. Infant botulism occurs through intestinal colonization by BoNT-producing clostridia and the subsequent \textit{in situ} production of BoNT in infants (1). Diagnosis is based on clinical presentation (1, 2). In the United States, botulism immune globulin human (intravenous) (BIG-IV) is available for the treatment of infant botulism through the California Department of Public Health (CDPH) Infant Botulism Treatment and Prevention Program (IBTPP) (2). Laboratory confirmation is performed at the Centers for Disease Control and Prevention (CDC) and state public health laboratories (1, 2).

\textit{Clostridium botulinum} types A and B are the serotypes most frequently associated with infant botulism worldwide, accounting for ~98% of reported infant botulism cases (2). Infant botulism caused by \textit{C. butyricum} type E was first reported in 1987 (3). Since then, this organism has been linked to infant botulism cases in Italy, Japan, the Republic of Ireland, and England (3–6). Additionally, \textit{C. butyricum} type E has been associated with food-borne botulism in China, Italy, and India (4, 7, 8). We report here the laboratory investigation of the first case in the United States of botulism caused by \textit{C. butyricum} type E.

A stool sample collected from a 7-day-old infant with suspected botulism was submitted to the Centers for Disease Control and Prevention (CDC) National Botulism Laboratory for laboratory testing. The sample was tested for BoNT by mouse bioassay (1). Procedures were conducted in accordance with Association for Assessment and Accreditation of Laboratory Animal Care guidelines and institutionally approved protocols for the ethical use of laboratory animals. The amount of BoNT type E detected in the stool was 126,400 mLDoS/g (mLD50, 50% mouse lethal dose), which is >150 times higher than that previously reported for type E infant botulism cases (4). The stool sample was cultured for the growth of BoNT-producing clostridia using standard laboratory methods (9). McClung-Toabe egg yolk agar (EYA) plates were streaked for isolation from stool and from enriched broth cultures. Plates were incubated for 2 to 3 days, and broth cultures

<table>
<thead>
<tr>
<th>Organism</th>
<th>BoNT type</th>
<th>Lipase</th>
<th>Aerobic growth</th>
<th>Nitrate reduction</th>
<th>Spore formation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reference clostridia(^a)</td>
<td></td>
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<tr>
<td>\textit{C. botulinum}</td>
<td>E</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>ST</td>
</tr>
<tr>
<td>\textit{C. sporogenes}</td>
<td>NT(^d)</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>ST</td>
</tr>
<tr>
<td>\textit{C. butyricum}</td>
<td>NT</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>ST</td>
</tr>
<tr>
<td>\textit{C. tertium}</td>
<td>NT</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>T</td>
</tr>
<tr>
<td>Isolates from infant</td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>Toxicogenic Lip(^{−}/\text{Lec}^{−}) \textit{(CDC51208)}</td>
<td>E</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>ST</td>
</tr>
<tr>
<td>Nontoxicogenic Lip(^{−}/\text{Lec}^{−})</td>
<td>NT</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>T</td>
</tr>
<tr>
<td>Nontoxicogenic Lip(^{+})</td>
<td>NT</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>ST</td>
</tr>
</tbody>
</table>

\(^a\) +, positive; –, negative; +\(^{+}\), most strains positive, some strains negative.
\(^b\) ST, subterminal; T, terminal.
\(^c\) Species characteristics as described in the VPI Anaerobe Laboratory Manual (10).
\(^d\) NT, nontoxic.
were incubated for 4 to 5 days. All broth cultures and plates were incubated under anaerobic conditions at 35°C.

Plate growth of stool and stool culture was heavily mixed with lipase-positive (\(\text{Lip}^+\)) colonies and lipase-negative/lecithinase-negative (\(\text{Lip}^-/\text{Lec}^-\)) colonies. Because both \(\text{C. botulinum}\) (\(\text{Lip}^+\)) and \(\text{C. butyricum}\) (\(\text{Lip}^-/\text{Lec}^-\)) are known to produce BoNT type E, both \(\text{Lip}^+\) (\(n = 11\)) and \(\text{Lip}^-/\text{Lec}^-\) (\(n = 25\)) colonies were selected for further culture and characterization. BoNT type E was detected in the cultures of two \(\text{Lip}^-/\text{Lec}^-\) isolates. The phenotypic characteristics (Table 1) and 16S sequence data were consistent with \(\text{C. butyricum}\). This strain was designated \(\text{C. butyricum}\) CDC51208.

Eleven \(\text{Lip}^+\) colonies and 23 \(\text{Lip}^-/\text{Lec}^-\) colonies were negative for BoNT by mouse bioassay and/or for the presence of the \(\text{bont/E}\) gene by PCR. Further investigations were conducted to identify the associated nontoxic bacteria that impeded rapid identification of \(\text{C. butyricum}\) type E. A representative \(\text{Lip}^+\) colony was identified as \(\text{Clostridium sporogenes}\) by biochemical analysis (Table 1). A representative nontoxic \(\text{Lip}^-/\text{Lec}^-\) colony was identified as \(\text{Clostridium tertium}\) by biochemical (Table 1) and 16S sequence analyses.

\(\text{C. tertium}\) is an aerotolerant anaerobe common in the fecal flora of infants (10, 11) that can be readily distinguished from the obligate anaerobe \(\text{C. butyricum}\) by growth under aerobic conditions. Further, \(\text{C. tertium}\) produces oval terminal spores, whereas \(\text{C. butyricum}\) produces subterminal spores (10). Out of the 25 \(\text{Lip}^-/\text{Lec}^-\) isolates examined, 23 isolates showed growth on blood agar under aerobic conditions and/or terminal spores by Gram stain, suggesting that the majority of these isolates were \(\text{C. tertium}\). Direct stool and enrichment cultures were heat shocked (80°C for 15 min.) and treated with alcohol (equal parts sample and 100% ethanol for 1 h) to eliminate vegetative growth and select for coccidia. However, these methods were not useful in eliminating these associated nontoxic bacteria from the stool culture.

Strain CDC51208 was characterized by molecular analysis. The \(\text{bont/E}\) gene nucleotide sequence was determined using previously reported primers that amplified overlapping regions of the gene (12) and was submitted to GenBank under accession number KP455988. Of the 11 currently recognized \(\text{bont/E}\) subtypes, only E4 and E5 are known to be produced by \(\text{C. butyricum}\) (13). The nucleotide sequence of \(\text{bont/E}\) in strain CDC51208 clustered with other \(\text{bont/E}\) sequences (Fig. 1). All previously reported strains containing \(\text{bont/E}\) are associated with botulism cases in Italy. \(\text{C. butyricum}\) strains containing \(\text{bont/E}\) are associated with a foodborne outbreak and soil samples from China (12). \(\text{C. butyricum}\) strain CDC51208 was compared by pulsed-field gel electrophoresis (PFGE) (14) to two available \(\text{C. butyricum}\) type E strains, 5262 and 5520, from the first and second Italian infant botulism cases, respectively (3). Although the \(\text{bont/E}\) sequences of strains 5262, 5520, and CDC51208 were identical, the Smal and Xhol PFGE profiles of strains 5262 and 5520 (>93% similar to each other) were distinct from that of CDC51208 (<66% similar) (Fig. 2A). These results indicate that while the toxin gene sequence is stable among the strains examined, differences in the genomic background of these strains appear to be associated with their origin (i.e., CDC51208, which was isolated in the United States, was genetically distinct from the Italian strains 5250 and 5262).

Identification of antimicrobial resistance genes provided another way to characterize and compare strain CDC51208 to \(\text{C. butyricum}\) type E isolates from Italy. The genes for \(\beta\)-lactamase (\(\text{bla}\)), tetracycline resistance [\(\text{tet(P)}\)], and lincomycin resistance protein (\(\text{linB}\)) were previously identified in \(\text{C. butyricum}\) type E isolates from Italy (15). Using primer sequences reported by Franciosa et al. (15), we searched for these three genes by PCR in strain CDC51208, and for comparison, in strains 5252 and 5520. Ampli-

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**FIG 2** Molecular characterization of \(\text{C. butyricum}\) type E strains 5520, 5262, and CDC51208. (A) Smal and Xhol pulsed-field gel electrophoresis (PFGE) patterns, with dendrograms constructed by unweighted pair group method using average linkages (UPGMA) analysis. PFGE patterns were compared by the Dice coefficient with a tolerance window of 1.5% and an optimization of 1.5%. (B) PCR products (≈400 bp) for \(\text{bla}\), \(\text{tet(P)}\), and \(\text{linB}\). Products were run on a 1% agarose gel and stained with ethidium bromide. The results for strains 5520 and 5526 are consistent with previous findings by Franciosa et al. (15).
fication for \textit{bla} and \textit{tet(P)} was observed in all three \textit{C. butyricum} type E strains (Fig. 2B). \textit{bla} was previously identified in \textit{C. butyricum} type E clinical isolates from Italy and China but was not found in soil isolates from China (15). The presence of \textit{bla} in strain CDC51208 lends additional support for the presence of this gene among clinical isolates. Although \textit{lmrB} was present in strains 5262 and 5520, it was not detected in strain CDC51208 (Fig. 2B). Interestingly, this gene also was not detected in any isolates containing \textit{bont/E5} isolated in China (15), suggesting that the presence of \textit{lmrB} is varied among \textit{C. butyricum} type E strains.

To our knowledge, this is the first case of botulism caused by \textit{C. butyricum} type E in the United States and the first identification of a strain outside Italy containing \textit{bont/E4}. The isolation of \textit{C. butyricum} type E in the United States suggests that this strain may be more widely distributed than was previously recognized. The presence of numerous nontoxicogenic \textit{Lip} and \textit{Lip}/\textit{Lec} bacteria in this infant stool sample and the rarity of \textit{C. butyricum} type E significantly complicated the identification of this rarely identified cause of infant botulism. Increased awareness of these concomitant strains in infant stool may help investigators more readily identify \textit{C. butyricum} type E in suspected infant botulism cases.

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REFERENCES


