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Characterization of a Neurotoxigenic *Clostridium butyricum* Strain Isolated from the Food Implicated in an Outbreak of Food-Borne Type E Botulism

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Neurotoxigenic *Clostridium butyricum* was isolated from the food implicated in an outbreak of clinically diagnosed type E botulism in China. PCR assay showed that the isolate (LCL 155) contained the type E botulinum toxin gene. This appears to be the first report of neurotoxigenic *C. butyricum* causing food-borne botulism.

The species *Clostridium botulinum* is divided into seven toxigenic types, A through G, on the basis of the serological specificity of the neurotoxins they produce. Three of these types, A, B, and E, are the ones most frequently associated with botulism in humans. Additionally, type F has been associated with several outbreaks among humans, and type G has also been involved in several human cases. Botulism in humans occurs in three forms: food-borne botulism, wound botulism, and infant botulism. Recently, three infant botulism cases, two in Rome, Italy (1, 5), and one in New Mexico (3), have been reported in which clostridial species other than *C. botulinum* produced botulinum toxin. *Clostridium butyricum* producing type E botulinum toxin was isolated from the cases in Rome, and *Clostridium baratii* producing type F botulinum toxin was isolated from the case in New Mexico.

In January 1994, six cases of clinically diagnosed food-borne type E botulism occurred in Guanyun, Jiangsu province, China. These people ingested salted and fermented paste made of soybeans and wax gourds (9). In this outbreak, the toxin, which was neutralized by type E botulinum antitoxin, was detected in the implicated food. However, *C. botulinum* type E could not be isolated, although numerous lipase-positive colonies were examined. These findings prompted us to examine the implicated food for neurotoxigenic organisms other than *C. botulinum.*

In this paper we describe the characteristics of a neurotoxigenic *C. butyricum* isolate from the implicated food in the food-borne botulism cases, which seem to be the first outbreak due to the organism.

Direct inoculation of the implicated food on agar plates was not performed. To isolate the causative organism from the food, we employed a method for isolating *C. botulinum* from soil reported by Yamakawa and Nakamura (8). In this method, each test material is examined with a small amount of inoculum and multiple cultures. In this study, several 1-g portions of the implicated food, salted and fermented paste made of soybeans and wax gourds, were inoculated into tubes (13 by 150 mm) containing 10 ml of chopped meat-glucose medium and incubated at 30°C for 5 days. The supernatants from the cultures were examined for botulinum toxin by a mouse toxicity test. Several 0.02-ml portions of the culture containing type E botulinum toxin were then spread on Trypticase peptone-yeast extract-glucose (20 g of Trypticase peptone [Becton Dickinson Microbiology Systems, Cockeysville, Md.]/liter, 5 g of yeast extract [Difco Laboratories, Detroit, Mich.]/liter, 5 g of glucose/liter, 0.9 g of NaCl/liter, 20 g of agar/liter [pH 7.2]) containing 5% egg yolk (egg yolk TYG) agar plates and incubated anaerobically for 2 days. Many lipase-positive colonies, which are produced by all known *C. botulinum* type E strains, several lecithinase-positive colonies, and several lipase- and lecithinase-negative colonies were selected, inoculated into chopped meat-glucose medium, and incubated anaerobically at 30°C for 5 days. The supernatants of the cultures were examined for botulinum toxin by a mouse toxicity test, and toxigenic colonies were purified on egg yolk TYG agar plates. Tests for biochemical properties were performed according to the methods of Yamakawa and Nakamura (8). Detection of the type E botulinum toxin gene by PCR was performed according to the procedure described by Szabo et al. (7): PCR templates were prepared from boiled cell lysates with the primers E1 (5′-TA TATATTAAACCAGGCGG-3′) and E2 (5′-TAGAGAA ATATTGGAACTG-3′).

A total of 57 colonies were examined, 43 lipase positive, 9 lecithinase positive, and 5 lipase and lecithinase negative. All of the cultures from lipase-positive and lecithinase-positive colonies were nontoxigenic. However, two of the cultures from lipase- and lecithinase-negative colonies produced a toxin that was neuroparalytic and lethal to mice. This toxin was neutralized by monovalent type E botulinal antitoxin, but not by monovalent antitoxins for types A, B, C, D, F, and G. The culture had a toxin titer of 10^3 minimum lethal doses/ml, which increased to 10^6 minimum lethal doses/ml after treatment with trypsin.

One isolate, LCL 155, was further tested for biochemical properties in comparison with neurotoxigenic *C. butyricum* BL 6340 (kindly provided by C. L. Hatheway, Centers for Disease Control, Atlanta, Ga.), which had been isolated in Rome (1), *C. butyricum* NCIB 7423, and *C. botulinum* type E Iwanai. Colonies of this isolate on blood agar plates showed white-to-cream color, like *C. butyricum*. Cultural and biochemical properties of the isolate were consistent with those of neurotoxigenic *C. butyricum* BL 6340, except for arabinose fermentation,

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TABLE 1. Biological and biochemical properties of the isolate

Property	Isolate LCL 155	*C. butyricum* BL 6340	*C. butyricum* NCIB 7423	*C. botulinum* type E Iwanai
Lecithinase	−	−	−	−
Lipase	−	−	−	+
Motility	+	+	+	+
Liquefaction of gelatin				
2%	−	−	−	+
10%	−	−	−	−
Digestion of casein	−	−	−	−
Digestion of meat	−	−	−	−
Milk reaction	Curd	Curd	Curd	−
Indole production	−	−	−	−
Nitrate reduction	−	−	−	−
Acid production from:				
Dextrin, fructose, glucose, maltose, mannose, ribose, starch, sucrose, trehalose	+	+	+	+
Amygdalin, arbutin, cellobiose, esculin, galactose, glycogen, lactose, melibiose, raffinose, salicin, xylose	+	+	+	−
Arabinose	−	+	−	−
Inulin	−	−	+	−
Adonitol, sorbitol	−	−	−	+
Dulcitol, erythritol inositol, mannitol, rhamnose, sorbose	−	−	−	−
Esculin hydrolysis	+	+	+	−
Starch hydrolysis	+	+	+	−
Sodium chloride tolerance	2%	2%	2%	2%
Product from peptone-yeast extract-glucose medium	ABls[a]	ABls	ABls	ABls

[a] A, acetic acid; B, n-butyric acid; l, lactic acid, s, succinic acid. Capital letters, ≥10 mM; small letters, <10 mM.

and with those of *C. butyricum* NCIB 7423, except for inulin fermentation, but were quite different from those of *C. botulinum* type E Iwanai (Table 1). On the basis of these findings the isolate was identified as neurotoxigenic *C. butyricum.*

To confirm the presence of the type E botulinum toxin gene, PCR was performed with primers specific for that gene. A product of the expected size (446 bp) was amplified from the isolate as well as neurotoxigenic *C. butyricum* BL 6430 and *C. botulinum* type E Iwanai (Fig. 1).

In the present study we showed that a neurotoxigenic *C. butyricum* organism was present in the food implicated in the outbreak of clinically diagnosed botulism which occurred in Jiangsu province, China, in 1994 (9). A causative organism linked to the outbreak has not been determined, since no clinical materials, such as sera, contents of the gastrointestinal tract, or feces, were examined for the presence of the botulinum toxin or organisms. However, the fact that toxic activity neutralizable by type E botulinal antitoxin was detected in the implicated food strongly suggests that the botulism cases were caused by food contaminated with neurotoxigenic *C. butyricum*. To the best of our knowledge, this is the first report of food-borne botulism caused by neurotoxigenic *C. butyricum.*

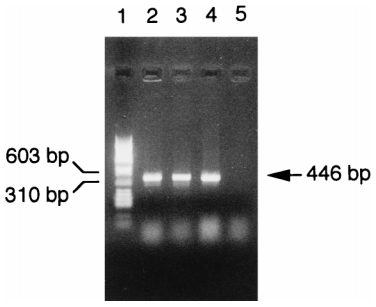

FIG. 1. Agarose gel electrophoresis of PCR products amplified with primers specific for the type E botulinum toxin gene. Lane 1, *Hae*III-digested ϕX174 DNA; lane 2, *C. botulinum* type E Iwanai; lane 3, neurotoxigenic *C. butyricum* isolate LCL 155; lane 4, neurotoxigenic *C. butyricum* BL 6340; lane 5, *C. butyricum* NCIB 7423.

Neurotoxigenic *C. butyricum* was first reported in 1986 in two cases of infant botulism in Rome (1). Since then, the properties of a neurotoxin from this organism have been extensively studied physicochemically, immunologically, and genetically (2, 4, 6), but the environmental distribution of the organism still remains unclear. In food-borne botulism, toxin types causing disease are usually consistent with the *C. botulinum* toxin types found locally in the soil. The food, salted and fermented paste made of soybeans and wax gourds, from which neurotoxigenic *C. butyricum* was isolated in this study was homemade, suggesting that the organism may exist in the soil in the area where the food was prepared. A survey for the organism in the soil is in progress in our laboratories.

Our isolate was different from neurotoxigenic *C. butyricum* BL 6340 in arabinose fermentation and from *C. butyricum* NCIB 7423 in inulin fermentation, suggesting that these properties may be useful in epidemiological respects.

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