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Examination of Feces and Serum for Diagnosis of Infant Botulism in 336 Patients

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In the 12-year period 1975 to 1987, feces from 336 infants were examined for botulinal neurotoxin and *Clostridium botulinum*. All the infants had illnesses which prompted their physicians to consider infant botulism in the diagnosis. Stool specimens from 113 of the infants yielded organisms that produced botulinal neurotoxins assumed to be responsible for the illness. The types of botulinal toxin in the confirmed cases were distributed as follows: 38 A, 69 B, 2 atypical B, 1 E, 1 F, 1 A + B, and 1 B + F. The type A and B toxins in a single infant were produced by two different strains of organism, and the type B and F toxins in another infant were produced by a single strain. The physiological characteristics of all the isolated toxigenic organisms except two were consistent with those of group I (proteolytic) *C. botulinum*. The toxigenic isolate from the infant with type E botulism was identified as *C. butyricum*, and that from the infant with type F botulism was identified as *C. barati*. Toxin of the same type as produced by the isolated organisms was identified in feces of 98 of 111 culture-positive infants. Botulinal toxin was identified in the serum of 9 of 67 culture-positive infants (8 of 22 infants with type A organisms; 1 of 43 infants with type B organisms; neither of 2 infants with A + B or atypical type B organisms). Botulinal toxin was not detected in feces (206 infants) or in serum (114 infants) of the culture-negative infants. The culture-positive infants had clinical features and a course of illness consistent with those of infant botulism. Most of the culture-negative infants probably had illnesses other than botulism, but specimens might have been obtained late in some infants' illnesses, when the organism had disappeared.

Infant botulism, first described in 1976 (16), usually affects infants up to the age of 6 months. The infants often present with general weakness, poor feeding, and constipation, and most require hospitalization. Confirmation of the diagnosis of infant botulism relies on the detection of botulinal toxin or *Clostridium botulinum* in the stool (10); serum from these patients is rarely reported to have detectable levels of toxin. Stools from affected infants usually contain moderate to high toxin levels (10^1 to 10^5 mouse lethal doses per g; Centers for Disease Control [CDC], unpublished observations), and they readily yield *C. botulinum* by direct streaking of specimens on agar medium (8) or from broth enrichment cultures (10). Stool specimens obtained in some cases weeks and even months after the onset of illness continue to be positive for the organism and its toxin (2).

In our laboratory from 1975 through April 1987, we examined fecal specimens from 336 ill infants less than 1 year of age who had symptoms suggestive of botulism. Serum samples from the patients were also examined when available. The details of the laboratory findings in the investigation of infant botulism are presented in this article.

MATERIALS AND METHODS

Infants and specimens. Specimens pertaining to approximately 1,750 investigations of suspected botulism incidents were received in the CDC Botulism Laboratory between 1975 and April 1987. Among these were 507 fecal samples from 336 infants up to the age of 1 year from 47 states, the District of Columbia, and one foreign country. Sera were available in sufficient quantities for toxin tests from 181 of these infants. All of the infants had illnesses characterized by severe weakness and clinical features which prompted physicians to request tests for botulism. Specimens from the infants were submitted through the various state health departments, federal agencies, and in one case, a foreign health ministry, to the CDC Botulism Laboratory. At least one fecal specimen from each infant was cultured for *C. botulinum*.

Testing of fecal samples for botulinal toxin. All fecal samples of more than 1 g were tested for botulinal toxin by using procedures described elsewhere (6, 10). Fecal samples were suspended in gelatin diluent (0.2% gelatin, 0.2% disodium phosphate, pH 6.2). The ratio of specimen weight to diluent volume varied from 1:1 to 1:10, depending on the dryness of the specimen or sometimes on the quantity of specimen available for testing. The suspensions were kept overnight at 4 to 8°C and centrifuged, and the resulting supernatant (extract) was tested for toxin by injecting 0.4 ml intraperitoneally into mice and observing for signs of botulism and death. The lethal (and paralytic) factor in the extract was identified as botulinal toxin by demonstrating neutralization by specific botulinal antitoxin, type A, B, E, or F, and polyvalent ABCDEF antitoxin. Toxin titers of the extracts were determined in many cases by testing the lethality of 10-fold dilutions of the extract (1:10 through 1:100,000). If specific neutralization was not obtained with any of the antitoxins, neutralization tests were repeated, using higher dilutions of the extract when feasible. Nonbotulinal lethality was often observed with undiluted fecal extract or at a 1:10 dilution maximum titer, while botulinal toxin was often demonstrated in confirmed cases at extract dilutions of 1:100 to 1:10,000. Nonbotulinal lethality, or nonspecific toxicity, is defined as the lethal effect in mice which could not be prevented by treatment of the extract with polyvalent or any of the monovalent antitoxins. Nonspecific mouse deaths were generally preceded by signs in the mice which were not consistent with botulism.

Culturing fecal specimens to recover *C. botulinum*. All fecal specimens, including rectal swabs in a few instances, were

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TABLE 1. Results of examining feces and sera from 336 infants for *C. botulinum* and botulinal toxin

Results	No. of infants	
	113 Culture positive	223 Culture negative
Infants tested for fecal toxin		
Toxin positive	98	1[a]
Toxin negative	4	153
Nonspecific	9	52
Infants tested for serum toxin		
Positive	9	0
Negative	58	114

[a] Probably false-positive result in view of negative cultures and the clinical features of the illness.

inoculated into cooked meat-glucose-starch medium (CMGS) (6, 10) in duplicate, and one of the pair of inoculated tubes was heated for 10 min at 80°C to eliminate vegetative organisms and thus select for sporeforming organisms. The presence of the causative organism in the cultures after at least 4 days of incubation (25 to 30°C in an anaerobic chamber) was demonstrated by testing culture supernatants for botulinal toxin in mice. Organisms were isolated from CMGS cultures by streaking on egg yolk agar or egg yolk agar containing antibiotics (8), incubating the plates for 2 days at 35°C in the anaerobic chamber, and picking lipase-positive colonies, typical of *C. botulinum*. Isolates inoculated into CMGS were confirmed as the causative agent by demonstrating their ability to produce botulinal toxin of the same type as that found in the feces. In two cases, the toxigenic organisms were lipase negative.

Characterization of isolated organisms. Isolated organisms were evaluated for their ability to produce botulinal neurotoxin by testing CMGS cultures in mouse toxicity and neutralization tests. Toxigenic organisms were observed for their colonial and cellular morphology, Gram reaction, and ability to grow in the presence or absence of oxygen; for their ability to metabolize certain carbohydrates and to hydrolyze gelatin and casein; for production of lipase, lecithinase, urease, catalase, and indole; and for production of various metabolic end products by gas-liquid chromatography, according to methods described by Dowell and Hawkins (5).

Testing serum samples for toxin. Sera were tested in mice for botulinal toxin in the same manner as that used for testing fecal extracts and culture supernatants. The amount injected was usually 0.4 ml per mouse but varied depending on availability. If less than 0.8 ml was available, half the total amount was injected into each of two mice. If larger amounts were available, up to 0.8 ml was injected if 0.4 ml had no effect on the mice.

RESULTS

The overall results of the toxin and culture studies are summarized in Table 1. Infants (n = 113) had neurotoxigenic clostridia in their stool cultures (Table 1). Toxin was also identified in the stools of most (87%) of the culture-positive infants; only 3.5% were negative, and about 8% were inconclusive because of nonspecific toxicity. Among stools of the culture-negative infants, 74% were negative for fecal toxin and 25% were nonspecifically toxic. The reason that the nonspecific percentage was lower with the culture-positive infants is that this problem is resolved when botulinal toxin can be identified at a dilution of the extract at which the nonspecific factor is not effective.

One false-positive toxin test result occurred when initial mouse test results on the stool from a Delaware infant indicated the presence of type B toxin. Deaths occurred on day 2 in mice receiving extract treated with trypsin and type A antitoxin, whereas mice receiving extract treated with type B and polyvalent antitoxins remained healthy. Tests on a second stool were equivocal; two mice receiving extract treated with type A antitoxin died, one on the first day and one on the second, whereas mice receiving extract treated with type B and polyvalent antitoxins and trypsin survived. Trypsin does not inactivate type B toxin under conditions of the test. No signs suggestive of botulism were noted in any of the mice in these tests before death. The mouse dying on day 2 of the test of the second specimen was noted to be less active but did not show typical signs on day 1. No lipase-positive organisms were found in any of the enrichment cultures, nor was botulinal toxin detected, although there was some nonspecific toxicity in the cultures. No growth was obtained in enrichment cultures after heat and alcohol treatment of the two specimens for spore selection. When reporting the initial toxin test results to the hospital, the clinicians were skeptical of the diagnosis of botulism because the infant had no paralytic signs. The final diagnosis of this case was gastroenteritis. The negative cultures, the inconsistencies of the toxicity tests, and the absence of typical features of the illness led us to conclude that the toxin test results were deceptive.

Toxin was detected in the sera of 9 of 67 culture-positive cases (Table 1). Eight of the nine serum-positive infants had type A botulism, although twice as many infants with type B

TABLE 2. Type of *C. botulinum* isolated from 113 infants confirmed to have infant botulism and demonstration of botulinal toxin in their feces and serum

Type of organism in feces	Test for toxin in feces				Test for toxin in serum[a]
	No. positive	No. negative	No. nonspecific	No. not tested	
A	31	1	5	1	8/22 (36.4)
B	61	3	4	1	1/43 (2.3)
A + B (mixed infection)	1	0	0	0	0/1
B (atypical toxin)[b]	2	0	0	0	0/1
Bf[c]	1	0	0	0	NA
E	1	0	0	0	NA
F	1	0	0	0	NA
Total					9/67 (13.4)

[a] No. positive/no. tested (percent); NA, serum not available for testing.
[b] Characteristics of toxin described in reference 12.
[c] Single strain which produces two types of toxin.

TABLE 3. Mouse toxicity test results on sera from nine infants with botulism

Date	State	Serum sample	Sample vol (ml)	Antitoxin treatment[a]	Test result[b] for mouse no.	
					1	2
9/79	Arizona	No. 1	0.4		D	D
			0.4	Poly	S	S
		No. 2	0.5		D	S(bot.)
			0.5	A	S	ND
12/81	Nebraska	No. 1	0.4		D	D
			0.5		D(bot.)	S
6/82	Florida	No. 1	0.4		D	D
			0.4	Poly	S	S
8/83	North Carolina	Day 1	0.5		D	ND
		Day 3	0.4		D	D
		Day 6	0.4		D	D
		Day 10	0.4	A	S	S
			0.4	B	D	S(bot.)
		Day 21	0.4		S	S
3/84	Indiana	No. 1	0.4	A	D	D
			0.4	B	S	S
6/84	Montana	No. 1	0.8		S(bot.)	S
9/84	Colorado	No. 1	0.8		S(bot.)	S
9/86	Oklahoma	No. 1	0.5	A	S	S
			0.5	B	S(bot.)	S(bot.)
3/87	Colorado	No. 1	0.6		D(bot.)	S(bot.)

[a] A, B, Poly: 0.1 ml of type A, type B, or polyvalent ABCDEF antitoxin, respectively, added to the amount injected.

[b] D, Mouse died; S, mouse survived; (bot.), signs of botulism, i.e., abnormal breathing and wasp-shape body conformation, observed before death [D(bot.)] or in surviving mice [S(bot.)]; ND, not done.

were tested (Table 2). The levels of toxin were sometimes low, not always causing death in the injected mice (Table 3).

The causative organisms in 111 of the 113 confirmed cases were proteolytic, group I (17) *C. botulinum*, toxin type A or B, or an unusual form of type B. One patient had *C. botulinum* of toxin types A and B isolated from each of two fecal specimens. The toxin in the fecal samples was completely neutralized with polyvalent antitoxin but not with monovalent type A or B antitoxin alone; type B organisms predominated in untreated enrichment cultures, whereas type A organisms predominated in cultures heat treated (10) after inoculation. Two patients had *C. botulinum* isolated that produced toxin neutralized with type B antitoxin only at a high antitoxin/toxin ratio (approximately 1 IU:10 mouse 50% lethal doses) (11). A *C. botulinum* strain recovered from one infant required a mixture of type B and F antitoxins for neutralization. A single strain was found which produced both toxins at an approximate mouse lethal dose ratio of 10 B:1 F, determined by neutralization of culture toxicity with either type B or F antitoxin and titration of the residual toxin activities. This strain is analogous to the type Af strain isolated in Argentina by Gimenez and Ciccarelli (7). The cultural and physiologic characteristics of the isolated organisms which produced A, B, atypical B, or Bf toxin (Table 2) were identical to those of proteolytic type A and B strains from foodborne and infant botulism studied by Dezfulian and Dowell (4).

The single cases of type E and type F infant botulism were caused by clostridia quite distinct from group I as well as from the other three groups of *C. botulinum* (17). The toxin that caused the type F case (12) was produced by an organism identified on the basis of its cultural and physiologic characteristics as *C. barati* (9), and the toxin that caused the type E case (3) was produced by an organism identified as *C. butyricum* (13). The toxigenic organisms from the type F and type E cases were found to be closely related genetically to the type strains of *C. barati* and *C. butyricum*, respectively, by DNA homology studies (Jane C. Suen, dissertation, Dr. of Public Health, University of North Carolina, Chapel Hill, 1986).

In the geographic distribution of the infants investigated in these studies (Table 4), the state with the highest number was Hawaii with 14, followed by Arizona with 12; Colorado, Delaware, and Pennsylvania with 10 each; and Louisiana with 6. The majority of the confirmed cases were type B infant botulism, although some variation in toxin type distribution occurred by state. Differences were also observed between states in the percentage of infants studied who were

TABLE 4. Location of infants tested for evidence of botulism

Location	No. tested	No. culture positive	No. confirmed as toxin type:		
			A	B	Other
Alabama	3	0			
Alaska	10	1	1		
Arizona	20	12	6	6	
Arkansas	12	3	1	2	
California	4	1	1		
Colorado	14	10	7	3[a]	
Connecticut	2	1		1	
Delaware	19	10		10	
Florida	23	1	1		
Georgia	4	1		1	
Hawaii	23	14	1	12	1 (A+B)[b]
Idaho	1	0			
Illinois	15	2	1	1	
Indiana	4	1		1	
Iowa	1	1		1	
Kansas	14	3	2	1	
Kentucky	1	0			
Louisiana	18	6	3	3	
Maine	7	0			
Maryland	3	1		1	
Michigan	1	1	1		
Minnesota	2	0			
Mississippi	1	0			
Missouri	7	1		1	
Montana	8	2	1	1	
Nebraska	9	1	1		
Nevada	4	0			
New Hampshire	4	0			
New Jersey	3	3		3	
New Mexico	3	3	2		1 (F)
New York	2	1		1	
North Carolina	15	2	1	1	
North Dakota	8	1	1		
Ohio	2	0			
Oklahoma	9	2	1	1	
Oregon	5	2	2		
Pennsylvania	14	10	1	9	
Rhode Island	1	0			
South Carolina	4	0			
South Dakota	3	0			
Tennessee	2	2		2	
Texas	5	4	1	2[a]	1 (Bf)
Utah	3	3	2	1	
Vermont	1	0			
Virginia	10	2		2	
West Virginia	7	2		2	
Wisconsin	1	0			
District of Columbia	3	2		2	
Foreign (Italy)	1	1			1 (E)

[a] One type B case due to atypical type B toxin (12).

[b] Two strains of organisms isolated.

confirmed. Of 7 patients from Maine, none were positive for toxin or organism, whereas 10 (71%) of 14 infants from Colorado were confirmed.

DISCUSSION

The results reported here indicate that the recommended laboratory approach to confirm infant botulism (10) has been reliable for investigating suspected cases over most of the United States. All the infants yielding positive cultures had clinical features consistent with botulism, and the course of the illness and subsequent recovery (except for two fatalities) gave additional confidence in the diagnoses. Most culture-positive infants also had botulinal toxin in their feces, so recovery of the organisms was not due to culturing a few spores that passed through the gastrointestinal tract or to contamination of the specimens. Of the 13 tested cases in which toxin was not identified, 9 could not be adequately evaluated because of nonspecific toxicity. In one toxin-negative, culture-positive case, specimens were not obtained until more than 3 months after the onset of illness. At least 60 (21 type A, 38 type B, and 1 type A + B) of the culture-positive infants had multiple culture-positive specimens (usually also toxin positive) over periods of 1 week to as long as 3 months. Negative follow-up results were found in six cases (2 type A and 4 type B) on specimens obtained within 1 month of the original specimen. Based on these observations, it could not be established whether type A or type B colonization persisted longer.

The negative results with 223 infants in this study suggest that there are other illnesses that may mimic infant botulism. Physicians with an awareness of infant botulism may be somewhat likely to consider that diagnosis for any infant showing pronounced weakness. Some of the infants showing negative results may have been botulism cases whose specimens were obtained too late in the course of illness. A case may not have been confirmed because the particular specimen examined had low numbers of organisms and little toxin; a second specimen might have provided confirmatory evidence. We have noted a fluctuation in toxin and culture results when examining serial specimens from an infant being treated with antibiotics. The treatment did not eradicate the organism, however (CDC, unpublished data). We believe that finding *C. botulinum* and botulinal toxin in the feces of an infant showing neurological signs consistent with botulism reliably confirms the diagnosis. Failure to obtain these results with multiple specimens in the acute phase of the infant's illness makes the diagnosis unlikely.

Thompson et al. (18) reported 20 instances of recovery of *C. botulinum* type A from feces of children of ages between 0.5 month and 11 years without clinical features compatible with botulism. There may be asymptomatic cases in which the organism multiplies but little toxin is absorbed. It has been shown that high levels of toxin and organism sometimes are present in stools of infants during recovery (2); apparently, little of the toxin is absorbed at that time. Some infants may experience this later phase without experiencing the earlier phase in which intoxication occurs. The largest study on the presence of *C. botulinum* in the feces of normal infants took place in California, where the greatest portion of infant botulism cases have been confirmed. Of 395 infants studied, only one yielded the organism but no toxin (1).

The documentation in this report of toxemia in nine cases of infant botulism (13.4% of the confirmed cases tested) is interesting in view of the negative results usually reported. Sera were positive in 36.4% of the type A cases but only in 2.3% of the type B cases. This difference is statistically significant ($P = 0.0029$; Fisher's exact test [14]). Conceivable explanations might include greater absorbability of type A into the bloodstream or more rapid disappearance of type B from the circulation. Botulinal toxin in the serum of these nine infants whose stools also contained toxin and organisms of the same type helps to affirm the causal relationship between the presence of the organism in the feces of infants and the illness of the patients under investigation. This relationship has been questioned by Pickett (15). If asymptomatic carriers (whose numbers must be quite small in view of the California experience [1]) do exist, the coincidence between the carrier state and a neuroparalytic illness mimicking botulism should be rare. Thus, the chance of a false-positive confirmation should be quite small.

Overall, type B botulism predominated while type A was more prevalent in Colorado; in Arizona and Louisiana, the cases were equally divided between A and B, whereas the cases in Delaware were exclusively type B. The geographic variation in percent positive infants in this study may be due to environmental factors, not just recognition by the physician. There may be more cases overlooked in the areas with higher confirmation rates than in the areas where few cases are confirmed in spite of aggressive submitting of specimens from infants with suggestive symptoms.

The knowledge gained from investigations of infant botulism during the past 12 years, including the results presented here, affirms the causal relationship between toxigenic organisms in the feces and illness of the infants. Investigators should know that the toxins or toxigenic organisms involved may have some unexpected features. Although toxigenic *C. barati* has been encountered only once, two type E cases caused by toxigenic *C. butyricum* (13) were identified in Italy (3). Two cases reported here were caused by strains producing similar atypical type B toxin (one in Texas and the other in Colorado). One case confirmed in New Mexico in April 1980 (not included in our data) was due to a strain which produced a mixture of type B and F toxins (CDC, unpublished information), similar to the Texas case reported here (Table 4). Testing of serum may offer more promise of confirmatory evidence than was previously thought.

Examination of multiple stool specimens and enumeration of lipase-positive organisms on selective egg yolk agar (8) may improve the reliability of the investigations. These methods would show that the infant is actually colonized by the organism and would lessen the probability of encountering an intermittent specimen in which low numbers of the organism might result in negative tests.

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