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NOTES

Dual Toxin-Producing Strain of *Clostridium botulinum* Type Bf Isolated from a California Patient with Infant Botulism

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A retrospective study of *Clostridium botulinum* strains isolated from patients from California with infant botulism identified the fourth known *C. botulinum* strain that produces both type B and type F botulinum toxins. This unique strain represented 0.12% of the California infant botulism case isolates from 1976 to 2003. The relative concentrations of type B and F toxins produced were temperature dependent.

Most strains of *Clostridium botulinum* produce a single toxin of the seven known botulinum toxin types (A to G). Toxin type is identified and classified by toxin neutralization with the homologous type-specific monovalent antitoxin (3). However, rare strains that produce two types of toxins, i.e., Ba, Ab, Bf, and Af (capital letter denotes predominant toxin type), are known (5). All three previously identified Bf strains were discovered because they were the causative agents of three geographically separate cases of infant botulism (7–9). Hatheway and McCroskey (7, 8) discovered the first two Bf strains in the United States. Strain 3281 (ATCC 43757) was isolated in 1980 from an infant in New Mexico (8), and strain 4013 (ATCC 43758) was isolated in 1981 from an infant traveling from California who was hospitalized in Texas (7, 8). Smith et al. discovered the third Bf dual toxin-producing strain in England in 1989 (9). This strain was found to produce primarily botulinum toxin type B at 37°C and primarily botulinum toxin type F at 30°C.

Infant botulism results when botulinum neurotoxin is produced in vivo after spores of *C. botulinum* are ingested, germinate, and colonize the infant large intestine (1, 2). The neurotoxin is absorbed across the intestinal mucosa and binds at the neuromuscular junction, where it blocks acetylcholine release, thereby causing flaccid paralysis (1, 2). The diagnosis of infant botulism is established by demonstration of botulinum neurotoxin and/or *C. botulinum* organisms in a fecal specimen. We now report the fourth instance of infant botulism caused by a dual toxin-producing, type Bf strain of *C. botulinum.* The patient had resided exclusively in California before the onset of illness, thereby establishing the westernmost-known occurrence of this rare organism. Like the British type Bf strain (9), the predominant toxin type (B or F) produced depended on whether the California strain was cultivated at 30 or 37°C.

All previously archived *C. botulinum* isolates from infant botulism patients in California were recultured to check for purity and to verify reported toxin type by a mouse bioassay and then frozen (3). All relevant institutional policies and federal guidelines for the ethical use of animals in research were followed. Monovalent botulinum antitoxins were used to verify the identification of all archived strains. *C. botulinum* strain 93-197 was listed in archived records as a type B strain. As with all archived strains, strain 93-197 was subcultured onto 4% egg yolk agar for the isolation of lipase-positive colonies. Isolated lipase-positive colonies were subcultured onto chopped-meat glucose starch broth, and incubated for 48 h at 35°C. An aliquot of chopped-meat glucose starch broth was diluted 1:20 with gelatin phosphate diluent and filter sterilized for neutralization studies using the mouse bioassay.

The diluted toxic culture filtrate of strain 93-197 unexpectedly failed to neutralize with monovalent botulinum antitoxin B. The toxin types produced by all other strains in the entire collection were verified per laboratory records. Accordingly, further dilution and neutralization studies were performed with both trivalent (ABE) and monovalent (A, B, E, and F) antitoxins. In addition, several colonies from the original 1993 stool culture were isolated and examined. Toxin neutralization studies were conducted with cultures incubated at 30 and 37°C.

All mice that were injected with a 1:20 dilution of untreated culture filtrate died. In contrast, all mice that were injected with culture filtrate that had been pretreated with a mixture of type B and F monovalent antitoxins survived. Neutralization of the toxic culture filtrate did not occur when either type B or type F monovalent antitoxin was used alone (Table 1). These results indicated that strain 93-197 produces two serologically distinct botulinum toxin types, specifically, types B and F. All of the toxic culture filtrates from the five additional single-colony isolates subcultured from the original 1993 stool culture

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TABLE 1. Bioassay determination of dual botulinum neurotoxin production by strain 93-197

Isolate no.[a]	No. of mice alive/no. tested after injection with:				
	Unheated filtrate	Heat-treated filtrate[b]	Unheated filtrate + antitoxin B	Unheated filtrate + antitoxin F	Unheated filtrate + B-F antitoxin mixture
1	0/4	4/4	2/4[c]	0/4	4/4
2–6	0/4	4/4	0/4	0/4	4/4

[a] Isolate 1 is the index isolate; isolates 2 through 6 are replicate isolates from the original fecal culture.
[b] Heat treatment consisted of 10 min in a boiling water bath. Botulinum toxin, a simple protein, is denatured by this treatment.
[c] Two of four mice survived but displayed typical signs of botulism.

exhibited a neutralization pattern identical to that of the index isolate (Table 1). These observations excluded the possibility that the findings in this report resulted from a mixed culture of *C. botulinum* type B and *C. botulinum* type F. In addition to demonstration of dual toxin production by the mouse bioassay, use of an enzyme-linked immunosorbent assay system (4) confirmed the presence of both toxin type B and toxin type F in a culture filtrate of the index isolate.

The British *C. botulinum* Bf strain was found to produce principally toxin type B when incubated at 37°C and principally toxin type F when incubated at 30°C (9). The California Bf strain was grown at three different incubation temperatures and tested for principal toxin production (Table 2). Like the British Bf strain, most isolates of the California Bf strain produced principally type B toxin at 37°C and principally type F toxin at 30°C. Incubation at 35°C produced both toxins at concentrations lethal for mice (Tables 2 and 3). The first two U.S. strains were not tested for temperature-dependent principal toxin production (7, 8).

Also noted was a difference in the mouse 50% lethal doses (LD_{50}) of toxic culture filtrates depending upon the incubation temperature. The LD_{50} of toxic culture filtrate from the 30°C culture was established to be 1:5,120, while the LD_{50} for the 37°C culture was 1:20,480 (Table 3). Therefore, a fourfold-greater concentration of botulinum toxins was produced at 37°C than at 30°C. Because toxin B was produced in a greater concentration than toxin F at 37°C, the strain was designated as being toxin type Bf.

C. botulinum strain 93-197 was initially isolated in 1993 from a fecal specimen submitted for diagnostic testing from a 4-week-old female clinically suspected of having infant botulism. This patient was a suburban resident of Southern California who had not traveled out of the state. The patient was

TABLE 2. Temperature dependence of principal toxin production by *C. botulinum* strain 93-197

Temp (°C)	No. of mice alive/no. tested after injection with[a]:				
	Untreated filtrate	Filtrate plus antitoxin B	Filtrate plus antitoxin F	Filtrate plus B-F antitoxin mixture	Botulinum toxin type detected
30	0/2	0/2	2/2	2/2	F
35	0/2	0/2	0/2	2/2	B and F
37	0/2	2/2	0/2	2/2	B

[a] All neutralizations were performed at a 1:2,560 dilution to optimize neutralization.

TABLE 3. Comparison of LD_{50} of botulinum toxins produced at 30 and 37°C by *C. botulinum* strain 93-197

Dilution	No. of mice alive/no. tested after incubation of culture at:	
	30°C	37°C
1:1,280	0/2	0/2
1:2,560	0/2	0/2
1:5,120	1/2[a]	0/2
1:10,240	2/2	0/2
1:20,480	2/2	1/2[b]
1:40,960	2/2	2/2

[a] The LD_{50} of type F toxin was 1:5,120 at 30°C; at this dilution and temperature, the LD_{50} of type B toxin was <1.
[b] The LD_{50} of type B toxin was 1:20,480 at 37°C; at this dilution and temperature, the LD_{50} of type F toxin was <1.

hospitalized for a total of 6.0 weeks, in contrast to the median 3.4-week hospital stay of other California infant botulism patients (1976 to 1993; $n = 215$). A review of the original laboratory records disclosed that it was necessary to dilute the broth culture to 1:500 in order to achieve monovalent toxin neutralization with type B antitoxin. Consequently, the original laboratory report concluded that *C. botulinum* type B organisms were present in the fecal specimen. Strain 93-197 was misidentified in 1993 because in order to achieve the expected results (monovalent toxin type), culture filtrates were diluted until toxin neutralization could be achieved with a single monovalent antitoxin.

Rare strains of *C. botulinum* that produce two toxins simultaneously may be more prevalent than realized (5), and the possibility exists that they may be overlooked or misidentified during routine botulism analysis. Insufficient anticipation and lack of flexibility in diagnostic procedures may also account for the infrequent recognition of the rare nonbotulinum clostridia that produce botulinum neurotoxins, i.e., *Clostridium baratii* type F and *Clostridium butyricum* type E (5, 6). Strain 93-197 is the first known strain of *C. botulinum* to be found in California that produces two distinct serotypes of botulinum toxin and is the fourth such strain ever reported. This single type Bf strain constitutes 0.12% of the archived collection of California infant botulism isolates and 0.33% of the California case isolates that produce type B toxin. The global distribution of dual toxin-producing *C. botulinum* Bf isolates is now known to extend from southwestern England to the western coast of the United States. To date, no environmental source of Bf dual toxin-producing strains has been identified. All four reported Bf strains were recovered from patients with infant botulism, and because of this, the human infant has thus far served as the "environmental sensor" for this microorganism.

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