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Clostridium botulinum Type E in Fish from the Great Lakes[1]

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ABSTRACT

BOTT, THOMAS L. (University of Wisconsin, Madison), JANET S. DEFFNER, ELIZABETH McCOY, AND E. M. FOSTER. *Clostridium botulinum* type E in fish from the Great Lakes. J. Bacteriol. **91**:919–924. 1966.—The intestinal contents of more than 3,000 fish from Lakes Erie, Superior, Huron, and Michigan were examined for *Clostridium botulinum* type E. Demonstration of the organism was accomplished by identifying its toxin in liquid cultures inoculated with material from the alimentary tract. Incidence figures, expressed as per cent of the fish tested, were: Lake Erie, 1%; Lake Superior, 1%; Lake Huron, 4%; the main body of Lake Michigan, 9%; and Green Bay (on Lake Michigan), 57%. Thus, *C. botulinum* type E appears to be widely but unevenly distributed in the Great Lakes, and fish from all areas are potential carriers of it.

All known outbreaks of type E botulism have occurred in the Northern hemisphere, and with few exceptions have been associated with the consumption of fish or other animal products from the sea (4, 10). Early suggestions that *Clostridium botulinum* type E is limited to the marine environment have been refuted by reports of its occurrence in soils and fresh waters of France (17), Japan (12), Sweden (9), and British Columbia (4). The organism now is known to be widely distributed in both marine and terrestrial environments, a notable exception to date being the British Isles (Hobbs, *personal communication*; 10).

The first indication that this organism may occur in the Great Lakes came in 1960, when an outbreak of type E botulism was traced to smoked fish from Lake Superior (13, 16). Further evidence was provided in 1963, when two additional outbreaks were caused by smoked fish known or believed to be processed in the Great Lakes area (1). These three episodes involved a total of 21 cases with 9 deaths.

The original objective of this study was to see whether *C. botulinum* type E is a common inhabitant of the Great Lakes and therefore a potential hazard to consumers of fish from these waters. It was soon learned that the organism can readily be demonstrated in and on a significant percentage of the fish from Lake Michigan (6). Extension of these observations to the other Great Lakes (excepting Lake Ontario) revealed wide variations in the incidence of the organism in fish from different lakes and even from different parts of the same lake.

Ecological studies were begun in an effort to understand the organism's irregular distribution. This report deals only with its occurrence in fish.

MATERIALS AND METHODS

Fish were taken by gill net, trap net, or trawl from commercial fishing waters at the locations shown in Fig. 1. Most of the specimens were packed in ice for transportation to the laboratory, but a few were frozen. Comparison of frozen and iced fish from the same catch showed a higher incidence of type E in the latter, indicating that freezing may kill some of the cells.

Within 48 hr after the fish were caught, the abdominal cavity was opened aseptically and the intestinal contents were transferred to tubes of Difco Brain Heart Infusion or Trypticase-peptone-glucose broth (19). These media were equally satisfactory for demonstration of toxin. Intestinal contents were used as the test material because of the higher incidence of type E there than in other parts of the fish (6).

The cultures were incubated at 30 C for 3 days or at 25 C for 7 days, the latter conditions giving a slightly higher percentage of positive cultures. After incubation, the cultures were chilled in ice water and were centrifuged in the cold at 800 × *g* for 30 min.

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A 0.5- or 0.25-ml amount of the supernatant fluid was injected intraperitoneally into a 20-g white mouse. The remainder of the culture was frozen immediately to minimize destruction of toxin before typing with antisera.

The mice were observed closely for symptoms of botulism throughout the first 10 hr. This was necessary to differentiate botulinal deaths from those caused by other toxins in the crude mixed cultures (8). Characteristic symptoms of type E botulism (indrawn flanks and labored breathing) usually appear between 1 and 4 hr after injection. Mice with these symptoms often enter a stage of frenetic activity just before death. They jump wildly about the cage and then expire within a few seconds.

If a mouse survived for at least 24 hr, or if it died earlier without symptoms of botulism, the culture was considered to be negative for type E. Attempts to demonstrate type E toxin in such cultures almost invariably failed.

If the mouse died within 24 hr and showed typical botulinal symptoms, three additional mice were injected intraperitoneally with 0.5 ml or less of the culture supernatant fluid. One mouse was protected with 1 international unit of type E antitoxin and another with type A. (With some samples, a fourth mouse was protected with type B antitoxin.) Type E toxin was considered to be present in the culture only if the mouse protected with type E antitoxin survived without apparent illness for at least 48 hr, and all others died with symptoms of botulism within 24 hr.

RESULTS

Figure 1 shows the locations that produced fish carrying *C. botulinum* type E, and Table 1 summarizes the overall incidence for each lake. The organism was found at four widely separated locations in Lake Superior, but in a very low percentage of the fish. Over 75% of the specimens from this lake were caught in the Keweenaw Bay area during a single 2-day period in August 1964.

Samples were collected from Lake Erie during July and October 1964. Type E was found at two locations, but again in a small percentage of the fish.

All of the specimens from Lake Huron were taken during the first half of June 1964. Type E

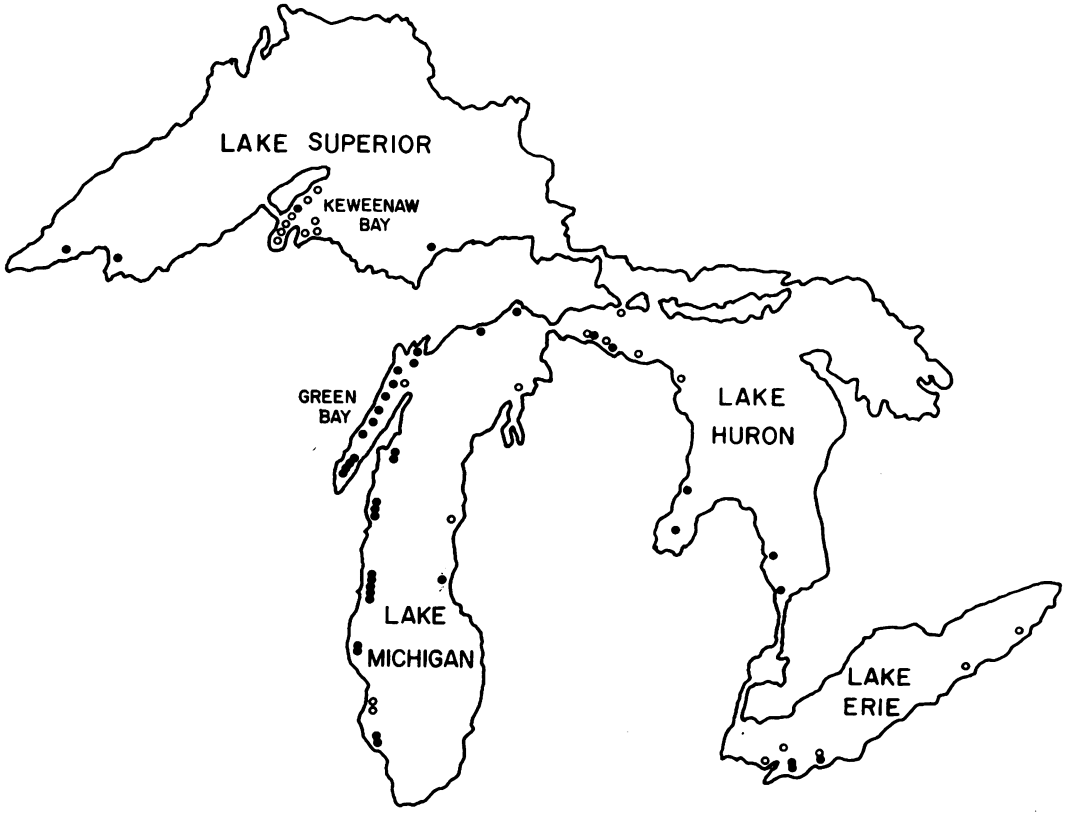

FIG. 1. *Sources of fish examined for Clostridium botulinum type E. Each symbol represents one lot of 20 or more specimens collected in a single day. Symbols: closed circles = type E present; open circles = type E absent. Joined circles represent repeated samplings of the same area at least 1 month apart.*

TABLE 1. *Occurrence of Clostridium botulinum type E in the intestinal contents of fish from the Great Lakes*

Lake	No. of fish tested	No. with type E	Per cent with type E
Superior	602	6	1
Erie	437	4	1
Huron	464	17	4
Michigan	1,009	93	9
Green Bay on Lake Michigan	728	416	57
Total	3,240	536	

was demonstrated in fish from 6 of the 11 locations, the highest incidence for a single sampling station being 13%.

Lake Michigan has been studied more thoroughly than any of the others. Eleven locations were examined, several of them two or more times. All but three gave at least one culture of type E each time they were sampled. The three negative locations were represented by small lots of fewer than 50 specimens each. The incidence figures for the five positive locations along the western shore ranged from 4 to 13% of the fish. At least 100 specimens were examined from each of these stations.

The unusually high incidence in Green Bay first was observed in May 1964, when more than half of 72 fish from the southern part of the bay were shown to harbor type E. This observation was confirmed 1 month later, when three-fourths of the fish in another collection from the same area were found to carry the organism. Fish taken in August 1964 from nine separate locations (Fig. 2) showed the organism to be present all over the bay (Table 2). Only the specimens from station 9 at the extreme northern end of the bay failed to show an incidence of at least 25%.

The differences between sampling stations in Table 2 suggest irregular distribution of type E in the environment, but more likely they merely reflect differences in the amounts of material in the intestinal tracts of the fish. Throughout most of this work the intestinal tracts were classified as full, half-full, almost empty, or empty at the time the cultures were prepared. These judgments obviously were imprecise, but they provided a rough measure of the size of the inoculum. Intestines judged to be empty yielded little more than mucus, whereas those classed as full were stuffed with material undergoing digestion.

For the fish from stations 1 through 7 there was reasonably good agreement between the amount of material in the intestinal tract and the incidence of type E (Table 3). Almost 75% of the specimens classed as full or half-full carried the organism, whereas only 12% of the fish judged to be empty or almost empty produced cultures of type E.

This relationship did not hold with fish from stations 8 and 9 nor with fish from the main bodies of the lakes, where the incidence of the

TABLE 2. *Incidence of Clostridium botulinum type E in fish from different parts of Green Bay (August 1964)*

Station no.*	No. of fish tested	No. with type E	Per cent with type E
1	40	35	88
2	50	20	40
3	50	43	86
4	50	17	34
5	40	30	75
6	49	13	26
7	40	27	68
8	50	14	28
9	49	2	4

* See Fig. 2 for location of sampling stations.

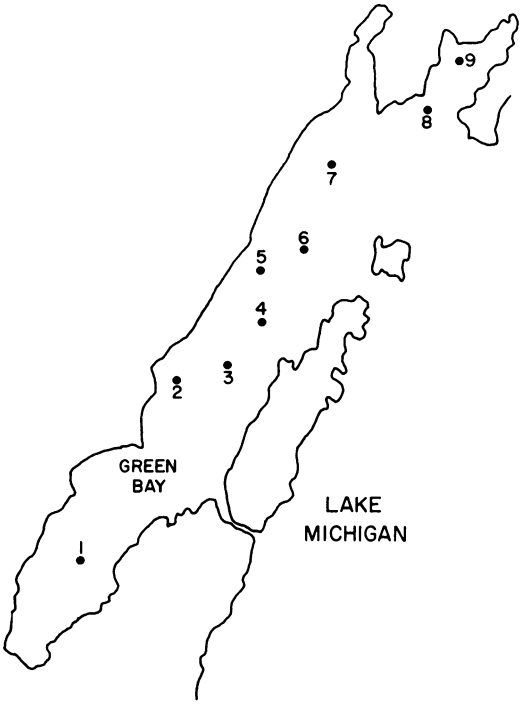

FIG. 2. *Locations sampled in Green Bay, August* 1964.

TABLE 3. *Relation of amount of material in the intestinal tract to presence of Clostridium botulinum type E in cultures prepared from fish of Green Bay (August 1964)*

Station no.*	(A) Fish with intestinal tract half full or more	(B) Cultures with type E	Ratio (A/B)
	%	%	
1	94	88	1.05
2	56	40	1.40
3	98	84	1.16
4	54	34	1.59
5	84	73	1.15
6	57	26	2.19
7	100	68	1.47
8	92	32	2.88
9	86	4	21.60

* See Fig. 2 and Table 2.

organism apparently was lower than that in the main part of Green Bay.

Repeated collections from a single location indicate that type E may be found in fish at all seasons of the year if it is present in the environment (Table 4). For example, the organism was demonstrated in more than 75% of the specimens taken from the southern part of Green Bay in March, June, and August. The temperature of the water ranged from 64 F (18 C) in August to 39 F (3 C) in March.

About 25 species of fish have been examined in this study, but most of the specimens were of the following species: alewife (*Pomolubus pseudoharengus*), creek chub (*Semotilus atromaculatus atromaculatus*), Great Lakes bloater chub (*Coregonus hoyi*), Great Lakes cisco (*Coregonus artedii artedii*), smelt (*Osmerus mordax*), sucker (*Catostomus commersonnii commersonnii* and *Moxostoma macrolepidotum macrolepidotum*), trout perch (*Percopsis omiscomaycus*), and yellow perch (*Perca flavescens*). There was no indication that type E is restricted to certain species.

Type E was found in 59% of the alewife specimens from Green Bay and in 10% of those from Lake Michigan, but not in the ones from Lake Superior. It was common in yellow perch from Green Bay, but not in those from Lake Erie. A number (11%) of the chubs from Lake Michigan carried the organism, but those from Lake Superior did not. Type E has been found in lake trout and brown trout as well as in carp, catfish, and suckers. Thus, it may appear in any species of fish if it is present in the environment, and therefore on the food of the fish.

TABLE 4. *Incidence of Clostridium botulinum type E in fish at different seasons of the year*

Source of fish	Date sampled	No. of fish tested	No. with type E	Per cent with type E
Southern part of Green Bay	May 1964	72	40	56
	June 1964	94	72	77
	August 1964	40	33	83
	March 1965*	121	111	92
Point on western shore of Lake Michigan	October 1963	31	12	39
	April 1964	24	5	21
	July 1964	73	8	11
	September 1964	48	7	14

* Fish taken through the ice.

DISCUSSION

The problem of demonstrating *C. botulinum* in samples from nature is well known (2, 8, 14). Lacking a selective medium for this organism, the usual procedure is to prepare an enrichment culture and then to look for botulinum toxin. Unequivocal demonstration of the toxin is adequate evidence that *C. botulinum* is present (3), although the evidence is strengthened by isolating the organism in pure culture.

Failure to find toxin in the enrichment culture does not necessarily prove the absence of *C. botulinum* from the original sample. Other organisms may inhibit its growth or they may destroy its toxin (8). To minimize competition in tests for *C. botulinum* types A and B, investigators usually heat the samples at 80 C for a few minutes. However, this selective procedure cannot be employed with type E, because of the extreme thermal sensitivity of its spores (15). In our experience, a treatment even as mild as 60 C for 30 min often renders the organism undetectable in mud samples from which it can readily be cultured without heating.

Identification of type E toxin by the mouse-protection test is a relatively simple matter when no other lethal agent is present in the culture. Mice receiving several MLD of type E toxin usually show characteristic symptoms within 1 to 4 hr and die within 10 hr. Homologous antitoxin gives excellent protection.

But other toxic bacterial products may be present and complicate the identification of type E (2, 12, 14). One such material in our experience kills mice in 10 to 15 min, clearly too soon for botulinum toxin to act. This substance is troublesome only because it kills the animal before botulinum toxin, if present, can exert its effect. At least two other nonbotulinal toxins have been recognized in this work. One typically kills mice

in about 4 to 8 hr, and the other, in 10 hr or more. Neither causes symptoms resembling those of botulism. These toxic substances were much more common in cultures from Lake Huron than from the other lakes.

The concentration of nonbotulinal toxins usually is less than 20 MLD/ml, and their effect can be eliminated by simple dilution. Unfortunately, this also eliminates many samples that contain type E. The titer of type E toxin in cultures prepared from fish intestinal contents has been found to range from 3 to 2,000 MLD/ml.

Thus, for routine use with thousands of samples, the choice of a challenge dose must be somewhat arbitrary. The larger the dose, the more interference there will be from nonbotulinal toxins, and the smaller the dose, the more false negatives there will be. In this study, a fairly large challenge dose was employed in the initial toxicity test and then reduced in a second trial if there was difficulty with extraneous toxins.

Another problem is related to the instability of type E toxin due, apparently, to the action of proteolytic enzymes produced by other organisms. This problem was minimized by centrifuging the cultures in the cold, by keeping them frozen at all times after centrifugation, and by typing them as quickly as possible after toxin had been demonstrated. Attempts to increase the toxin titer by activation with trypsin (5) resulted in a decrease rather than an increase, possibly because the toxin already had been activated by proteolytic enzymes in the culture (18), and the trypsin merely degraded the toxin molecule.

In view of these problems, rigid criteria were established for the identification of type E toxin: (i) unprotected mice had to die with typical symptoms of botulism within 24 hr, and (ii) protected mice had to survive without apparent illness for at least 48 hr. Death times in excess of 24 hr were ignored, because of the possibility of slow-acting toxins or bacterial infections.

With these criteria, there can be little doubt about the validity of the positive samples in this study. Therefore, the incidence figures in Table 1 can be considered as minimal. The true percentages might actually be higher, because some samples containing type E probably were missed under the criteria imposed. Nevertheless, it is clear that *C. botulinum* type E is widely distributed in the Great Lakes and that fish from these waters are potential carriers of the organism.

Toxigenic isolates of *C. botulinum* type E have been obtained from only a few of the enrichment cultures. Colonies resembling Dolman's (3) "Tox" form on blood-agar and Brain Heart Infusion Agar proved on subculture to be nontoxigenic. Streaking alcohol-treated toxic cultures on egg yolk-agar (11) gave rise to typical colonies with zones of opalescence characteristic of type E, but rarely did the subcultures produce toxin. Only three toxigenic isolates were obtained by this procedure from one group of 60 Green Bay cultures, all of which gave unequivocal evidence of type E toxin. As was observed by Hobbs, Roberts, and Walker (7), many of the nontoxigenic isolates appear to be similar in every way to *C. botulinum* type E, except for their inability to produce toxin.

It is premature at this time to attempt to explain the irregular distribution of type E in the Great Lakes. Its occurrence in fish seems to be related to its presence in the environment rather than to the species of fish or their feeding habits. Tests for the organism in water and bottom sediments have been uniformly negative for Lake Superior and Lake Erie, and only a few positive samples have been obtained from Lake Huron and Lake Michigan. But the organism can be demonstrated at will in water and mud from Green Bay. These observations correspond roughly with the incidence in fish.

The high incidence of type E in Green Bay resembles that found by Johannsen (8, 9) in the Baltic Sea. Green Bay is approximately 125 miles long by less than 20 miles wide, with a maximal depth of 20 fathoms. Communication with Lake Michigan is limited to a few narrow channels between islands near the northern end of the bay (Fig. 2); hence, there is little intermixing of lake and bay waters.

Like the Baltic Sea, Green Bay receives the drainage from a considerable area of farm and forest land. In addition, it collects the wastes from several sizable cities and from a large number of paper pulp mills and other industrial establishments. As a result, the water of the bay is rich in nutrients. It has been estimated that Green Bay produces a greater tonnage of fish than all the remainder of Lake Michigan (Hasler, *personal communication*).

It is tempting to ascribe the high incidence of type E in Green Bay to pollution with agricultural, industrial, and domestic wastes. But such a conclusion is not in accord with the results for Lake Erie, which receives huge quantities of waste material and is rapidly undergoing eutrophication. Certain areas of Lake Erie remain anaerobic the year around, and seemingly should provide ideal conditions for growth of clostridia.

Further ecological studies are in progress in an attempt to explain the irregular distribution of type E in Great Lakes waters.

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