Recovery of a Strain of *Clostridium botulinum* Producing Both Neurotoxin A and Neurotoxin B from Canned Macrobiotic Food

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A rare strain of *Clostridium botulinum* subtype Ab was isolated from a canned macrobiotic food suspected of being linked to a fatal case of food-borne botulism. The strain was recovered and identified by conventional methods modified by the inclusion of a PCR assay (G. Franciosa, J. L. Ferreira, and C. L. Hatheway, *J. Clin. Microbiol.* 32:1911–1917, 1994). The titers of neurotoxins produced by the strain were evaluated by a mouse bioassay.

Seven serologically distinct botulinum neurotoxins (BoNTs), A to G, capable of causing the serious illness of botulism in humans and animals have been recognized to date (16). *Clostridium botulinum* along with peculiar strains of *C. butyricum* (1) and *C. baratii* (9, 15) generally produce one BoNT, which is identified by neutralization with monospecific antitoxins. On the basis of the toxin produced, clostridium strains are traditionally classified as types. Besides the strains of *C. botulinum* types C and D, which respectively produce minor amounts of BoNT D and BoNT C in addition to their main toxins (14), there are rare members of the species which are known to synthesize a mixture of two BoNTs in different proportions and which are grouped in subtypes (7) (Table 1). Interestingly, all of them share the ability to produce either BoNT A or BoNT B, which are the serotypes most commonly involved in human botulism (13). Their serological identification has been troublesome, because they generally produce in low amounts one of the two toxins that are not detectable after dilution; furthermore, doubts about whether two distinct toxins or a single molecule having two BoNT antigenicities is actually formed by these strains have persisted (7, 18).

Some strains classified as subtypes (*C. botulinum* Ba 657 and *C. botulinum* Ab 588) have recently been shown to possess the two different BoNT A- and BoNT B-encoding genes in their genomes by PCR (4).

Here, we describe the detection by PCR of a new strain of *C. botulinum* producing both BoNT A and BoNT B from a canned macrobiotic food of commercial origin suspected to have caused a fatal case of food-borne botulism. In brief, a 76-year-old woman was hospitalized in July 1995, after presenting with symptoms such as vomiting and diarrhea. Two days after admission, symptoms evolved into severe constipation and respiratory failure. At that time, botulism was suspected and the woman was treated with polyvalent antiserum; she was also supported by respiratory aid. Nevertheless, she died 2 days later. Unfortunately, neither clinical nor autopsy specimens were available for analysis.

However, some of the foods consumed by the patient before the onset of the illness were sent to the Food Microbiology

Laboratory of the Istituto Superiore di Sanità. Among others, there was a canned macrobiotic food made of gluten, seaweed, herbs (rosemary and ginger), soybean sauce, and water, based on a traditional Eastern recipe ("Seitan").

Additionally, 30 samples of the same brand item were collected from the retail supplier and sent to our laboratory. Some samples of the ingredients (i.e., two bottles of soybean sauce and three cans of seaweed, imported from Japan), taken randomly from the producing plant, were also received at our laboratory.

The pH values of the food samples were measured with a pH meter (model micro 2001; Crison, Modena, Italy); all their physical characteristics (color, texture, turbidity, odor, etc.) were also recorded.

First, the remainder of the product actually consumed by the patient was analyzed for the presence of any BoNT by a mouse neutralization bioassay (10). One-milliliter samples of the food liquid and its 10-fold dilutions in 0.2 M gelatin-phosphate buffer (pH 6.5) were intraperitoneally inoculated into pairs of mice (0.5 ml per mouse), either alone or, alternatively, mixed with 0.25 ml of polyvalent ABE antiserum (750 IU of type A antitoxin/ml, 500 IU of type B antitoxin/ml, 50 IU of type E antitoxin/ml; Behring, Marburg, Germany) or type A, B, or E monovalent antiserum (horse serum antitoxins, 10 IU/ml; Centers for Disease Control and Prevention). In some experiments, 0.25 volumes of monovalent antitoxins to type A and B BoNTs were added to 1 ml of the dilutions to be tested.

Recovery of the toxigenic microorganism from the food sample was then performed by a modification of the conventional methodologies (10). About 5 g of the food was seeded into cooked meat broth (Oxoid, Basingstoke, Hampshire, United Kingdom) tubes and incubated at 37°C for 5 days, under anaerobic conditions (Whitley anaerobic cabinet MKIII). The enrichment cultures were then tested for toxigenicity by mouse bioassay. Two or three egg yolk agar (EYA) plates (2) were streaked with cultures toxic to mice and incubated at 37°C for 5 days under anaerobic conditions. The modification consisted of testing single colonies for the presence of BoNT A, B, or E genes by PCR: 10 colonies, randomly selected, were picked from the plates by capillary pipette and transferred into TPGY broth (5% Trypticase, 0.5% peptone, 0.4% glucose, 2% yeast extract, 0.1% sodium thioglycolate [pH 7.0]). After heat shock treatment at 70°C for 10 min, the broth cultures were incubated at 37°C overnight, under anaerobic conditions. One milliliter of the TPGY cultures was then used

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TABLE 1. Strains of *C. botulinum* producing two BoNTs

<i>C. botulinum</i> strain	<i>C. botulinum</i> subtype ^a	Origin, botulism type (country)	Yr of recovery	Toxins, their ratio
84	Af (6)	Soil sample (Argentina)	1970	A/F, 10:1
657	Ba (5, 12)	Stool specimen, infant botulism (United States)	1981	B/A, 20:1
2712	Ab (17)	Food sample, food-borne botulism (France)	1983	A/B, 10:1
	Af (3)	Stool specimen, food-borne botulism (Argentina)	1986	A/F, 10:1
4013	Bf (11)	Stool specimen, infant botulism (United States)	1987	B/F, 10:1
588	Ab (4)	Stool specimen, food-borne botulism (United States)	1976	A/B, 10,000:1

^a Numbers in parentheses are references for each strain.

for PCR experiments, according to the protocol previously described (4). PCR amplification products were analyzed by gel electrophoresis on 0.7% agarose (Pharmacia, Uppsala, Sweden).

Finally, the colonies yielding positive PCR products were tested for production of the corresponding BoNTs by mouse neutralization bioassay.

Analysis for the presence of BoNTs and toxigenic microorganisms in the other food samples were performed as described above.

The remains of the food sample actually consumed by the patient appeared organoleptically deteriorated. Its pH was 5.2, and it was found to contain BoNT; indeed, the undiluted food liquid was lethal to mice and could be neutralized only by addition of the polyvalent antiserum. However, after the food extract was diluted 1:100, neutralization occurred with the addition of the type A monovalent antiserum. Therefore, the presence of BoNT A in the food was initially supposed. Detection of the toxigenic microorganisms from EYA plates was attempted by PCR assay, as a preliminary screening for the presence of BoNT A, B, or E genes in the genomes of organisms isolated in pure culture, with the purpose of limiting the use of mice and antiserum reagents. Human botulism is commonly caused by the ingestion of BoNT type A, B, or E, generally produced in food by the lipase-positive *C. botulinum*.

All single colonies tested by PCR showed two amplification bands of ~2,300 and 1,300 bp, which correspond to BoNT A and BoNT B gene fragments, respectively (4). It had previously been demonstrated by PCR that an unexpressed type B toxin gene was present in 43 of 79 (50%) strains of *C. botulinum* type A, and one of them (strain 588) actually produced both toxins (4); moreover, strain 657, already known as *C. botulinum* subtype Ba (5), also possessed both BoNT A and BoNT B genes. Therefore, production of both neurotoxin A and B by the

strain isolated from the canned macrobiotic food could not be excluded. Results of the mouse neutralization test performed with a TPGY broth culture of one of the single colonies positive for type A and B PCR products are summarized in Table 2. Dilutions of the culture supernatant of up to 1:10,000 were still lethal to mice, either alone or when mixed with type A or B monovalent antitoxins; however, toxicity was neutralized when the culture supernatant was mixed with both antisera, suggesting the presence of the two toxins together. Type A monovalent antiserum alone completely neutralized the toxicity of the culture supernatant diluted 1:100,000; therefore, BoNT B was absent from this dilution while it was still present in the previous 1:10,000 dilution. We assumed, therefore, that the strain produced BoNT B at levels of ~20,000 mouse lethal doses (LD)/ml of TPGY culture. In addition, the strain produced ~200,000 mouse LD of BoNT A per ml. According to the conventional methodologies (10), the strain was classified as *C. botulinum* subtype Ab (A/B ratio, 10:1). The same results were obtained following reisolation of the single colony from EYA plates after 10 recultures.

The original food was analyzed again for the presence of both BoNT A and BoNT B, by mouse bioassay (Table 3). The type A monovalent antitoxin completely neutralized the toxicity of the food liquid diluted 1:25. Therefore, we concluded that the titer of BoNT B in the food was about 50 mouse LD/ml. The last dilution lethal to mice of the food liquid neutralized by the addition of type A antitoxin was 1:650; the amount of BoNT A in the food was thus estimated to be 1,300 mouse LD/ml, with a ratio of toxins (A/B, ~1:25) similar to that calculated for the TPGY culture of the isolated strain.

The analysis carried out with 30 samples (which represented all the product on the market and came from the same production lot) and with ingredients obtained from the processing plant gave the following results: 10 samples were positive for toxin detection (4 for BoNT B and 6 for both BoNT A and B).

TABLE 2. Identification and quantitation of the BoNTs produced by the strain of *C. botulinum* isolated from the food sample, by the mouse neutralization bioassay

Sample ^a	No. of mice of indicated status ^b after administration of sample at dilution:									
	1:10		1:100		1:1000		1:10,000		1:100,000	
	D	S	D	S	D	S	D	S	D	S
Without antiserum	2		2		2		2		2	
With antiserum A	2		2		2		2		2	
With antiserum B	2		2		2		2		2	
With antiserum A and antiserum B		2		2		2		2		2

^a The supernatant of a 5-day-old TPGY broth culture was diluted, and 1 ml of each dilution was then mixed with 0.25 ml of each monovalent antiserum (see the text).

^b Two mice were inoculated intraperitoneally with 0.5 ml of each test solution. D, mice died; S, mice survived.

TABLE 3. Identification and quantitation of BoNTs in the food sample related to the fatal case of botulism

Sample ^a	No. of mice of indicated status ^b after administration of sample at dilution:							
	1:5		1:10		1:20		1:25	
	D	S	D	S	D	S	D	S
Without antiserum	2		2		2		2	
With antiserum A	2		2		2			2
With antiserum B	2		2		2		2	
With antiserum A and antiserum B		2		2		2		2

^a The aqueous supernatant of the food was diluted, and 1 ml of each dilution was then mixed with 0.25 ml of each monovalent antiserum (see the text).

^b Two mice were inoculated intraperitoneally with 0.5 ml of each test solution. D, mice died; S, mice survived.

Three more samples, negative for the presence of BoNTs, were found to contain *C. botulinum* spores. Interestingly, the majority of the toxigenic strains (7 of 13) isolated from the food samples and cultured in TPGY broth produced both type A and type B toxins, at levels similar to those produced by the strain of *C. botulinum* subtype Ab isolated from the sample involved in the case of botulism. The other six strains were identified as *C. botulinum* type B; no strain of *C. botulinum* type A was isolated from any sample. The ingredients were negative for both the toxin and toxigenic organisms.

The pH values of these products were all in a range (5.2 to 6.8) allowing the growth of *C. botulinum* as well as of other microorganisms.

Preparation of the macrobiotic food in the production plant consisted of washing whole-meal flour with water, to reduce the starch and concentrate the gluten. This was then boiled for 2 h, cooled under tap water, cut in slices, and placed in glass jars. Finally, soybean sauce, herbs, seaweed, and water were added. Heat treatment of the canned products was carried out by placing the jars in boiling water for 20 min.

Contamination of the original ingredients at the source, improper handling, especially inadequate heat processing, and low acid content were all critical points in the production chain; lack of instruction labels for refrigerated storage also contributed to the high rate of detection of toxins and *C. botulinum* spores in the food samples. The recovery of a rare strain of *C. botulinum* subtype Ab in such a large number of food samples (23% of the items examined) remains inexplicable, even if the similar levels of BoNTs produced by the strains suggest a single source of contamination.

As other authors have pointed out (7), the existence of *C. botulinum* subtypes may have been underestimated because of the difficulty of detecting the minor toxin, which is masked by the major one. The same authors also proposed a method for BoNT typing based on extensive use of mice and antitoxins (8).

In this study, we used PCR technology to preliminarily detect type A, B, or E botulin neurotoxin genes in the genomes of the organisms isolated from the foods. This method proved useful; indeed, only after amplification of the two BoNT A and BoNT B gene fragments from the genomes of single organisms did we suspect the possible production of the corresponding BoNTs. Moreover, our modification of the conventional protocol, including the PCR assay, allowed us to shorten analysis time and use fewer mice and reagents. However, the mouse test remained necessary to establish the relative levels of production of the two neurotoxins by the isolated strain and their respective amounts in the broth culture as well as in the food samples.

We thank Charles L. Hatheway, Director of the Botulism Laboratory, Centers for Disease Control and Prevention, for confirming the toxin titration results.

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