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Cultural and Physiological Characteristics and Antimicrobial Susceptibility of *Clostridium botulinum* Isolates from Foodborne and Infant Botulism Cases

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Isolates of *Clostridium botulinum* from foodborne and infant botulism cases in the United States were compared on the basis of toxigenicity, cultural and biochemical characteristics, metabolic products, and susceptibility to antimicrobial agents. Seventy-eight strains, including 42 from foodborne and 36 from infant botulism sources, were examined. Cultures on anaerobic blood agar exhibited circular, spindle, and rhizoid (medusa head) colonies. Overall, the characteristics of isolates from foodborne and infant botulism cases were quite similar. We concluded that it was not possible to differentiate *C. botulinum* isolates associated with foodborne botulism from those recovered from infant botulism cases. All of the 78 strains produced an unidentified indole derivative(s), detected with paradimethylaminocinnamaldehyde reagent, and hydrocinnamic acid, detected by gas-liquid chromatography; all exhibited a high degree of resistance to cycloserine, sulfamethoxazole, and trimethoprim. These characteristics should prove to be useful in the isolation and identification of *C. botulinum* from mixed microbial populations.

Three clinical forms of botulism are now recognized: foodborne, infant, and wound botulism (3, 9). Foodborne botulism is associated with the ingestion of neurotoxin released by *C. botulinum* in contaminated food. Infant and wound botulism result from neurotoxin produced by organisms growing in the gut and in injured tissue, respectively (1-4, 9, 11). Another category of botulism for which no vehicle is identified (classification undetermined) was established in 1977 (3).

Colonization and establishment of *C. botulinum* in the gut that leads to infant botulism may depend on the conditions in the gastrointestinal tract, specific characteristics of the causative strains, or both. On the basis of the second possibility, we raised the question of whether *C. botulinum* strains isolated from infant botulism differed in their phenotypic characteristics from those associated with foodborne disease. We now report the results of our comparative studies of the cultural and physiological characteristics and antimicrobial susceptibilities of *C. botulinum* strains isolated from foodborne and infant botulism cases.

MATERIALS AND METHODS

Organisms. Seventy-eight *C. botulinum* strains were studied. These included 42 strains (20 type A, 22 type B) from foodborne botulism outbreaks (1 isolate per outbreak) and 36 strains (15 type A, 21 type B) isolated from the feces of infants with botulism in the United States. All of the strains were proteolytic. Two reference strains, the Hall strain of type A and the Beans strain of type B, were included in the foodborne botulism group. Ten isolates from infant botulism cases were obtained from the California Department of Health, Laboratory Services Branch, and the remainder were from the collection of the Center for Disease Control, Anaerobe Section. Working cultures were maintained in tubes of chopped-meat-glucose-starch medium at ambient temperature. The purity of each strain was checked by examining single-colony isolates from egg yolk agar and anaerobe blood agar.

Media. All media used in this study, with the exception of the Schaedler broth (BBL Microbiology Systems) and the Mueller-Hinton broth (Difco) for determining susceptibility to antimicrobial agents, have been described previously (7); all were purchased from the Nolan Biological Laboratories, Tucker, Ga.

Characterization of cultures. The organisms were subcultured in chopped-meat-glucose medium and incubated at 35°C inside a glove box. After 24 h, plates of blood agar and egg yolk agar were streaked and incubated anaerobically for 24 to 48 h. The resulting colonies were examined with a hand lens, and a well-isolated colony was further studied with a stereomicroscope. Gram-stained smears from 48-h cultures in chopped-meat-glucose-starch medium and growth on chopped-meat agar slants and blood agar were studied under the microscope.

Biochemical tests performed in characterizing the strains are listed in Table 1. Conventional tests in-

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cluded those for fermentation of carbohydrates; production of indole (and indole derivatives), H_2S, catalase, and urease; hydrolysis of esculin, gelatin, and starch; reduction of nitrate; motility; and action in milk (5). Some of the tests were done in quadrant plates containing various differential agar media (6). The conventional tests were performed with tubes of anaerobic differential media used by the Center for Disease Control Anaerobe Reference Laboratory (7) as follows. (i) Each isolate was heavily streaked on a blood agar plate to obtain confluent growth. (ii) After 24 to 48 h of incubation anaerobically at 35°C, the growth was suspended in a tube containing 7 ml of Lombard-Dowell broth without glucose (7) to a turbidity equal to or greater than that of a McFarland no. 5 nephelometer standard. (iii) The conventional differential media were inoculated with this suspension of bacteria (approximately 0.3 ml per tube). (iv) Tubes were incubated at 35°C in a glove box and observed after 24 and 48 h and 7 days of incubation. With the exception of carbohydrate fermentation tests, the tests were recorded as described by Dowell and Hawkins (5).

Fermentation tests were read as follows. (i) All fermentation tubes appearing green or greenish-blue to blue were marked as negative (−) (pH 6.10 or above). The pH of the uninoculated media was about 6.75. (ii) The fermentation media developing a greenish-yellow color were recorded as very weak (pH 5.80 to 6.05). (iii) Media turning yellow with a slight green tinge were marked weak (pH 5.55 to 5.75). (iv) A definite yellow or orange was considered as positive (+). The pH of the contents of these tubes ranged between 5.10 and 5.50.

The quadrant plates were incubated and read as described by Dowell and Lombard (6) and Story and Dowell (S. Story and V. R. Dowell, Jr., Abstr. Annu. Meet. Am. Soc. Microbiol. 1978, C24, p. 281).

Indole and derivative(s) were detected with the method described by Lombard and Stargel (G. L. Lombard and M. D. Stargel, Abstr. Annu. Meet. Am. Soc. Microbiol. 1977, C174 p. 64) and, in addition, Ehrlich reagent (5). A drop of 48-h culture in indole-nitrite medium (BBL) was placed on a filter paper saturated with 1% paradimethylaminocinnamaldehyde reagent. A lavender to violet color indicated a positive reaction for indole derivative(s); blue indicated indole.

Reactions on the quadrant plates were interpreted as follows: (i) Catalase, esculin hydrolysis, indole, lecithinase, lipase, and growth on bile agar were read as described by Dowell and Lombard (6). The presence of indole derivative(s) was interpreted as described above after paradimethylaminocinnamaldehyde reagent was added to the disk on the Lombard-Dowell agar quadrant. (ii) Clearing of the medium around the bacterial growth showed digestion of milk. (iii) When the starch agar quadrant was flooded with Gram iodine solution, absence of a dark blue color (clear zone) around the bacterial growth showed starch hydrolysis. (iv) If glucose was fermented, the growth on glucose agar turned yellow. (v) A change of the deoxyribonucleic acid medium from royal blue to pinkish violet around the bacterial growth showed deoxyribonuclease production.

Gas-liquid chromatography. For detection of volatile and nonvolatile acids, the general procedure described by Dowell and Hawkins (5), with some modifications, was followed. For volatile acids, 2 ml of a 48-h culture of bacteria in peptone-yeast extract-glucose broth was transferred to a screw-cap tube (13 by 100 mm) and acidified to approximately pH 2 by adding 0.2 ml of 50% (vol/vol) H_2SO_4. A 1-ml volume of ethyl ether was then added to the acidified culture and mixed by gently inverting the capped tube about 20 times. The ether extract containing volatile acids was separated by centrifuging and freezing. To detect nonvolatile acids, we mixed 1 ml of a 48-h-old peptone-yeast extract-glucose culture with 0.4 ml of 50% (vol/vol) H_2SO_4 and 2 ml of methanol in a screw-cap tube (13 by 100 mm) and incubated the mixture in a 55°C water bath overnight. One milliliter of distilled water and 0.5 ml of chloroform were added to the tube, which was inverted about 20 times. The tube was then centrifuged, and the extract at the bottom of the tube was used to identify nonvolatile acids.

The gas-liquid chromatography analysis was performed with a Dohrman (Ana Bac) instrument (Dohrman Environtech, Mountain View, Calif.) equipped with a thermoconductivity detector and two columns. A column containing 15% SP-1220 (a low-polarity stationary phase) and 1% H_3PO_4 on 100/120 Chromosorb W/AW was used to detect volatile acids, and another column packed with 10% SP-1000 (a moderately polar stationary phase) and 1% H_3PO_4 on 100/120 Chromosorb W/AW (Supelco Inc., Bellefonte, Pa.) was used for nonvolatile acids. The injection port temperature during operation was 200°C; that of the column was 130°C. The carrier gas was helium, with a flow rate of 100 ml/min. Fourteen microliters of ether or methylated extract was injected into the appropriate column. The fatty acids were identified by comparing their elution times with those of known acid standards, which were analyzed in the same manner. The volatile acid standard contained 1 meq each of acetic, propionic, isobutyric, butyric, isovaleric, valeric, isocaproic, and caproic acids per 100 ml. The nonvolatile acid standard contained the same amount of pyruvic, lactic, and succinic acids and hydrocinnamic acid (HCA).

Toxicity and toxin neutralization tests. The toxin type of each strain was confirmed with toxin neutralization tests. A 4-day-old culture of each organism in chopped-meat-glucose-starch medium was spun at 10,000 × *g* for 30 min in a refrigerated centrifuge (4°C), and the culture fluid was used for toxicity and toxin neutralization tests. Then 0.4-ml samples of diluted (1:10 and 1:100) or undiluted culture fluid were injected intraperitoneally into each of two 15- to 20-g Swiss white mice. In the neutralization test, 0.25 ml of antitoxin (A or B) was mixed with 1 ml of undiluted or appropriately diluted culture fluid and incubated at room temperature for 1 h. Mice were injected intraperitoneally with 0.5 ml of culture fluid-antitoxin mixture. The mice were observed at 4, 24, 48, and 72 h after injection for signs of botulism and death (which usually occurred 4 to 48 h after culture fluid injection).

Antimicrobial agents. The manufacturers supplied the antimicrobial agents as powders suitable for susceptibility tests. These included penicillin G (Pfizer,

TABLE 1. *Biochemical reactions of 78 strains of C. botulinum from foodborne and infant botulism sources*

Test	% Positive[a]			
	Foodborne		Infant	
	Type A(20)	Type B(22)	Type A(15)	Type B(21)
Conventional tests				
L-Arabinose	0	0	0	0
D-Glucose	100 (15)^w	100 (5)^w	100 (7)^w	100 (5)^w
Glycerol	0	0	0	0
Lactose	0	0	0	0
Maltose	95 (5)^w	96 (5)^w	100 (7)^w	95 (5)^w
Mannitol	0	0	0	0
Mannose	0	0	0	0
Rhamnose	0	0	0	0
Salicin	0	0	0	0
Sucrose	0	0	0	0
Trehalose	85 (45)^w	91 (37)^w	87 (20)^w	100 (43)^w
D-Xylose	0	0	0	0
Catalase	0	0	0	0
Esculin hydrolysis	95 (15)^w	100 (9)^w	87	90 (10)^w
Gelatin	100	100	100	100
H_2S	100	100	100	100
Indole	0	0	0	0
Indole derivatives	100	100	100	100
Milk digestion	100	100	100	100
Motility	95	95	100	95
Nitrate reduction	0	0	0	0
Starch hydrolysis	0	0	0	0
Urease	0	0	0	0
Quadrant plates				
Catalase	0	0	0	0
Esculin hydrolysis	95 (15)^w	100 (9)^w	97	90 (10)^w
Indole	0	0	0	0
Indole derivatives	100	100	100	100
Milk digestion	100	100	100	100
Starch hydrolysis	0	0	0	0
Glucose fermentation	100	100	100	100
Deoxyribonuclease	55 (20)^w	86 (32)^w	80 (20)^w	48 (14)^w
Lecithinase	0	0	0	0
Lipase	100	100	100	100
Bile agar, growth	100	100	100	100

[a] Parentheses indicate number of strains tested. ( )^w, Percent of strains showing weak or very weak reactions. The carbohydrate fermentation tests showing very weak reactions included glucose (4% of all strains), maltose (2% of all strains), and trehalose (10% of all strains).

Brooklyn, N.Y.), cefoxitin (Merck, Sharp and Dohme, Rahway, N.J.), tetracycline (Pfizer), rifampin (Ciba-Geigy, Summit, N.J.), clindamycin (Upjohn, Kalamazoo, Mich.), erythromycin (Abbott, North Chicago, Ill.), metronidazole (Searle, Chicago, Ill.), sulfamethoxazole and trimethoprim (Burroughs Wellcome, Research Triangle Park, N.C.), chloramphenicol (Parke Davis, Detroit, Mich.), nalidixic acid (Sterling-Winthrop, New York, N.Y.), and cycloserine (ICN Pharmaceuticals, Cleveland, Ohio).

The antimicrobial agents were dissolved in the appropriate solvents (13), sterilized by membrane filtration, and frozen at −70°C. They were thawed as needed and diluted in sterile distilled water to the final desired concentration.

Antimicrobial susceptibility testing. Antimicrobial susceptibility of the isolates was tested by a broth microdilution method (13). Serial twofold dilutions of antibiotics were prepared in Schaedler broth (BBL) supplemented with 10 μg of vitamin K_1 and 5 μg of hemin per ml, or in supplemented Mueller-Hinton broth (Difco) (sulfamethoxazole and trimethoprim), and dispensed into microdilution trays by means of an automated system (Dynatech Laboratories, Inc., Alexandria, Va.). The trays were placed in plastic bags and stored at −70°C. At 3 to 4 h before the trays were inoculated, they were removed from the freezer and placed in the glove box. The bacterial inoculum was prepared as follows. A 24-h culture of the organism on blood agar was suspended in supplemented Schaedler broth to a turbidity equal to half that of a McFarland no. 1 standard. This suspension was further diluted 1:1,000 (in supplemented Schaedler or Mueller-Hinton medium), and 0.05 ml of the inoculum was added to each well of the microdilution tray. The trays were inoculated inside the glove box. The inoculated trays

were sealed in plastic bags and incubated at 35°C for 48 h. These were read macroscopically, and the minimal inhibitory concentration (the lowest concentration of antibiotic that prevented growth of the organism) was determined. Control cultures of *Bacteroides fragilis*, *Clostridium perfringens*, *Bacteroides thetaiotaomicron*, *Streptococcus faecalis*, and *Streptococcus aureus* were run in parallel with test cultures each time the antimicrobial susceptibility tests were performed.

RESULTS

Colonies. No distinct differences were observed in colonies produced by the *C. botulinum* strains from foodborne and infant sources regardless of whether the toxin type was A or B. On anaerobe blood agar, the cultures formed three distinct colony shapes: circular, spindle, and rhizoid. Most of the circular and spindle-shaped colonies appeared smooth, with an entire or undulate margin. The rhizoid colonies, on the other hand, were mainly rough with an undulate or a filamentous edge. On anaerobe blood agar all colonies exhibited hemolysis, which appeared as a clear zone around or underneath the colonies.

Colony variation. With respect to density, two distinct types of colonies appeared in cultures of several of the *C. botulinum* strains on blood agar. These were designated opaque (Op) and translucent (Tr). The Op type appeared as raised, opaque white colonies, which could easily be distinguished from translucent, flat, spreading, gray Tr-type colonies. The organisms in each type colony (Op and Tr) had similar toxigenic and biochemical characteristics (indicating a common origin) but differed in their growth kinetics, as determined by observation of growth in Schaedler broth (13), and their ability to form heat-resistant spores with time. These observations will be described in detail in a separate publication.

The biochemical characteristics of the strains were largely uniform (Table 1). Nearly all strains fermented glucose and maltose and, to a lesser degree, trehalose. On the other hand, none fermented arabinose, glycerol, lactose, mannitol, mannose, rhamnose, salicin, starch, sucrose, or xylose (Table 1). All of the strains, regardless of toxin type or source, gave positive reactions for gelatin hydrolysis, H_2S, indole derivative(s), digestion of milk, lipase, and growth on bile agar. Nearly all of the strains were motile and hydrolyzed esculin, but they varied in deoxyribonuclease production.

Other negative tests shown by all strains included those for catalase, indole, urease, and lecithinase production and nitrate reduction (Table 1). Although none of the 78 strains exhibited indole when tested with Ehrlich reagent (conventional tube test) or paradimethylaminocinnamaldehyde reagent (conventional tube and quadrant plate), all gave a positive test for an indole derivative(s) with the latter reagent. All strains showed growth on Lombard-Dowell agar and the 20% bile agar, and, with the heavy inoculum described, bile neither inhibited nor stimulated the growth.

Results of biochemical tests performed by conventional tube and quadrant plate techniques (both recorded after 48 h of incubation) agreed satisfactorily. Tests performed by both methods included those for esculin and starch hydrolysis, catalase, indole and derivative(s), digestion of milk, and glucose fermentation. The results of the conventional differential tests read after 48 h and 7 days of incubation agreed quite well.

Metabolic products. Volatile and nonvolatile acids produced by the *C. botulinum* strains are summarized in Table 2. Nearly all strains produced acetic, butyric, isobutyric, and isovaleric acids. On the other hand, isocaproic acid was formed in only 50 to 62% of the isolates, depending on toxin type and source of the isolate. Our search for nonvolatile acids in methylated extracts of the culture fluids led us to detect HCA in all *C. botulinum* strains tested, but we detected no other nonvolatile acids (pyruvic, lactic, succinic). Although the nonvolatile acids were not quantitated, it was obvious from the large peaks, as compared to that of the standard, that HCA was present in large concentrations in all of the *C. botulinum* strains tested. The retention time of HCA in the SP-1000 column was greater than that of succinic acid. Under the chromatographic conditions we used, the retention time of HCA was around 21 min and that of succinic acid was about 7.5 min (the ratio of the retention times of HCA and succinic acid was about 2.7 to 2.8).

TABLE 2. *Metabolic products identified by gas-liquid chromatography*

Fatty acid	% Positive			
	Foodborne		Infant	
	A(20)[a]	B(22)	A(15)	B(21)
Acetic	100	100	100	100
Propionic	0	0	0	0
Isobutyric	100	95	100	95
Butyric	95	100	100	95
Isovaleric	95	100	100	100
Valeric	0	0	0	0
Isocaproic	55	50	53	62
Caproic	0	0	0	0
HCA	100	100	100	100

[a] The numbers in parentheses indicate number of organisms used. A, Type A; B, type B.

Antimicrobial susceptibility. Results of susceptibility tests are shown in Table 3. Most of the organisms were highly susceptible to penicillin, cefoxitin, tetracycline, rifampin, clindamycin, erythromycin, and metronidazole. In contrast, moderate resistance to chloramphenicol and nalidixic acid and a high level of resistance to cycloserine, sulfamethoxazole, and trimethoprim were observed.

TABLE 3. *Susceptibility of C. botulinum types A and B to antimicrobial agents*

Drug	MIC (mcg/ml)	% of isolates susceptible			
		Foodborne		Infant	
		A(20)[a]	B(22)	A(15)	B(21)
Penicillin G	≤0.125	45	36	13	29
	0.25	20	50	67	33
	0.50	30	9	20	38
	>0.50	5	5	0	0
Cefoxitin	≤0.125	5	0	7	29
	0.25	25	36	40	29
	0.50	60	59	46	42
	>0.50	10	5	7	0
Tetracycline	≤0.125	90	100	100	81
	0.25	10	0	0	19
Rifampin	≤0.125	15	27	0	19
	0.25	40	55	40	76
	0.50	10	0	27	0
	>0.50	35	18	33	5
Clindamycin	≤0.25	70	68	93	67
	0.50	15	9	0	9
	1	5	0	7	14
	>1	10	23	0	10
Erythromycin	≤0.125	40	50	60	62
	0.25	35	41	33	38
	0.50	25	9	7	0
Metronidazole	≤0.125	85	95	93	90
	0.25	15	5	7	10
Chloramphenicol	≤1.25	5	9	0	9
	2.5	60	73	86	62
	5.0	30	18	7	24
	>5.0	5	0	7	5
Nalidixic acid	32	50	50	33	38
	64	45	45	54	62
	128	5	5	13	0
Cycloserine	>256	100	100	100	100
Sulfamethoxazole	>302	100	100	100	100
Trimethoprim	>32	100	100	100	100

[a] Figures in parentheses indicate number of isolates tested. A, Type A; B, type B.

DISCUSSION

Biochemical tests and gas-liquid chromatography of metabolic products showed uniformity among the strains of *C. botulinum* (types A and B) isolated from various parts of the United States. Regardless of the toxigenic type, source, or geographical location, the same acid products were produced, and similar reactions were observed with regard to carbohydrates fermented, gelatin and milk digestion, production of H_2S, lipase, and indole derivative(s), esculin hydrolysis, and other biochemical reactions (Table 1). Variation in deoxyribonuclease activity between type A and B strains from foodborne and infant botulism sources needs further study. Production of an unidentified derivative of indole by all of the strains of *C. botulinum* (types A and B) tested may be of diagnostic significance and could prove to be a useful characteristic for presumptive identification of these organisms. Lombard observed that dilute solutions of the indole derivatives—indole propionic acid, indole butyric acid, and indole-3,methyl (skatole)—give a similar reaction with paradimethylaminocinnamaldehyde reagent (G. L. Lombard, personal communication).

Detection of HCA in methylated extracts of the culture supernatants of all the *C. botulinum* (types A and B) strains by using a gas chromatograph with a thermal conductivity detector is significant because the finding has the potential of being useful in diagnostic work. This acid was previously identified in culture fluids of *C. botulinum* types A, B, D, and F (none was found in types C and E strains examined) and certain other clostridia by Moss et al. in 1970 (10) using a chromatograph with a hydrogen-flame detector. However, detection of HCA has not been routinely used as a criterion for identifying these organisms. Production of HCA as the only nonvolatile acid by some toxigenic types of *C. botulinum* and its absence in other types might be useful in further characterizing and identifying organisms in the *C. botulinum* group. It has recently been demonstrated by L. M. McCroskey and C. L. Hatheway (personal communication) that *C. botulinum* type G does not produce HCA.

The high level of resistance of *C. botulinum* types A and B to cycloserine, sulfamethoxazole, and trimethoprim may prove to be useful in the development of a selective medium for isolation of the organism from mixed microbial populations. Furthermore, the resistance of *C. botulinum* types A and B to these antimicrobial agents may prove to be helpful in identification of these microorganisms.

In conclusion, our results showed no pheno-

typic difference between foodborne botulism strains of *C. botulinum* and those from infant botulism cases. We have by no means used all of the tests that could be used to study the strains. Phage typing, deoxyribonucleic acid hybridization, immunological techniques, and other methods may prove useful in distinguishing the agents of infant botulism from those associated with foodborne cases, if indeed they are different.

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