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Clostridium botulinum IN TVA LAKES[1]

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ABSTRACT

Evidence was obtained to establish that *Clostridium botulinum* exists in muds of certain areas of the Tennessee Valley Authority lakes. Types E and B were obtained from a single embayment on Douglas Lake and type B alone was isolated from two areas of the French Broad River. Fort Loudon Lake, except for one highly suspicious culture, was negative for the anaerobe. The incidence of *C. botulinum* may be rare due to the environmental conditions that exist in these lakes. The bottom muds in the channels are mainly mineral. It is only in some of the coves and embayments that sufficient organic matter has been deposited to provide food for development of most kinds of microorganisms, including anaerobes such as *C. botulinum*. Unless hundreds of samples were collected, the organism could easily be missed. Furthermore, during the procedure for isolation, the presence of proteolytic anaerobes and acid producing aerobes can destroy botulinus toxin and cause one to overlook the existence in the mixed culture of *C. botulinum*. Failure to find the organism in several hundred fish suggests that neither the individual fisherman nor the commercial fishing industry needs to be greatly concerned over the possibility of a potential health hazard on the basis of data obtained.

Research on botulism is now progressing at an unusual pace as evidenced by the abundance of literature appearing within the past three years. Thorough reviews have been published in the Proceedings of a Symposium sponsored by the U.S. Public Health Service, and edited by Lewis and Cassel, (1964), by Meyer and Eddie, (1965) by Foster and Sugiyama (1966) and by the contributors to a Symposium on Botulism at the IX International Congress for Microbiology, Moscow, U.S.S.R., including, Holtman, *et al.* (1966).

The outbreak of botulism Type E in the Tennessee Valley in 1963 focused our attention on the need for an intensive ecological study of the causative agent. The present contribution covers our investigation of bottom muds and fish of some of the man-made lakes of the Tennessee Valley Authority. It provides evidence that at least two types of *Clostridium botulinum* exist in the area and there is reason to believe that other types may also exist here.

MATERIALS AND METHODS

Collection and Treatment of Samples

Mud samples used for experimentation were obtained from the lake and river bottoms by the use of a special core sampling device. Approximately 150 grams of sample were collected at a time depending on the consistency and composition of the mud in the area. Most of the samples were composed of red clay, or red clay mixed with varied amounts of sand, fine chipped shale, and loam. The amount of organic content, in the form of decayed plant matter, varied greatly from one sample to another. The organic content of the channel muds was low compared to that of mud from embayments. Mud samples were also taken from the shore in some areas. Additional samples were collected from the littoral zone of one embayment during the winter months while the lake level was low. All samples were placed in sterile plastic Whirl-pak Bags (Scientific Products) and transported to the laboratory where they were tested or frozen until required.

Procedure A. Several portions of each sample were treated by transferring 1.0 to 1.5 gm quantities to separate tubes containing 5.0 ml of 50% ethanol. These were incubated at room temperature with intermittent shaking for 1 hour, this being a modified procedure of that described by Johnston, Harmon, and Kauter (1964) in which 50% ethanol was used to destroy non-sporeforming bacteria. Remaining portions of each sample were not given the alcohol treatment, so as to permit vegetative cells of *C. botulinum* to develop if present in the sample on occasions when spores might be absent.

Procedure B. A second procedure was employed, wherein the total sample was suspended in isotonic saline. The mud was allowed to settle out, and the supernatant fluid was filtered through Whatman No. 1 filter paper by gravity filtration. The resultant filtrate was passed through a Millipore HA 0.45 u filter. Half of this filtrate was treated with 50% ethanol for one hour at room temperature and was again filtered through a Millipore HA 0.45 u filter to trap any spores present. The untreated half of the first Millipore filtrate served as a control.

Some of the fish tested were obtained from local sportsmen, but by far the greatest number was made available to us through the courtesy of the Tennessee Game and Fish Commission and the Tennessee Valley Authority during a survey of the fish population in Douglas Lake. Numerous specimens representing all of the species of fish occurring in the lake were obtained from this latter source. The fish were gutted, and the entrails were placed in sterile plastic bags which were then frozen until tested. Portions of the intestinal content were treated as described in Procedure A for the mud samples, or the entire gut was macerated with sterile mortar and pestle in an equal volume of saline and then treated as in Procedure B.

Media

Various commercially prepared media were examined for their ability to promote germination of spores, growth of cells, and production of toxin from stock cultures, mud samples, and fish intestines. Best results were obtained with Cooked Meat Medium enriched with 0.2 per cent starch, 0.5 per cent dextrose, and 1.0 per cent N-Z Case.[2] This enrichment medium is a modified version of that described by Lewis and Angelotti (1964). The medium was dispensed in 150 x 20 mm screw-capped tubes to which were added 2.5 grams of Cooked Meat Medium and 20 ml of a distilled water solution of the other components. These were sterilized in the autoclave at 121C for 30 minutes and stored at room temperature until needed. Before the tubes were inoculated, they were placed in boiling water to remove dissolved oxygen and cooled to room temperature under running tap water.

Egg yolk agar medium for plating was prepared according to the formula of McClung and Toabe (1947) as in the procedure described by Lewis and Angelotti (1964). A loopful of each toxic enrichment culture was streaked on egg yolk agar plates and incubated four to six days at 30C in a National Appliance anaerobic incubator under an atmosphere of 90% H_2 and 10% CO_2 in the presence of palladium catalysts. Isolated colonies were picked and transferred to tubes of the enrichment medium.

Test for Production of Toxin

Inocula of 0.5 ml of the mud-alcohol mixtures or fish intestinal content-alcohol mixtures, and 1.0 to 1.5 gm portions of the samples not treated with alcohol were transferred to tubes containing 20 ml of the enrichment medium. The part of the Millipore filtrate that had been treated with alcohol and the untreated part was placed aseptically into different tubes of the enrichment medium. After four to six days of incubation at 30C, the tubes were centrifuged to remove the cells and solid material, and the supernatant fluid was removed with a pipette. Part of the supernatant fluid from each culture was injected by intraperitoneal route in 0.5 ml amounts into three mice, each weighing approximately 25 grams, for toxicity testing. To activate any Type E toxin present, another portion was treated with 1.0 per cent trypsin in 0.2 *M* phosphate buffer at pH 6.5 and incubated at 37C for 1 hour (Duff *et al.* 1956). Then, lots of three mice were injected with 0.5 ml amounts each. The tests for evidence of toxins of *C. botulinum* were considered to be positive if the mice survived more than 45 minutes but succumbed within 72 hours with typical symptoms of botulism. The symptoms observed were essentially the same as those reported by Bott *et al.* (1966). Tests in which the mice died in less than 45 minutes were repeated using 1:10 and 1:100 dilutions of the toxic fluid in gelatin-phosphate buffer.

Toxin Neutralization Tests

Toxic culture supernatants, trypsinized or non-trypsinized, were subjected to toxin neutralization tests in mice. To determine whether the toxicity was due to botulinal toxin or other toxins or compounds that might be toxic to mice (Bott *et al.* 1966), a polyvalent antitoxin was used. The antitoxins, one polyvalent and the others specific for Types A through F, were obtained from the Communicable Disease Center, Atlanta, Georgia. The polyvalent antitoxin contained 2 standard units per ml of each of the six types of antitoxins in gelatin-phosphate buffer at pH 7.0. A mixture of equal quantities of a 1:10 dilution (in gelatin-phosphate buffer) of the toxic fluid (trypsinized or non-trypsinized) and the polyvalent antitoxin were incubated for one hour in a 37C water bath. A group of three mice was injected with 0.5 ml quantities each of the mixture, and three mice received 0.5 ml each of the diluted fluid to serve as controls. The animals were observed for 72 hours for symptoms of botulism and for mortality. If the control mice died and the protected mice survived, monovalent toxin neutralization tests were conducted to determine the type of toxin involved.

One ml quantities of each toxic fluid were mixed with 1.0 ml of a different antitoxin which had been diluted to contain 2 standard units per ml. The mixtures were incubated 1 hour at 37C in a water bath. Six groups of three mice each were inoculated. Each mouse received 0.5 ml of the toxic fluid mixed with antitoxin, and a different antitoxin was employed in separate groups. An unprotected control group was included in each instance. The cultures were identified as being the type of *C. botulinum* corresponding to the type of homologous antitoxin employed in the group of mice surviving 72 hours after inoculation. Confirmation of the identification was accomplished by demonstrating the toxin in enrichment medium cultures from isolated colonies picked from egg yolk agar plates. Figure 1 presents the overall scheme for demonstration of the organism in samples.

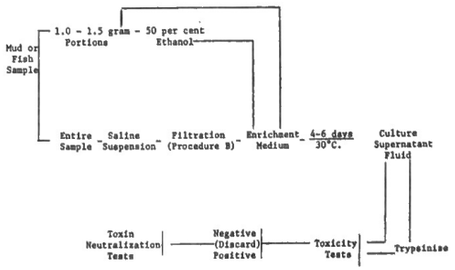

Figure 1. A schematic representation of the overall treatment of mud and fish samples leading to the demonstration of the presence of C. botulinum.

Controls

Sterilized mud samples; non-sterilized mud samples containing the normal flora, but no *C. botulinum* (as determined by toxicity tests of their cultures); and fish intestinal contents that were negative for the organism were inoculated with isotonic saline spore suspensions

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[2] A casein digest and partly synthetic medium of the Sheffield Chemical Co., Norwich, N. Y. 13815.

(10 and 1,000 spores per ml) and non-sporulated cell suspensions (10,000 cells per ml) prepared from stock cultures of Types A, B, and E. The latter were from the collection of the Types A through F obtained from the Communicable Disease Center, Atlanta, Georgia. Counts of washed cell and spore suspensions were conducted by using a hemacytometer, and the appropriate dilutions were made. One group of the inoculated samples was incubated at room temperature over night, another group for six days, and then frozen. Specimens of each type of these samples were tested (according to the procedures described above) before freezing and at weekly intervals for 10 weeks. These were tested for the effect of freezing on the ability of the organisms to survive.

Groups of five samples each of non-sterilized mud and fish specimens were inoculated with 10 spores per gram of sample using different types of *C. botulinum* (types A, B, and E) in separate groups. Half of each sample was treated with alcohol, and half not treated, before transferring to enrichment broth. The supernatant fluids of the cultures after four days of incubation at 30C were tested by toxicity tests and toxin neutralization tests in mice. This work was done to determine the effect of treatment of a sample with alcohol on our ability to demonstrate the presence of *C. botulinum*.

Part of the culture supernatant fluid from each of the above cultures was removed after 18 hours of incubation. Half of the fluid from each culture was trypsinized and the other half was not. The effect of trypsinization was determined by toxicity tests in mice.

Results

Controls

A qualitative study on the samples which had been inoculated with spores and vegetative cells of *C. botulinum* showed 100 per cent recovery of the organisms from frozen mud samples and fish specimens. Toxin was detectable in enrichment cultures after four days of incubation at 30C from samples frozen up to ten weeks.

Samples which had been inoculated with 10 spores per gram of sample with types A, B, and E were tested for the effect of treatment with alcohol. Toxin was detectable after four days of incubation at 30C in all cultures from samples that were treated wtih alcohol, while toxin was not detectable in the majority of cultures from samples that were not treated (Table I). This establishes clearly that detection of *C. botulinum* toxin in cultures is enhanced by treatment of a sample with 50% ethanol.

Supernatant fluids from two of five of the *C. botulinum* type E cultures were toxic only when treated with trypsin. Trypsin had no effect on the toxicity of supernatant fluids from cultures of types A and B (Table II).

The presence of trypsin in the toxic supernatant fluids from cultures of all six types of *C. botulinum* had no effect on the monovalent or polyvalent toxin neutralization tests.

TABLE I

THE EFFECT OF ALCOHOL ON THE DETECTION OF TOXIN IN CULTURES FROM SAMPLES INOCULATED WITH SPORES OF C. BOTULINUM TYPES A, B, AND E.

Sample	Test Organism Type	Treated with Alcohol No. Samples	Treated with Alcohol No. Toxic Cultures	Not Treated with Alcohol No. Samples	Not Treated with Alcohol No. Toxic Cultures
Fish	A	5	5[a]	5	3
	B	5	5	5	2
	E[b]	5	5	5	2
Mud	A	5	5	5	2
	B	5	5	5	2
	E	5	5	5	0

[a]Toxicity tests were done on four day culture supernatant fluids by injecting three mice with 0.5 ml. amounts.

[b]Type E culture supernatant fluids were trypsinized.

TABLE II

THE EFFECTS OF TRYPSIN ON THE DETECTION OF TOXINS OF C. BOTULINUM IN CULTURE SUPERNATANT FLUIDS[a]

Test Organism Type	Treated with Trypsin[b] No. Samples	Treated with Trypsin[b] No. Toxic[c]	Not Treated with Trypsin No. Samples	Not Treated with Trypsin No. Toxic[d]
A	3	3	3	3
B	5	5	5	5
E	5	5	5	3

[a]Eighteen hour enrichment medium cultures were employed.

[b]Samples of culture supernatant fluids with 1.0 per cent trypsin added were incubated at 37°C. for one hour in a water bath.

[c]Three mice per sample were injected with 0.5 ml. of trypsinized fluid.

[d]Three mice per sample were injected with 0.5 ml. of the supernatant fluid.

TABLE III

THE OCCURRENCE OF CLOSTRIDIUM BOTULINUM IN THE BOTTOM MUDS

Geographical Location of Sample	No. of Sites Sampled	No. of Toxic Cultures	No. with Typical Typing Patterns	Classification of Samples
Fort Loudon Lake	135	52	0	---*
French Broad River	10	4	2	type B
Douglas Lake	240	84	2	type E

*No positive toxin neutralization tests

Examination of Bottom Muds

The two lakes and two rivers from which bottom muds were obtained are shown in Figure 2. One hundred thirty-five mud samples from Fort Loudon Lake yielded 52 toxic cultures of which none gave typical patterns for toxins of *C. botulinum* by toxin neutralization tests in mice (Table III). Ten samples from the French Broad River gave 4 toxic cultures of which two produced typing patterns for type B toxin. Two hundred forty samples from Douglas Lake yielded 84 toxic cultures. Two of these presented typical patterns for type E toxin. Both samples were obtained from two different locations in the same large embayment.

Selected results of toxin neutralization tests are given in Table IV. A toxic culture obtained from one sample from Fort Loudon Lake provided a strange result in the toxin neutralization tests (sample FLP-1). Although no deaths occurred in mice protected with type E antitoxin, one of each group of three mice survived among those receiving the other types of antitoxin. It was not possible by subsequent tests to confirm this. The original culture lost its toxicity after being frozen for several

TABLE IV

RESULTS OF TOXIN NEUTRALIZATION TESTS IN MICE

Sample	Unprotected[a] Controls	Homologous Antitoxin A[b]	B	C	D	E	F	Classification of Sample
FLP-1[c]	+++[d]	++-	++-	++-	++-	---	+++	Type E (?)
FB-1	+++	+++	---	+++	+++	+++	+++	Type B
FB-2	+++	+++	---	+++	+++	+++	+++	Type B
D-12	+++	+++	+++	+++	+++	+--	+++	Type E
D-12-13A	+++	+++	+++	+++	+++	---	+++	Type E
D-12-9	---	---	---	---	---	---	---	Lost Toxicity
D-23	+++	+++	---	++-	+++	---	+++	Non-specific
FB3-1	+++	+++	+++	+++	+++	+++	+++	Atypical

[a]Unprotected controls received 0.5 ml. supernatant without antitoxin.

[b]Mice received 0.5 ml. antitoxin-supernatant mixture containing 1 standard antitoxin unit per ml.

[c]Code designating geographical location of source FL = Fort Loudon Lake FB = French Broad River, D = Douglas Lake

[d](+) = Death of mouse, (—) = mouse survived 72 hours.

(?) = Not confirmed.

weeks, and although microscopic examination revealed the presence of sporulating cells, no growth was obtained upon anaerobic plating on egg yolk agar.

Loss of toxicity was observed in a large number of cultures which had proved toxic in the initial tests one day earlier. Sample D-12-9 (Table IV) is an example of this. Also presented in Table IV are examples of toxin neutralization test patterns which were termed non-specific and atypical for *C. botulinum* toxins. Plating inocula from some of these cultures on egg yolk agar revealed no colonies typical of *C. botulinum*. No toxic cultures were recovered from shore mud samples taken from several areas corresponding to the sites of bottom mud sampling.

Examination of Fish Specimens

Seven hundred sixty-four fish specimens from a single embayment on Douglas Lake (Fig. 2) were negative for *C. botulinum* when cultures of intestinal contents were tested for the organism. Many non-specific death patterns in mice were obtained from these cultures, as well as the loss of toxicity from a few samples when Procedure A (of Methods Section) was employed. No toxic cultures were obtained from specimens which were treated according to Procedure B (of Methods Section).

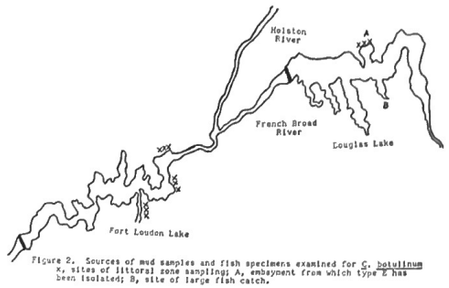

Figure 2. Sources of mud samples and fish specimens examined for C. botulinum x, sites of littoral zone sampling; A, embayment from which type E has been isolated; B, site of large fish catch.

Fish from other areas in the lakes were obtained from local sportsmen. These included 27 specimens from Fort Loudon Lake, 5 from the French Broad River, 12 from Norris Lake, 8 from Douglas Lake, and 10 from Cherokee Lake. None of these cultures were positive for *C. botulinum* by toxin neutralization tests.

Examination of Littoral Zone Mud

Mud banks and large areas of exposed bottomland were sampled during the lowered lake levels maintained by TVA on Douglas Lake and Fort Loudon Lake. The results of analysis of these samples are given in Table V. Two *C. botulinum* type E and one type B culture were obtained from the samples collected from the Douglas Lake littoral zone. All of these latter samples were taken from the same embayment on Douglas Lake that had yielded type E in the bottom muds (Fig. 2).

Isolation

Pure cultures of the organism have been isolated from sample cultures which exhibited positive toxin neutralization tests. Isolated colonies were obtained by streaking a loopful of the toxic culture on egg yolk agar. Colonies having the appearance and reaction of *C. botulinum* colonies, as described by McClung and Toabe (1947), were picked and transferred to tubes of enrichment medium. Positive identification was made by the neutralization of toxin in these cultures with specific monovalent antitoxins. In this way, *C. botulinum* type E and *C. botulinum* type B were positively identified in mud cultures.

TABLE V

RESULTS OF TESTS FOR THE DETECTION OF C. BOTULINUM IN LITTORAL ZONE MUD SAMPLES FROM FORT LOUDON LAKE AND DOUGLAS LAKE

Geographical Location	No. of Samples	No. of Toxic Cultures	No. of Positive Polyvalent Neut. Tests	Results of Monovalent Neut.
Fort Loudon	32	6	0	--
Douglas	45	4	4	1B & 2E

Discussion

Evidence has been obtained to indicate that *C. botulinum* is present in muds of certain areas of the TVA lakes. Types E and B were obtained from a single embayment on Douglas Lake and type B alone was found in two areas of the French Broad River. Fort Loudon Lake, except for one suspicious culture, was negative for *C. botulinum*. This information, together with the negative results from a large number of fish, would seem to indicate quite a low incidence of this anaerobe in the TVA system. However, such an interpretation can hardly be made until all the lakes in the system have been examined. It is conceivable that a high incidence of *C. botulinum* of any type might exist in one of the lakes not yet sampled. It is also possible that a high incidence of type E might be found in fish intestinal contents, especially bottom feeders, if a large number could be obtained from the embayment on Douglas Lake which has been shown to harbor the organism in its bottom muds.

The incidence of *C. botulinum* in TVA lakes may be rare due to the environmental conditions that exist in these lakes. The bottom muds in the channels are purely mineral, for the most part, due to erosion, but small pockets exist which are rich in deposited decomposed organic matter. Such pockets provide a good environment for saprophytic clostridia. This leaves to chance the possibility of locating one of these deposits by random sampling, although the coves and embayments, generally, should provide the most promising results. The lake level fluctuations during different seasons when they are manipulated for flood control could have an effect on the occurrence of *C. botulinum* in some areas. Thousands of acres are drained seasonally so that the anaerobic conditions of the lake bottoms in these areas undergo marked alteration.

Certain cultural conditions interfere with the recovery of *C. botulinum* from samples and with the detection of toxin in cultures which contain other microflora. In mixed cultures certain proteolytic anaerobes and acid-producing aerobes destroy *C. botulinum* toxins. Another problem has been the possibility that in some samples the organism may have been present as vegetative cells but not as spores. This would have prevented the recovery of *C. botulinum* from samples which had been treated with alcohol. These problems have been circumvented to some degree by the multiple procedures employed in the treatment of each sample.

Loss of toxicity from toxin cultures and specific and atypical results in toxin neutralization tests or mouse protection tests have also been reported by Foster *et al.* (1965) and Bott *et al.* (1966). Atypical monovalent toxin neutralization patterns can be explained by the presence of other sporeforming anaerobes that produce toxins which are lethal to mice and by the presence of toxic chemicals from fish. An adequate interpretation is needed for loss of toxicity from cultures which were initially toxic.

This study should be continued to provide more concrete information on the relative incidence of *C. botulinum* in the TVA system. It is quite significant that at least two types of the botulinum group are present in bottom muds of these waters. As for the fish of the area, it can only be said that the organism is not common to them.

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