Original Article

Identification of Botulinum Toxin Type in Clinical Samples and Foods in Iran

Fereshteh Shahcheraghi PhD•[1], Saman Nobari MSc[1], Hossein Masoumi Asl MD[2], Mohammad Mehdi Aslani PhD[1]

Abstract

Background: Botulism is a serious neuroparalytic disease caused by toxins of *Clostridium botulinum*. Botulinum toxin is produced under anaerobic conditions and is one of the most dangerous toxin in the world. Rapid diagnosis of botulism is very essential for successful therapy. In this study, we reviewed data of cases of botulism in Iran from April 2004 through March 2010.

Materials and Methods: From a total of 1140 samples of suspected botulism samples, 477 serum, 294 stool, 111 gastric secretions, and 258 food samples were collected from 21 provinces. These samples belonged to 432 distinct patients. All samples were tested for botulism by mouse bioassay, a gold standard method for detection of botulism.

Results: From 1140 received samples, 64 (5.6 %) positive samples of botulism were identified. Of these, 14 (21.8 %) cases had toxin type A, seven (11 %) cases had toxin type B, 22 (34.3 %) cases had toxin type E, and seven (11 %) cases had toxin type AB. The toxin type could not been identified in 14 (21.8 %) cases. The highest positive results were in Gilan, Tehran, Golestan, and Hamedan provinces. Seafoods and locally- made cheese were the most implicated foods in type E and type A botulism, respectively.

Conclusion: Accurate and rapid diagnosis of botulism is very important because every case of botulism can be a public health emergency. During the study period, the median number of positive cases per year was 2.7 (range: one to18). Therefore, it is suggested that all clinicians are required to submit the collected samples from patients with botulism symptoms to the botulism reference laboratory for specific diagnosis and confirmation of botulism.

Keywords: Botulism, Iran, mouse bioassay

Cite this article as: Shahcheraghi F, Nobari S, Masoumi Asl H, Aslani MM. Identification of botulinum toxin type in clinical samples and foods in Iran. *Arch Iran Med*. 2013; **16(11):** 642 – 646.

Introduction

Botulism is a serious disease caused by botulinum toxin produced by the *Clostridium botulinum (CB)*, an anaerobic, gram-positive, spore-forming bacillus which exists in the environment.[1] Botulism was first described in Europe in the 18th century following the consumption of sausage. Before the application of methods for inactivating spores in cans, canned foods caused outbreaks in the 19th and early 20th centuries.[2] In recent years, botulism is a rare disease and physicians are unfamiliar with its clinical manifestations.

There are five clinical forms of botulism: foodborne botulism, wound botulism, infant botulism, iatrogenic botulism, and adult intestinal toxemia botulism.[1] Foodborne botulism is the classical form of botulism that is caused by consumption of foods containing botulinum toxin.[3] Production of botulinum toxin occurs only under special conditions which include an anaerobic, low-salt, low-acid environment, and sugar content. Canned foods and native foods are the major source of intoxication in the world, because of creating anaerobic conditions that allow *CB* spores to germinate.[2,4] In Asia, consumption of fish is a leading cause of foodborne botulism.[5] Wound botulism is caused by colonization of wound by *CB* and production of botulinum toxin. Infant botulism results from ingestion of spores that germinate colonize the infant intestine, and produce toxin within the lumen.[6] Adult intestinal toxemia botulism is caused by absorption of toxin produced in situ by rarely occurring intestinal colonization in a few adults by botulinum toxin produced by *CB*. Iatrogenic botulism results from injection of botulinum toxin for cosmetic or therapeutic purposes.[7]

CB produces seven immunologic toxins, designated A through G. Types A, B, E, and rarely F cause most human cases in the world.[1] Botulinum toxins are heat labile. All of toxins are genetically distinct, and they have similar weight and subunit structure. The sequence homology among the botulinum toxins suggests that all of them employ similar mechanisms of action.[8] Botulinum toxin binds to the presynaptic nerve endings of peripheral cholinergic nerves, and blocks acetylcholine transmission across the neuromuscular junction.[1, 2]

Clinical illness is characterized by cranial nerve palsies, followed by descending flaccid muscle paralysis, which can involve the muscles of respiration. Ocular muscle paralysis is due to paralysis of cranial nerves and manifests as blurry vision or diplopia and ptosis. Paralysis of cranial nerves produces dysphagia which may present as regurgitation of foods or beverages. These symptoms often progress to weakness of jaw muscles and dysarthria.

Authors' affiliations: [1]Department of Bacteriology and Microbiology Research Center, Pasteur Institute of Iran, Tehran, Iran, [2]Foodborne and Waterbone Diseases, and Nosocomial Infection Program, Center for Communicable Disease Control, Ministry of Health and Medical Education, Tehran, Iran.
•Corresponding author and reprints: FereshtehShahcheraghi PhD, Department of Bacteriology and Microbiology Research Center, Pasteur Institute of Iran, Tehran, Iran. Telfax: 0098-21-66405535,
E-mail: shahcheraghifereshteh@yahoo.com.
Accepted for publication: 2 October 2013

Dry mouth has also been reported.[3, 8,9,10]

Routine laboratory tests such as blood chemistry, hematology, and urinalysis are not useful for diagnosis of botulism.[11] Rapid diagnosis of botulism is required for successful and fast therapy because botulism is a life-threatening disease. The standard method for detecting botulinum toxin includes a mouse bioassay involving an intraperitoneal injection of toxin into mice and observation of botulism symptoms after 24 to even 48 hours. In recent years, tremendous progress occurred in the development of alternative tests for detection of botulism. Molecular and immunologic methods such as ELISA, PCR, and radioimmunoassay have high sensitivity and specificity in some studies[12–15] but mouse bioassay remains the most reliable method.1In this study, we reviewed the cases of botulism in Iran from 2004 through 2010.

Materials and Methods

This study involved all suspected botulism samples sent to the Botulism Laboratory of Microbiology Department of Pasteur Institute of Iran by Iranian health centers from 2004 through 2010. Botulism samples included serum, stool, gastric secretions, and food samples such as cans, fish and seafoods, cheese, yoghurt, meat, etc.

Preparation of samples

All identifying information, collection time of samples, and characteristics of the patients were recorded. For preparation of samples, sufficient volume of samples was placed in sterile tubes and a homogeneous suspension was obtained.

Toxin neutralization

Neutralization of toxins was done by using type A, B, and E antitoxins as described by Hatheway, et al.[16] Positive controls included a suspension of samples without antitoxins, and negative controls were prepared by heating the samples because botulinum toxins are heat labile.

Mouse bioassay and interpretation of results

Mouse injection was done intraperitoneally with prepared samples, alone or mixed with antitoxins. We used four mice for each antitoxin type. We observed the signs of botulism in the mice daily for a period of 24 to 48 hours, and four days occasionally. The final results were reported to health centers and recorded in special botulism files.

Results

A total of 1140 samples of suspected botulism were submitted to the Microbiology Department of Pasteur Institute of Iran from April 2004 through March 2010. These samples included 477 serum, 294 stool, 111 gastric secretions, and 258 food samples (each food sample had been related to one patient). These samples belonged to 432 distinct patients. Twenty-one Iranian provinces sent the samples of suspected botulism in this period.

From 1140 samples, after mouse bioassays, 64 (5.6 %) samples were contaminated with botulinum toxin. Of these, 14 (21.8 %), seven (11 %), 22 (34.3 %), and seven (11 %) botulism cases were caused by type A, type B, type E, and type AB botulinum toxins, respectively. Distribution of botulism toxin type and number of positive cases from 2004 through 2010 are shown in Figure 1. We could not identify toxin type in 14 (21.8 %) cases because the quantity of samples (especially serum samples) received from some health centers were very low. Botulism was confirmed in 42 of 432 patients.

The median number of positive cases per year was 2.7 (range: one to 18). The highest positive results were in Gilan, 14 (21.8 %); Tehran, 12 (18.7 %); Golestan, 11 (17.1 %); and Hamedan, 10 (15.6 %). Table 1 shows positive botulism cases and their types in different provinces. Positive botulism samples included four (6.2 %) serum, 23 (35.9 %) stool, and 37 (57.8 %) food samples (Table 2). Among positive cases, no clear seasonal pattern was observed.

During the study period, from 22 cases of botulism type E, 20 (91 %) cases were caused by fish and other seafoods. The most implicated food in type A botulism was homemade cheese with six (42.8 %) cases. All seven cases of botulism type B were identified in stool samples (Table 2).

During the study period, overall 15 outbreaks occurred in Iran. These outbreaks included Hamedan (three outbreaks; 2004, 2005, and 2007), Gilan (three outbreaks; 2008, 2009, and 2010), Golestan (three outbreaks; two of them in 2009 and one in 2010), Tehran (two outbreaks; 2005 and 2010), Kordestan (one outbreak; 2008), Khorasan Razavi (one outbreak; 2006), Eastern Azarbaijan (one outbreak; 2010) and Western Azarbaijan (one outbreak; 2008) provinces. Reported outbreaks of botulism were relatively small (involving two to five persons) and sporadic. In this period, five outbreaks (involving Khorasan Razavi, Hamedan, Kordestan, Western Azarbaijan, and Tehran) were reported which were associated with locally-made cheese.

Table 1. Distribution of positive botulism cases in Iran, 2004 – 2010

State	Average population1	Total number	Positive cases	Type A	Type B	Type E	Type AB	Unknown
Gilan	2430969	193	14	1	0	10	0	3
Tehran	11706008	214	12	3	1	0	2	6
Golestan	1685034	180	11	0	0	10	0	1
Hamedan	1716432	85	10	2	3	0	2	3
AzarbaijanWestern	2956178	76	6	4	1	1	0	0
Azarbaijan Eastern	3625944	69	5	2	0	1	1	1
Markazi	1370393	35	2	0	2	0	0	0
Khorasan Razavi	5755191	89	2	1	0	0	1	0
Qazvin	1164650	74	1	1	0	0	0	0
Kordestan	1454989	34	1	0	0	0	1	0

1Average population according to published data by Statistical Center of Iran. Available from: URL: http://www.amar.org.ir/Default.aspx?tabid=133.

Table 2. Positive cases of botulism by samples

Samples	Total number	Positive cases	Type A	Type B	Type E	Type AB	Unknown
Serum	477	4	0	0	1	1	2
Stool	294	23	4	7	0	4	8
Gastric secretions	111	0	0	0	0	0	0
Dairy products	57	12	8	0	1	2	1
Fish eggs	47	6	1	0	4	0	1
Fish	68	18	0	0	16	0	2
Meat	14	1	1	0	0	0	0

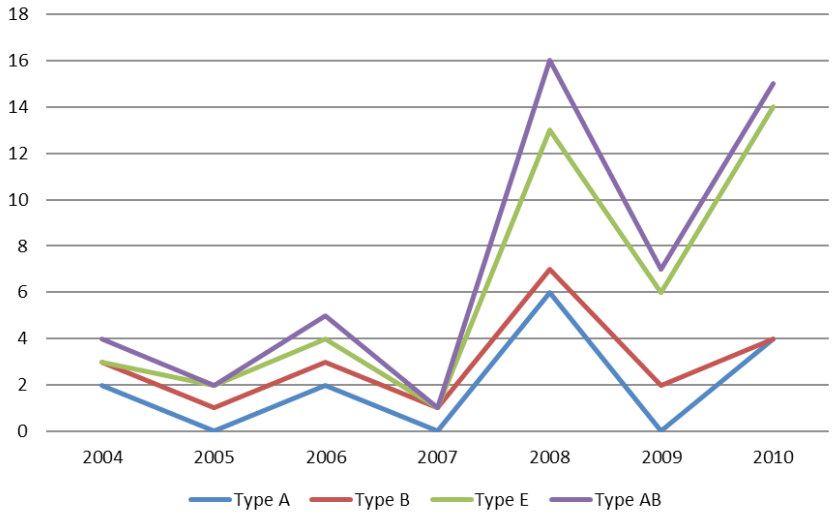

Figure 1. Positive botulism cases in Iran, 2004 – 2010.

Discussion

Worldwide, native foods are the most frequent cause of foodborne botulism and have caused many outbreaks; for example, bean curd in China,[17] canned vegetables in USA,[18] fish eggs in Canada,[19] locally- made cheese in Iran,[20] fish and fish eggs in Alaska,[3] and cream cheese in Italy.[21] Our results showed that cases of botulism in Iran have been prevalently associated with the consumption of seafoods and dairy products, respectively. Among dairy products, locally- made cheese was a major factor in the production of botulism, especially type A.

In Argentina, France, and the United States cases of botulism associated with dairy products have been reported.[22] Cherington, et al. recommended that non acidic foods need to be pasteurized twice, at 24 hour intervals, to kill the bacteria generated from the surviving spores.[23]

In this study, all cases attributable to consumption of fish, were caused by botulinum toxin type E. Seafoods especially sold fish contaminated with botulinum toxin type E, remained a leading cause of botulism in Iran. We suggested that health centers should warn people of botulism caused by consumption of contaminated fish. Botulism caused by consumption of fish is well documented in Egypt,[24] East Europe,[25] Hawaii,[26] and Alaska.[27]

In a previous study, of the 2622 outbreaks reported from 38 countries, 34 % were type A, 52 % were type B, and 12 % were type E.[28] In the United States, in 2008, toxin type A was found in 56 % of botulism cases and type E was 33 %.[27] In Asia, botulism is an important entity because of the popularity of fermented foods and seafoods. In Japan, during 1955 – 1998, 86 cases of botulism were reported that all of these cases were foodborne botulism.[29] Homemade fermented bean curd accounted for 74 % of outbreaks in China during 1958 – 1983. In this period, there were 4377 cases of botulism in China.[30] In the United States, during 1990 – 2000, the median number of foodborne botulism cases per year was 23 cases (range: 17 – 43) and according to the CDC's annual report of botulism cases in 2008, 18 cases of foodborne botulism were reported.[27] Similar to our investigations in Iran, most cases of botulism in the world are sporadic, involving two or three persons.[1]

In this study, we identified seven cases (11 %) of *CB* type AB. A few cases of *CB* strains have shown this type in the world. Kobayashi, et al. reported the first case of foodborne botulism by *CB* type AB in Asia in 2003.[31] Previous studies showed that *CB* type AB produced a large amount of type A toxin and a small amount of type B toxin.[31,32] In this study, interestingly, six cases of detected *CB* type AB were identified in stool and dairy products and only one case of type AB was identified in serum. It seems that in botulism cases caused by *CB* type AB, the samples of patient's serum detoxified type A antitoxin that it caused misidentification of type AB in serum.

Botulinum toxins disseminate easily and cause high mortality rates in the world. Therefore, botulism classified as a category A substance on the CDC's list of critical biologic agents.[33] Botulism toxin is produced usually during home canning and heating for less than 30 minutes at a low temperature. Heating for 30 minutes at 121 °C is needed to kill the spores. Botulinum toxin can be destroyed by boiling or heating to 80 °C for 10 minutes.[34]

Accurate and rapid diagnosis of botulism is very important because every case of botulism can be a public health emergency and clinicians should submit the collected samples from patients with botulism symptoms to the botulism laboratory for specific

diagnosis and confirmation of botulism.

Clinicians should suspect botulism if a patient simultaneously reveals acute onset of cranial nerve and gastrointestinal problems. Cranial nerves dysfunction includes diplopia, dysphagia, and ptosis, and gastrointestinal problems include nausea, vomiting, and paralytic ileus.[35,36] Patients who do not receive antitoxin within 12 hours after botulism presentation are three times more likely to develop respiratory failure which is a major cause of mortality of the disease.[37]

Our data have several limitations. Most of our clinical samples were collected only from provinces that they were responsible for sending suspected samples to the laboratory. Received samples from some health centers were very low in the study period, while samples of all suspected cases of botulism should be submitted to the botulism laboratory immediately and confirmation of botulism helps prevent additional cases. During the study period, 27 identified botulism cases had no contaminated food source. Because most clinicians are unfamiliar with botulism symptoms, some botulism cases, especially the cases with no implicated food, may have been misidentified as other neurologic diseases such as Guillain-Barre syndrome, myasthenia gravis, stroke syndromes, and Easton- Lambert syndrome.[1]

Confirmation of botulism usually takes time because the mouse bioassay requires approximately 48 hours to four days for final results. If suspected, botulinum antitoxin should be given immediately, particularly in the first 24 hours of symptoms onset after collection of required samples by clinicians. The Microbiology Department of Pasteur Institute of Iran provides equipment for accurate and rapid diagnosis of botulism by mouse bioassays in the botulism laboratory. Therefore, all Iranian health centers can submit the samples to Pasteur Institute of Iran. All samples should be refrigerated, but not frozen and examined as soon as possible after collection. The date of collection and specifications of patients should be indicated on the labels. Serum samples should be obtained immediately and always be collected before administration of antitoxins, because antitoxin will neutralize circulating toxin. Ideally 10 – 15 mL of serum and 25 – 50 gram of stool should be collected from patients. This quantity permits specific identification of botulinum toxin and repeat tests if necessary.[16]

The laboratory should be notified for any drug therapy that might interfere with toxin. All samples, especially serum, should be sent to the laboratory immediately, because ingested toxin is not demonstrable in serum after one week. The serum toxin isolation rate will decrease to 30 % from 35 % after two days of ingestion.[23] In another study, positive rate of serum toxin was only 13 % – 28 % after two days of ingestion.38 The positive rate of stool cultures will decrease to 36 % after three days; however, toxin is stable in many foods for a long period.[23]

We, therefore, suggest that hospitals and health centers personnel, responsible for sending the samples, should ensure that they will send appropriate and sufficient samples to the botulism laboratory. All health centers are recommended to initiate epidemiologic investigations to determine the source of infection.

Acknowledgments

We would like to thank all Iranian health centers for sending suspected botulism samples to Pasteur Institute of Iran in this period.

References

1. Sobel J. Botulism. *Clinical Infectious Diseases*. 2005; **41:** 1167– 1173.
2. Sobel J, Tucker N, McLaughlin J, Maslanka S. Foodborne botulism in the United States, 1990–2000. *Emerg Infect Dis* 2004; **10:**1606– 1611.
3. Houschild AHW. *Clostridium botulinum*. In: Doyle M, editor. *Foodborne Bacterial Pathogens*. New York: Marcel Dekker; 1989: 112– 189.
4. Middlebrook JL, Franz DR. Botulinum toxins. In: Sidell FR, Takafuji ET, Franz DR, editors.*Medical Aspects of Chemical and Biologic Warfare*. Washington: Borden Institute.Walter Reed Army Medical Center; 1998: 643– 654.
5. Lindstrom M, Korkeala H. Laboratory diagnostics of botulism. *ClinMicrobiol Rev*. 2006; **19(2):** 298– 314.
6. McCroskey L, Hatheway CL, Woodruff BA, Greenberg JA, Jurgenson P. Type F botulism due to neurotoxigenic*Clostridium baratii* from an unknown source in an adult. *J ClinMicrobiol*. 1991; **29:** 2618– 2620.
7. Arnon S. Infant botulism. In: Feigen RD, Cherry JD, editors. *Textbook of Pediatric Infectious Diseases*. 4th ed. Philadelphia: WB Saunders; 1998: 1570–1577.
8. Sugiyama H. *Clostridium botulinum* neurotoxin. *Microbiol Rev*. 1980; **44:** 419– 448.
9. Hatheway CL. *Clostridium botulinum* and other *clostridia* that produce botulinum neurotoxin. In: Hauschild AHS, Dodds KL, editors. *Clostridium botulinum: Ecology and Control in Foods*. New York: Marcel Dekker; 1992: 3–20.
10. Hatheway CL. *Clostridium botulinum*. In: Bartlett JG, Blacklow NR, editors.*Infectious Diseases*. Orlando: Saunders Company; 1991:1583–1586.
11. Centers for Disease Control and Prevention. Botulism in the United States, 1899–1996. *Handbook for Epidemiologists, Clinicians, and Laboratory Workers*. Atlanta, GA: CDC; 1998.
12. Poli MA, Rivera VR, Neal D. Development of sensitive colorimetric capture ELISAs for *Clostridium botulinum* neurotoxin serotypes E and F. *Toxicon*. 2002; **40:** 797–802.
13. Potter MD, Meng J, Kimsey P. An ELISA for detection of botulinal toxin types A, B, and E in inoculated food samples. *J Food Prot*. 1993; **56:** 856–861.
14. Boroff DA, Shu-Chen G. Radioimmunoassay for type A toxin of *Clostridium botulinum*. *ApplMicrobiol*. 1973; **25:** 545–549.
15. Keet CA, Fox CK, Margeta M, Marco E, Shane AL, Dearmond SJ, et al. Infant botulism, type F, presenting at 54 hours of life. *Pediatr Neurol*. 2005; **32:** 193–196.
16. Hatheway CL. Botulism. In: Balows A, Hausler WJ Jr., Ohashi M, Turano A, editors. *Laboratory Diagnosis of Infectious Diseases: Principles and Practice*. New York: Springer-Verlag; 1988; **1:** 111 – 133.
17. Ying S, Shuyan C. Botulism in China. *Rev Inf Dis*, 1986; **8(6):** 984 – 990.
18. MacDonald KL, Cohen ML, Blake PA. The changing epidemiology of adult botulism in the United States. *Am J Epidemiol*. 1986; **124(5):** 7949.
19. Hauschild AHW, Gauvreau L. Foodborne botulism in Canada, 1971 – 84. *Can Med Assoc J*. 1985; **133:** 1141 – 1146.
20. Pourshafie MR, Saifie M, Shafiee A, Vahdani P, Aslani MM, Salemian J. An outbreak of foodborne botulism associated with contaminated locally made cheese in Iran. *Scand J Infect Dis*. 1998; **30(1):** 92 – 94.
21. Aureli P, Di Cunto M, Maffei A, De Chiara G, Franciosa G, Accorinti L, et al. An outbreak in Italy of botulism associated with a dessert made with mascarpone cream cheese. *Eur J Epidemiol*. 2000; **16:** 913 – 918.
22. Collins-Thompson DL, Wood DS. Control in dairy products. In: Hauschild AHW, Dodds KL, editors. *Clostridium botulinum. Ecology and Control in Food*. New York: Marcel Dekker; 1992: 261 – 277.
23. Cherington M. Clinical spectrum of botulism. *Muscle Nerve*. 1998; **21:** 701 – 710.
24. Weber JT, Darwish A, Mishu B, Corwin AL, Rakha M, Hatheway CL, et al. A massive outbreak of type E botulism associated with traditional salted fish in Cairo. *J Infect Dis*. 1993; **167:** 451–454.
25. Centers for Disease Control and Prevention. Botulism associated with commercially distributed Kapchunka-New York City. *MMWR*. 1985; **34:** 546–547.
26. Centers for Disease Control and Prevention. Fish botulism-Hawaii. 1990. *MMWR*. 1991; **40:** 412–414.
27. Centers for Disease Control and Prevention. Summary of Botulism Cases Reported in 2008. National Botulism Surveillance; 2008.
28. Hatheway CL. Botulism: the present status of the disease. *Curr Top MicrobiolImmunol*. 1995; **195:** 55–75.

29. Infectious Disease Surveillance Center, National Institute of Infectious Diseases. Botulism, Japan. *Infectious Agents Surveillance Report (IASR)*. 2000; **21:** 49–50.
30. Shih Y, Chao SY. Botulism in China. *Rev Infect Dis* 1986; **8:** 984–990.
31. Kobayashi H, Fujisawa K, Saito Y, Kamijo M, Oshima S, Kubo M. A botulism case of a 12-year-old girl caused by intestinal colonization of *Clostridium botulinum* type AB. *Jpn J Infect Dis*. 2003; **56(2):** 73 – 74.
32. Kitamura M, Sakaguchi S, Sakaguchi G. Significance of 12S toxin of *Clostridium botulinum* type E. *J Bacteriol*. 1969; **98(3):** 1173 – 1178.
33. Prevention CfDCa. Biological and chemical terrorism: strategic plan for preparedness and response. Recommendations of the CDC Strategic Planning Workgroup. *MMWR recommendation Report*. 2000; **49(RR-4):** 1–148.
34. International Commission on Microbiological Specifications for Foods. *Clostridium botulinum*. In: *Micro-organisms in Foods 5: Characteristics of Microbial Pathogens*. New York: Blackie Academic & Professional; 1996: 68–111.
35. Armada M, Love S, Barrett E, Monroe J, Peery D, Sobel J. Foodborne botulism in a six-month-old infant caused by home-canned baby food. *Ann Emerg Med*. 2003; **42:** 226–229.
36. Lund BM. Foodborne disease due to Bacillus and *Clostridium* species. *Lancet*. 1990; **336:** 982–986.
37. Sandrock CE, Murin S. Clinical predictors of respiratory failure and long-term outcome in black tar heroin-associated wound botulism. *Chest*. 2001; **120:** 562–566.
38. Woodruff BA, Griffin PM, McCroskey LM, Smart JF, Wainwright RB, Bryant RG, et al. Clinical and laboratory comparison of botulism from toxin types A, B, and E in the United States, 1975–1988. *J Infect Dis*. 1992; **166:** 1281–1286.