JOURNAL OF BACTERIOLOGY, May 1968, p. 1542-1547
Vol. 95, No. 5

Possible Origin of the High Incidence of *Clostridium botulinum* Type E in an Inland Bay (Green Bay of Lake Michigan)[1]

THOMAS L. BOTT, JODIE JOHNSON, JR., E. M. FOSTER, AND H. SUGIYAMA
Department of Bacteriology and Food Research Institute, University of Wisconsin, Madison, Wisconsin 53706

Received for publication 14 February 1968

Bottom and shoreline sediments of Green Bay, northern Lake Michigan, and rivers of the Green Bay drainage basin, as well as soils of the surrounding land mass, were examined for *Clostridium botulinum* type E. Detection was based on identification of type E toxin in enrichment cultures and was influenced by many factors. Testing smaller amounts of sample in multiple cultures was more productive than examining large inocula in fewer cultures. Incubation at 30 C was unsatisfactory, but 14 days at 20 C or 7 days at 25 C gave good results. Mild heating (60 C for 30 min) of specimens reduced the incidence of positive findings. Freezing enrichment cultures prior to testing for toxicity eliminated many nonbotulinal toxic substances that killed mice. A control culture inoculated with type E spores was employed to show whether a specimen contained factors which could mask the presence of type E. Samples from 708 stations were tested in 2,446 cultures. Type E was found in nearly all underwater specimens of Green Bay and northern Lake Michigan but was present less frequently in samples taken along their shores. The incidence was still lower in the rivers emptying into Green Bay with the organism being rare on the shores of these rivers and in the soils of the land mass proper. Samples from the upper reaches of the rivers practically never contained type E. Runoff could deposit type E spores in Green Bay, but this is not considered to be the major factor in the high incidence of the organism. Multiplication in the bay itself is indicated.

An extensive survey for *Clostridium botulinum* type E in the fish of Lakes Erie, Huron, Superior, and Michigan was made after several type E botulism outbreaks in 1960 and 1963 (11) were traced to smoked fish prepared in the Great Lakes area. The organism was found in the digestive tract of fish of all the lakes, with incidences ranging from 1 to 9%. An exceptionally high (almost 60%) carrier rate was established for the fish of Green Bay on Lake Michigan (2). This high incidence corresponded to the frequency with which the organism could be demonstrated in Green Bay bottom sediments.

Foci of high concentrations of *C. botulinum* type E occur in certain marine areas of the northern latitudes. Explanations of this distribution have emphasized a terrestrial origin, with the organism being washed down from the surrounding land mass to the waters which serve as catchment basins (4). Thus, the presence of type E in the deposits of lakes and in soils of Hokkaido (7, 10), British Columbia (4), and Sweden (6) has been correlated with the frequent occurrence of type E in the northern Pacific and the Baltic Sea.

The distribution of *C. botulinum* type E in Green Bay and its drainage basin was studied to see whether similar reasoning could explain the high occurrence of the organism in Green Bay. Studies leading to the method finally adopted for the detection of the organism in mud and soil samples are also summarized.

MATERIALS AND METHODS

Collection stations were selected to give representative sampling of a given geographical unit. A single 400- to 500-g sample was obtained at each of these stations with a sterile spoon and stored in a plastic container (Spectrum Feacups; Aloe Co.,

[1] Published with permission of the Director, Wisconsin Agricultural Experiment Station.

Schiller Park, Ill.). Bottom deposits (mud and sand under water) and wet shoreline samples [taken from the shore within 6 inches (15.24 cm) of the water's edge] from rivers, lakes, and bays were iced immediately and refrigerated until tested. Dry samples (soil specimens from gardens, fields, wooded tracts, and shores) were held at ambient temperature. Shore specimens consisted of soil or sand taken 20 to 150 ft (6 to 45 m) inland from the shoreline of bays and lakes or 6 to 20 ft (1.8 to 6 m) from the rivers, depending on the topography of the particular location. Bottom deposits not accessible to direct collection were obtained with a Peterson dredge. The junctions where rivers emptied into Green Bay and where mixing of the waters was possible were considered part of the bay; this extended as much as 0.25 mile (0.4 km) upstream in some instances.

Presence of type E was based on the identification of the specific neurotoxin in at least one of the several (usually 3) 1-g amounts cultured in 10 ml of Johannsen's beef heart infusion-cooked meat medium containing 1.5% peptone and 0.5% NaCl (6). A control culture of each sample was prepared by adding 1 g of sand taken near Chamber's Island in Green Bay to an additional replicate. The sand served as a source of natural type E spores (approximately 150 spores/g), since enrichment cultures of this material alone invariably developed type E toxin. A negative control culture indicated that the test specimen contained factors which inhibited growth of the botulinal organism or destroyed the toxin that was formed. Thus, absence of type E from a sample could be accepted with confidence only when the corresponding control culture was positive for type E toxin. Incubation was under N_2 for 14 days at 20 C.

Fish were caught by trawl net or electric shock and iced until examined. The gastrointestinal contents were tested for the presence of type E as described previously (2).

Incubated enrichment cultures were frozen (−6 C) overnight. After thawing at room temperature, 0.25 ml of the uncentrifuged supernatant fluid was injected intraperitoneally into white mice without prior trypsinization. The unused portions were kept frozen until the test was completed. If the preliminary toxicity test killed mice with signs of botulism within 24 hr, the lethal material was identified by the use of type-specific antitoxins (2).

RESULTS

The method for detection of type E described in the previous section and used in the second half of this study evolved from a series of comparative tests.

(i) A culture medium giving the highest type E toxicity was desirable, since trypsinization (5) did not generally increase the low toxin titers commonly obtained in enrichment cultures of the types of samples being studied. The most productive medium was determined by inoculating 10^3 or fewer type E spores into media containing 1 to 10 g of a dried mud sample known to be free of the organism. The beef heart infusion-cooked meat medium of Johannsen (6) usually gave toxin levels about 10-fold higher than those obtained in Trypticase-peptone-glucose (12), glucose-peptone-beef infusion-meat (3), proteose peptone-Trypticase (1), fish infusion (7), Reinforced Clostridial Medium (Consolidated Laboratories, Chicago Heights, Ill.), or Brain Heart Infusion (Difco).

(ii) Type E spores are of low heat resistance, but heating at 60 C for 60 min has been employed to select forspore formers (7, 10). Pretreatment at 60 C for 30 min did not improve the detection of type E in our samples: 45 (23%) of 196 heated cultures developed type E toxin compared to 64 (33%) of the unheated duplicates. Likewise, drying mud samples at 37 C before inoculation into enrichment media could destroy some of the competing microflora (6), but type E was found in 115 (37%) of 308 dried specimens versus 107 (35%) of the undried duplicates.

(iii) Type E was found in only a low percentage of mud samples when the enrichment cultures were held at 30 C. Since detection of type E in fish intestinal contents was slightly higher after incubation at 25 C than at 30 C (2), the muds were retested at lower temperatures with better results. However, incubation at 20 and 25 C did not give a significant difference, toxin being demonstrated in 150 (36%) of 412 cultures incubated at 20 C for 14 days and in 131 (32%) duplicates held at 25 C for 7 days.

(iv) Use of larger sample portions to increase the initial numbers of type E introduced per enrichment culture did not improve the detection of the organism. Each of 15 mud samples was cultured as sets of six 1-g, three 5-g, and two 30-g amounts in 10-, 10-, and 50-ml volumes of Johannsen's medium, respectively. At least one type E toxigenic culture was obtained for 10 of the specimens tested with the 1-g inocula, for 6 tested with 5 g, and for only 2 with 30 g.

(v) Nonbotulinogenic lethal activity for mice of many enrichment cultures interfered with the type E detection procedure. Freezing the incubated enrichment cultures at −6 to −20 C eliminated much of this nonspecific activity. In an experiment with fish intestinal contents (Table 1), 118 cultures were lethal for mice when injected immediately after incubation; after freezing, only 43 killed mice. In the latter group were 14 which were lethal only after freezing; type E toxin was found in the supernatant fluid of 5 of these cultures. This could result from lysis of cells during freezing and thawing. Refrigeration for 1 to 2 days at 4 C did not have the same effect: of 64 cultures tested, 31 were lethal when injected without treatment, 15 after freezing, and 35 after

refrigeration. Although not specifically tested, the occasional loss of type E toxicity seemed less frequent after freezing than after refrigeration.

The mud, soil, and other samples collected in Green Bay and its environs (Fig. 1) during the summers of 1965, 1966, and 1967 were tested for the presence of *C. botulinum* type E by a procedure based on the studies described. Specimens collected at 708 stations were tested in 2,446 individual cultures (Table 2).

The organism was found extensively in the bottom and shoreline samples of northern Lake Michigan as well as in Green Bay and its northward extensions of Little Bay de Noc and Big Bay de Noc. Shore samples of all these waters except Big Bay de Noc contained type E, but at frequencies considerably lower than those found for the adjacent bottom and shoreline specimens. Distribution is given as percentages of samples (1/station) and of cultures (3 or more/sample) positive for type E, the latter giving some indication of the concentration of the organism. For reasons previously discussed, only samples giving positive control cultures are considered significant.

Exclusive of the Fox River system, type E was found in 22% of the cultures prepared with bottom and shoreline samples of the rivers emptying into Green Bay. However, only one of 21 samples obtained 7 miles (11 km) or more from the mouths of the rivers was positive. Significantly lower type E incidence was found for the shore samples taken along these watercourses than for the corresponding bottom and shoreline specimens. Three of the four positive shore samples were taken within 2 miles (3.2 km) of Green Bay at locations where the organism was present in the neighboring river or shoreline collection points.

The Fox River is the largest tributary of Green Bay, with the upper Fox draining a large agricultural area and the lower Fox an important industrial complex. Type E was demonstrated in only 2% of the cultures and in 4% of the samples obtained from bottom and shoreline collection sites. Corresponding to this low incidence, the type E carrier rates of fish of the Fox River caught in the summer of 1965 (Table 3) were significantly lower than those of Green Bay reported earlier (2).

TABLE 1. *Lethality for mice of supernatant fluids of enrichment cultures of Lake Erie fish as affected by freezing at −6 C for 1 to 5 days*

Lethality for mice		No. of cultures	
Before freezing	After freezing	In group	With type E toxin
+	+	29	16
+	−	89	0
−	−	88	0
−	+	14	5

The relatively rare occurrence of type E in the land mass around Green Bay was shown by the 2% incidence in the 78 miscellaneous soil specimens tested (Table 2); three of the four positive samples were taken within 0.5 mile (0.8 km) of Green Bay. The disappearance of the organism within a few hundred feet inland from the shoreline (Table 4) emphasized the difference between the bay and the land proper. Comparison of counts at the GB8 location (Table 4) at two different times suggests that the maxima found for the other transects may have resulted from previous high-water levels.

Negative control cultures were obtained with 50 (19%) of 265 bottom and shoreline samples of the various rivers. Only 5 (3%) of 174 similar specimens from Lake Michigan and Green Bay and 6 (2%) of the total 269 soil and shore samples tested gave this inhibition of toxin formation in the controls, which had an added inoculum of Chamber's Island sand (Table 2). The inhibitory factors were found most frequently in the lower Fox River (Table 5), control cultures of nearly half of the samples being nonbotulinogenic. Type E inocula as high as 10^6 spores were needed to produce demonstrable levels of toxin in 1-g enrichment cultures of some of these samples. Contrasted to this, 10 spores consistently gave type E toxigenic enrichment cultures if the muds had been autoclaved previously for 20 min at 15 psi.

The number of organisms capable of inhibiting type E growth was determined for mud specimens giving negative control cultures. Pour plates of beef infusion agar heavily seeded with type E spores were inoculated on the surface with dilutions of the mud samples and incubated anaerobically. The total organisms per gram of mud were determined from the nonbotulinal surface colonies distinguishable on the confluent type E lawn and the dilution of mud giving a plate suitable for counting of these colonies. Colonies surrounded by a clear zone indicated the organisms inhibiting type E growth. Of the 1.5×10^5, 5.2×10^4, and 1.4×10^6 organisms enumerated per gram of three mud samples tested, 16, 27, and 93%, respectively, prevented growth of type E. The degree of inhibitory activity seen with a solid medium may not be reached in liquid cultures where greater dilution of metabolic products and competition between nonbotulinal

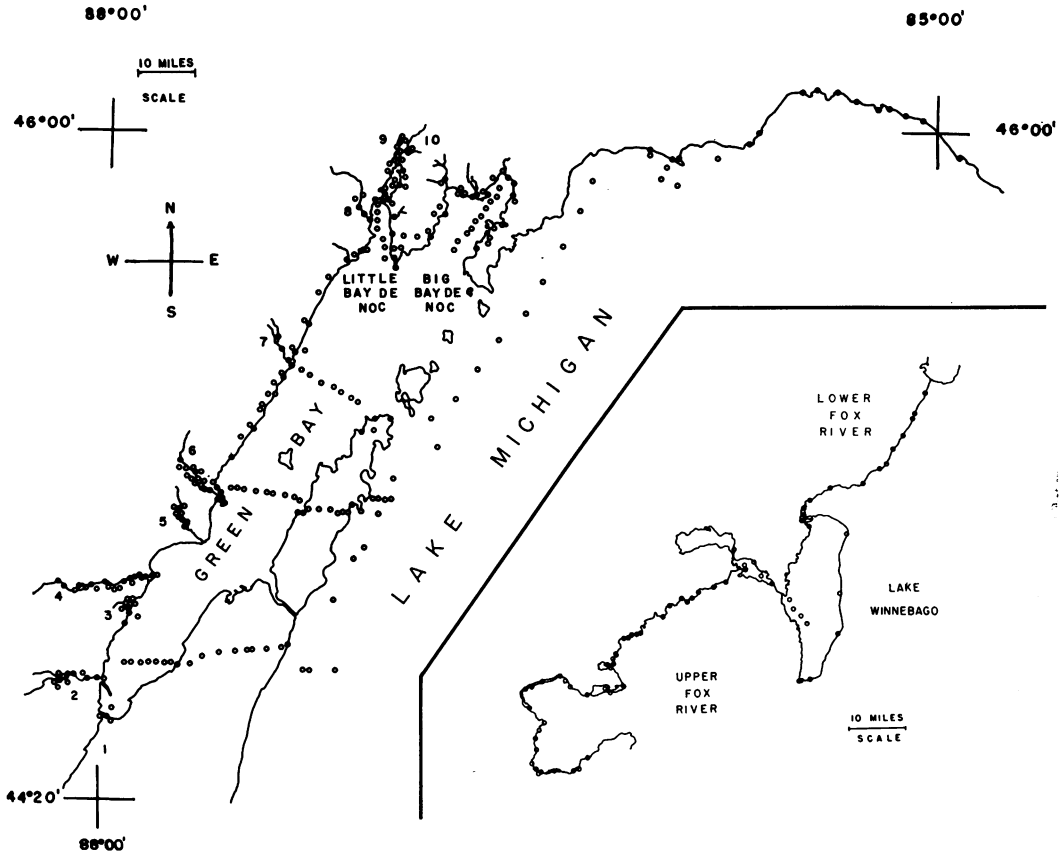

FIG. 1. *Sampling locations in Lake Michigan, Green Bay, and the surrounding land mass. Rivers sampled: (1) Fox, (2) Suamico, (3) Pensaukee, (4) Oconto, (5) Peshtigo, (6) Menominee, (7) Big Cedar, (8) Escanaba, (9) Rapid, (10) Whitefish. Inset: Fox River sampling locations. Three samples (bottom, shoreline, shore) usually taken at locations along rivers and on the shores of Green Bay and Lake Michigan.*

organisms would occur. However, the data show the difficulty of detecting type E in some specimens. The nontoxigenic type E-like organisms which produce a bacteriocin-like material active against *C. botulinum* type E (9) are undoubtedly important in this consideration.

DISCUSSION

A strong water outflow toward the northeast from Green Bay into Lake Michigan exists in the upper 60 ft (18 m); a compensatory return flow occurs in the deeper layers (13). Since water currents should carry microorganisms, a similar incidence of *C. botulinum* type E in Green Bay proper and in northern Lake Michigan was expected and confirmed in this study.

The presence of type E in the digestive tract must be due to some activity of the fish; otherwise, the prevalence of the organism in the main body of Green Bay would result in all the fish of this area becoming intestinal carriers. This is not the case, although the incidence rate is high. On the other hand, a good correlation is found between the presence of type E and of food in the alimentary tract (2). Also, type E does not multiply in the gut of fish maintained under laboratory conditions (*unpublished data*). These observations can be interpreted to support the previous suggestion that the botulinal organisms in the digestive tract of living fish are those ingested with the food.

In the earlier study, the intestinal tract of 86% of the fish of Big Bay de Noc were half or more full with food, but only 4% had type E. This may be due to the level of contamination of the environment with type E, the 68% incidence in the bottom and shoreline samples (Table 2) being significantly lower (99% confidence level) than the 96% incidence for the main body of Green Bay. It should also be noted that the fish and

TABLE 2. *Distribution of Clostridium botulinum type E in bottom, shoreline, shore, and soil samples taken at stations shown in Fig. 1*

Location	No. of samples[a]	Negative control samples[b]	Positive control samples[b]	
			Type E/total samples	Type E cultures/total cultures
Lake Michigan				
Bottom and shoreline	54	0	49/54 (91)[c]	124/206 (60)
Shore	18	0	10/18 (56)	20/59 (34)
Green Bay, main body				
Bottom and shoreline	66	2	62/64 (97)	199/257 (77)
Shore	22	0	8/22 (36)	19/67 (28)
Green Bay, Little Bay de Noc				
Bottom and shoreline	26	0	24/26 (92)	51/78 (65)
Shore	8	0	2/8 (25)	2/24 (8)
Green Bay, Big Bay de Noc				
Bottom and shoreline	28	3	17/25 (68)	46/75 (61)
Shore	9	0	0/9 (0)	0/27 (0)
Lakes and rivers				
Bottom and shoreline	99	14	33/85 (39)	64/285 (22)
Shore	56	3	4/53 (8)	9/164 (5)
Fox River System				
Bottom and shoreline	166	36	5/130 (4)	17/753 (2)
Shore	78	3	1/75 (1)	4/204 (2)
Miscellaneous soils	78	0	4/78 (5)	6/247 (2)

[a] Samples were taken one per station, and there were usually three cultures per sample.
[b] Absence or presence of type E toxin in enrichment culture of specimen inoculated with natural type E spores.
[c] Figures in parentheses represent percentages.

TABLE 3. *Comparison of Clostridium botulinum type E in the gastrointestinal tract of fish of Green Bay and the Fox River system*

Source	No. of fish tested	Fish with type E	Percentage of fish with type E
Green Bay[a]	728	416	57
Fox River system			
Lower Fox	569	34	6
Lake Winnebago	638	72	11
Upper Fox	438	20	5
Totals for Fox River System	1,645	126	8

[a] Data taken from Bott et al. (2).

environmental samples were collected at different times.

C. botulinum type E in Green Bay could originate in the surrounding land mass as suggested for the Baltic (6) and northern Pacific areas (4, 7, 10), but passive accumulation of the organism coming from the drainage basin seems inadequate to account for the present data. If most of the type E in Green Bay originated in the

TABLE 4. *Most probable numbers (five tubes per dilution) of Clostridium botulinum type E per gram of samples collected on transects heading inland from the Green Bay shoreline*

Inland from shoreline (ft)	Location and date of sampling			
	GB4 4/7/67	GB8 4/7/67	BG8 6/22/67	GB7 6/22/67
Under water	5		24	
Shoreline	17	620		140
25	18	470	490	
50	4	1,800	240	280
75	1	620	240	<1
100	35	35	490	2
125	330	18	35	0
150	0	6	35	0
175	0	0	<1[a]	0
200	0	1	0	0
225	<1			
250			0	
275			0	
300			<1	

[a] New shoreline at time of collection; all locations toward bay under 2 to 5 inches of water.

TABLE 5. *Distribution along the Fox River system (Fig. 1) of bottom and shoreline materials whose control cultures did not contain type E toxin (negative controls)*

Location	Negative controls/total samples	Percentage
Lower Fox	21/47	45
East River	0/12	0
Lake Winnebago	3/29	10
Upper Fox	12/78	15

drainage basin, the organism would be expected to be more uniformly distributed along the entire lengths of the rivers instead of being almost completely absent in their upper reaches.

Type E was found in 29 (11%) of 263 dry shore and soil specimens, but 20 of these were Green Bay and Lake Michigan shore samples exposed to contamination from the water by wind and wave actions. Consequently, the true incidence of type E in dry samples is closer to 9 of 243, or 4%, with 6 of these 9 positive samples being taken within 2 miles (3.2 km) of the Green Bay shoreline. The drop-off in numbers in the transect studies emphasizes its low incidence in the soil away from the bay. The presence of the organism in 103 (90%) of 115 samples from the aquatic environment of Green Bay contrasts sharply and suggests multiplication in the bay itself.

Temperature would not be limiting; at the southern end of Green Bay, the bottom levels have reached 16 C (13) and temperatures up to 25 C have been observed in the shallow shoreline areas. Multiplication of type E in bottom and shoreline mud samples under laboratory conditions has not been demonstrated as yet, but this does not necessarily mean inadequate nutrients. Factors such as the disturbance of the natural ecological system during manipulation need investigation.

Live fish harboring the organism would be active in its dissemination, and on death would be foci for its multiplication. Aquatic birds may act similarly, since type E toxin has been found in dead birds of several species (8). Smaller bottom animals can be expected to contribute in a similar manner to the maintenance of type E in nature.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service grant UI-00165 and by contracts 64-44 (Neg.) and 68-4 (Neg.) with the Food and Drug Administration

We especially thank the personnel of Great Lakes and Central Region, Bureau of Commercial Fisheries, Ann Arbor, Mich., and the Wisconsin Conservation Department, Oshkosh, Wis., for help in obtaining the samples. Technical assistance was provided by Janet Deffner, Ellen Hamilton, Richard Heimsch, Bonnie Sandusky, Marko Spalatin, Hugh Trenk, and Gary Wolfe.

LITERATURE CITED

1. APPLETON, G. S., AND P. G. WHITE. 1957. *Clostridium botulinum* type C. I. Studies on laboratory variables affecting toxin production and toxoid evaluation. Am. J. Vet. Res. **18**:942–946.
2. BOTT, T. L., J. S. DEFFNER, E. MCCOY, AND E. M. FOSTER. 1966. *Clostridium botulinum* type E in fish from the Great Lakes. J. Bacteriol. **91**:919–924.
3. DOLMAN, C. E. 1957. Recent observations on type E botulism. Can. J. Public Health **48**:187–198.
4. DOLMAN, C. E., AND H. IIDA. 1963. Type E botulism: its epidemiology, prevention, and specific treatment. Can. J. Public Health **54**:293–308.
5. DUFF, J. T., G. G. WRIGHT, AND A. YARINSKY. 1956. Activation of *Clostridium botulinum* type E toxin by trypsin. J. Bacteriol. **72**:455–460.
6. JOHANNSEN, A. 1963. *Clostridium botulinum* in Sweden and adjacent waters. J. Appl. Bacteriol. **26**:43–47.
7. KANZAWA, K. 1960. Ecological studies on *Clostridium botulinum* type E. Distribution of the organism in the soil of Hokkaido. Rept. Hokkaido Inst. Public Health **11**:161–173.
8. KAUFMANN, D. W., AND L. D. FAY. 1964. *Clostridium botulinum* type E toxin in tissues of dead loons and gulls. Quart. Bull. Mich. Agr. Expt. Sta. **47**:236–242.
9. KAUTTER, D. A., S. M. HARMON, R. K. LYNT, JR., AND T. LILLY, JR. 1966. Antagonistic effect on *Clostridium botulinum* type E by organisms resembling it. Appl. Microbiol. **14**:616–622.
10. NAKAMURA, Y., H. IIDA, S. SAEKI, K. KANZAWA, AND T. KARASHIMADA. 1956. Type E botulism in Hokkaido, Japan. Japan. J. Med. Sci. Biol. **9**:45–59.
11. OSHEROFF, B. J., G. G. SLOCUM, AND W. M. DECKER. 1964. Status of botulism in the United States. Public Health Rept. U.S. **79**:871–878.
12. SCHMIDT, C. F., W. K. NANK, AND R. V. LECHOWICH. 1962. Radiation sterilization of food. II. Some aspects of the growth, sporulation, and radiation resistance of spores of *Clostridium botulinum* type E. J. Food Sci. **27**:77–84.
13. U.S. DEPARTMENT OF THE INTERIOR. 1966. Comprehensive water pollution control program for the lake Michigan basin, Green Bay area. Federal Water Pollution Control Admin.