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Authors: Marion, Wayne R., O'Meara, Timothy E., Riddle, Gerald D., and Berkhoff, Herman A.

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PREVALENCE OF *CLOSTRIDIUM BOTULINUM* TYPE C IN SUBSTRATES OF PHOSPHATE-MINE SETTLING PONDS AND IMPLICATIONS FOR EPIZOOTICS OF AVIAN BOTULISM[1]

Wayne R. Marion,[2] Timothy E. O'Meara,[2] Gerald D. Riddle,[3] and Herman A. Berkhoff[3]

ABSTRACT: Prevalence and conditions for occurrence of *Clostridium botulinum* type C were examined on phosphate-mine settling ponds and a natural wetland in northern Florida between April 1981 and March 1982. Substrate samples were collected monthly (winter) and semi-monthly (summer) from 16 locations on seven ponds. Selected environmental parameters were measured at each location at the time of sampling. Mouse inoculation tests and toxin neutralization tests using enrichment culture filtrates were conducted to identify *C. botulinum* type C in the samples. The bacteria were identified in 26 (5.6%) of 467 sediment samples. Occurrences were distributed over four of the seven ponds and included nine of the 16 sample locations, but were restricted to the months April through October. The organism occurred over a wide range of ecological conditions found on the ponds during these months. If the presence of *C. botulinum* type C in the substrate is a prerequisite for botulism to occur, the prevalence and fairly wide distribution of this organism on settling ponds makes it difficult to predict where future outbreaks may occur.

INTRODUCTION

Waterfowl populations and wetland habitats in Florida have dwindled considerably over several decades for a variety of reasons (Chamberlain, 1960). Despite these reductions, rapid expansion of phosphate mining in Florida has resulted in establishment of new wetlands in the form of settling ponds. Settling ponds are diked impoundments used for settling and consolidating a clay/water slurry produced as a by-product of phosphate extraction. These ponds cover an area in excess of 24,000 ha (Hendry, 1978) and are attractive to large numbers of waterfowl (Montalbano et al., 1978) and shorebirds (Maehr, 1981). Phosphate-mine settling ponds typically have changing water levels, concentrations of birds, and abundant vegetation repeatedly inundated by phosphatic clays. These provide conditions for development of avian botulism in birds as described by Enright (1971), Rosen and Bischoff (1953) and Smith (1976).

Large mortality of waterfowl due to avian botulism has never been documented in the eastern United States, where it is considered rare (Richardson et al., 1965). However, two outbreaks of avian botulism in phosphate-mine settling ponds in northern Florida were recently documented (Forrester et al., 1980). Because these outbreaks occurred in man-made phosphate-mine settling ponds, it was suggested that ecological and microbiological conditions extant in these areas may be conducive to future outbreaks.

A number of prerequisites for development of avian botulism in an area have been proposed and debated in the last two decades. Most theories focus upon three major concepts: the sludge-bed theory, the microenvironment theory, and the bird carcass theory.

The sludge-bed theory requires the occurrence of *C. botulinum* type C in the immediate vicinity for an outbreak to occur (Haagsma et al., 1972). A possible repository for these bacteria is the aquatic substrate. According to this theory, the aquatic substrate acts as an incubator for bacterial growth and toxin production under favorable environmental conditions. The sludge-bed theory has been challenged by Coburn (1940) and more recently by Hunter (1970).

The microenvironment theory (Bell et al., 1955) contends that invertebrate carcasses are favorable sites for growth and toxin production by bacteria. According to this theory, toxin production would occur if *C. botulinum* type C organisms were present in the digestive tract of the invertebrates at the time of their death.

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[1] Contribution No. 4745 of the Journal Series, Florida Agricultural Experiment Station, Gainesville, Florida 32611, USA.

[2] School of Forest Resources and Conservation, University of Florida, Gainesville, Florida 32611, USA.

[3] School of Veterinary Medicine, North Carolina State University, Raleigh, North Carolina 27606, USA.

Widespread die-offs of invertebrates, such as may result along shorelines following rapid changes in water levels, would precipitate a botulism outbreak. A more recent report (Moulton et al., 1976) provided evidence that contradicts this theory.

The third theory does not require the presence of *C. botulinum* in the aquatic environment prior to an outbreak. If a bird with spores in its alimentary tract dies at a yet uncontaminated lake (death not necessarily caused by botulism, but by injury, old age, etc.), type C organisms are likely to invade the putrefying carcass and multiply profusely. Multiplication of *C. botulinum* type C in the carcasses of birds may result in high concentrations of toxin. The toxin may then be consumed by fly larvae, which are not themselves susceptible but can concentrate large amounts of toxin by ingesting decaying meat. The eating of such larvae by other waterfowl can result in botulism (Rosen, 1971; Smith et al., 1975). Duncan and Jensen (1976) and Malcolm (1982) reported that living aquatic and terrestrial invertebrates normally found in close association with dead, decomposing birds carried toxin and also they showed that aquatic birds dead of botulism had consumed these invertebrates.

The sludge-bed and microenvironment theories require the presence of *C. botulinum* type C in the substrate and/or in invertebrates associated with the substrate to foster an outbreak of botulism. Despite these theories, little information is available on the conditions under which *C. botulinum* type C occurs in nature, and few surveys have been conducted to determine the distribution of these bacteria in freshwater wetlands. Smith and Moryson (1975) identified *C. botulinum* type C in mud from 12 of 69 (17%) aquatic sites sampled in London. The bacterium was found in 129 of 230 (56%) soil samples taken from lakes in Japan (Serikawa et al., 1977). Smith (1978) found an average prevalence of 3% in 554 mud samples from Britain and Ireland with frequencies of up to 51% in some areas. Nine of 1,414 (1%) animal and sediment samples collected primarily in marine habitats along the Gulf Coast of the United States produced positive indications of *C. botulinum* type C (Ward et al., 1967).

Due to a general lack of information on the presence and distribution of *C. botulinum* type C in freshwater habitats of the southeastern United States, we undertook this study to determine the prevalence and year-round occurrence of this organism in two types of wetlands.

METHODS AND MATERIALS

Sample collection

Clay slurry and bottom sediment samples were collected from six phosphate-mine settling ponds and one natural pond in Hamilton County, Florida. Sixteen sampling locations representing a variety of ecological conditions on the ponds were selected. Each location was sampled semi-monthly between April and October 1981 (summer), and monthly between November 1981 and March 1982 (winter). Sampling consisted of collecting two bottom sediment samples of approximately 10 cc each from each location. One sample was collected at a water depth of 5 cm and one at a depth of 20 cm when sufficient water depths occurred at the location. Samples were stored in sealed, plastic tubes and refrigerated for subsequent laboratory analyses. Samples collected at both depths from each location during April and May were pooled.

Site characteristics at each depth were described when samples were collected at a sampling location. A portable meter was used to measure water temperature and dissolved-oxygen concentrations. Slope of the bottom was measured as the water depth in cm at 1 m from the water's edge. Turbidity was described on a scale of 1 to 3, where 1 = clear and 3 = opaque. Water movement was identified as either flowing or stagnant and depended on the proximity of the sampling location to an inflow or outflow point on the pond. Percent cover of detritus and living vegetation were estimated for a 0.5 m^2 area surrounding each sampling point and major plant species were identified.

Sample analyses

Mouse inoculation tests and toxin neutralization tests were conducted to identify the presence of *C. botulinum* type C in the samples. Two 1-gram subsamples were removed from each mud sample, added to 5 ml of sterile broth, and shaken to suspend the mud and sand. This suspension was then centrifuged at 1,500 rpm for 3 min to pellet larger particles, while leaving most bacterial cells and spores suspended. The supernatant was then filtered through sterile 0.45 μm membrane filters to collect the bacterial cells and spores. This membrane filter pad was then added to 10 ml prereduced chopped meat carbohydrate (CMC) broth tubes using the VPI anaerobic culture system to maintain anaerobiosis. This suspension-filtration step proved necessary since adding mud directly to CMC broth irreversibly oxidized the medium.

All inoculated CMC tubes were then heat-treated to kill non-spore forming organisms thereby reducing non-botulinic deaths during toxin tests. Tubes were treated by suspension for 20 min in an 80 C water bath followed by rapid cooling. The CMC tubes were incubated for 5 days at 37 C, then frozen for 24 hr.

After thawing, the supernatants from the duplicate subsamples were withdrawn, combined, and passed through sterile 0.45 μm membrane filters. Mouse inoculation tests were conducted by injecting 0.3 ml of the combined supernatant mixture into a pair of mice (Smith, 1977). Positive results were indicated by the mice demonstrating typical signs of botulinum intoxication including ruffling of fur, breathing difficulty, "wasp-waist," and finally, death due to respiratory failure.

Clostridium botulinum type C identification was accomplished for positive samples by toxin neutralization tests (Smith 1975). The toxic supernatant (1.2 ml) was combined with the specific antitoxin (0.3 ml) of *C. botulinum* type C. Two mice were each injected with 0.5 ml of the supernatant antitoxin mixture and two additional mice received 0.5 ml of untreated supernatant as controls. Identification of *C. botulinum* type C was denoted by the death of unprotected mice along with the survival of the protected mice. Nonbotulinic deaths did not occur.

Data analyses

Data for all continuous variables (water temperature, dissolved oxygen, detritus, vegetative coverage, and slope) were compared using a *t*-test (Snedecor and Cochran, 1967) by season between sites where *C. botulinum* was detected and where it was undetected. Also, *t*-tests were used to compare the means for continuous variables between seasons. Chi-square tests (Snedecor and Cochran, 1967) were used with discrete variables (flow and turbidity) to make seasonal comparisons of sites where *C. botulinum* type C was found with those where it was not found. The chi-square test also was used to evaluate differences in frequency of occurrence between seasons.

RESULTS

Clostridium botulinum type C was identified in 26 (5.6%) of 467 sediment samples. It was distributed over four of the six settling ponds. In the four settling ponds where the organism was found, it was identified in nine of 16 sampling locations. *Clostridium botulinum* was not found in samples from the natural wetland.

The organism occurred at both depths, with nine of 212 samples from 5 cm testing positive and nine of 192 samples from 20 cm testing positive for samples collected during all months except April and May. Results were subsequently pooled between depths for each date and location to allow comparison with results from April and May, where separate analyses were not available. Combining data for each date and location also avoided bias caused by interdependence of samples and facilitated use of parametric statistics to compare results.

Clostridium botulinum type C was identified only during the summer months (April through October) with greatest prevalence occurring in June (Fig. 1). Sampling intensity was twice as great during the summer as during the winter (November through March) and, as a result, frequency of occurrence of botulism would expectedly be greater. Frequency of occurrence was significantly greater during summer than during winter ($\chi^2 = 8.53$, 1 df, $P < 0.05$).

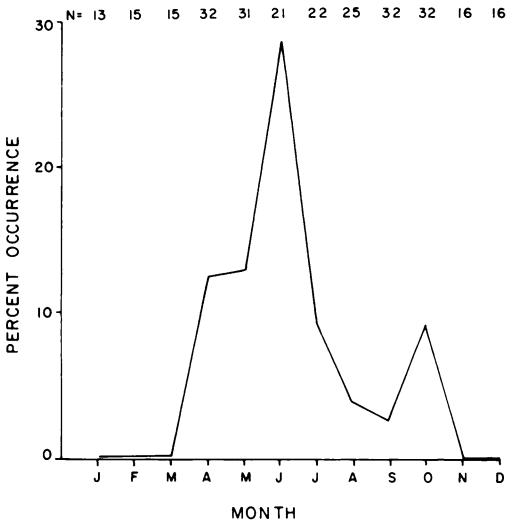

FIGURE 1. Frequency (N) and percent frequency of substrate samples from North Florida phosphate settling ponds that were positive for *Clostridium botulinum* type C by month.

The organism occurred over a wide range of ecological conditions found on the ponds. Water temperatures recorded at all sites averaged 16 C during the winter and 26 C during the summer with ranges of 8–29 C and 18–36 C, respectively. Dissolved oxygen concentrations averaged 6.1 ppm during the winter and 4.9 ppm during the summer with ranges of 0.4 to 13.5 ppm and 0.1 to 19.6 ppm, respectively. The combined data for summer mean water temperatures and dissolved oxygen concentrations differed ($P < 0.05$) from those for winter months. The bacterium was identified in substrate samples with water temperatures ranging from 18 to 33 C and dissolved oxygen concentrations between 0.7 and 19.6 ppm. Sampling sites where the organism was identified had slopes ranging from 1 to 54 cm/m within 1 m of the water's edge and vegetation or detritus coverage ranging from 0 to 100%.

T-tests performed on data obtained from months during which *C. botulinum* type C occurred indicated no difference ($P > 0.05$) between mean values of water temperature, slope, or percent coverage of detritus for samples with and without the organism. Mean values of dissolved oxygen and percent coverage of vegetation did differ ($P < 0.05$) between samples with and without the organism. Dissolved oxygen averaged 8.3 ppm for samples with *C. botulinum* and 4.5 for samples without *C. botulinum* ($P = 0.01$) during the summer months. Vegetative coverage averaged 34 and 53% ($P = 0.02$) for samples with and without the organism, respectively. Chi-square tests indicated that *C. botulinum* type C was equally likely ($P > 0.05$) to occur in association with the three turbidity classifications recorded and in substrates under flowing or stagnant water during the months April through October.

DISCUSSION

Our finding of *C. botulinum* in 5.6% of all samples taken from phosphate-mine settling ponds in northern Florida was comparable to frequencies observed in other studies. Mouse inoculation tests, as used in this study, indicated presence of *C. botulinum* type C, but did not differentiate between vegetative and endospore states of the bacterium. Several authors (Jensen, 1969; Smith, 1978) have hypothesized that *C. botulinum* may survive for several years as spores in aquatic substrates to grow and produce toxin when conditions become favorable. However, Ward et al. (1967) assumed that *C. botulinum* exists in the vegetative state in nature. The occurrence of *C. botulinum* type C in our substrate samples was independent of most of the ecological parameters measured within the season in which the organism occurred. Only dissolved oxygen concentrations and vegetation coverage differed between samples with and without *C. botulinum* type C. The mean dissolved oxygen concentration was greater in water where positive samples were collected, yet *C. botulinum* is an anaerobic bacterium that is sensitive to oxygen (Smith, 1976). Since dissolved oxygen was measured in the water column above the substrates, our measurements may not have been correlated with conditions within the substrate where the bacteria occurred.

Clostridium botulinum type C was very likely still viable in the settling ponds during the winter months. There are several possible explanations for our inability to detect *C. botulinum* during the winter, including:

a. The bacterium was reproducing in whatever media were available from April to October, thereby increasing numbers of vegetative cells and spores. The organism ceased reproduction when temperatures lowered, thereby decreasing the chance that any particular substrate sample tested would contain an adequate inoculum.
b. Lower temperatures might have favored the growth of organisms that inhibit the growth of *C. botulinum* or destroy its toxin. This topic has been discussed recently by Graham (1978).
c. Strains of *C. botulinum* type C can loose toxigenicity on serial subcultures as described by Oguma and Iida (1979). When the identification of this organism depends upon the demonstration of its toxin, this has to be taken into account. Loss of toxigenicity may occur in nature as the bacterium goes through "subcultures" in aquatic invertebrates or other media. Perhaps the organism was present in our samples but was not producing toxin.

Although the local distribution of the organism was not correlated with ecological parameters within the season in which it occurred, our inability to identify the organism during the winter months suggested effects of environmental factors. Mean water temperatures were higher for the combined data for summer, when *C. botulinum* was found, and winter months. Dissolved oxygen concentrations were greater in the winter when water temperatures were lower than during the summer. Haagsma et al. (1972) indicated that type C toxin can only be produced on an extensive scale at temperatures above 23 C. Also, Miyazaki and Sakaguchi (1978) reported that the optimal temperatures for the growth of *C. botulinum* type C is 40–42 C. These data suggest that temperatures, dissolved oxygen concentrations, or some other variable may seasonally limit the occurrence of bacteria during the winter months. Of the two botulism outbreaks reported on settling ponds, one occurred in May and June, the second in November and December during a period of abnormally high ambient temperatures

(Forrester et al., 1980). Serikawa et al. (1977) found *C. botulinum* type C year round in a lake in Japan with greater prevalence during the autumn seasons, but they did not attempt to correlate occurrence of the organism with environmental characteristics.

If outbreaks of botulism require toxin production in the substrate, outbreaks will most likely occur during the summer (April through October) on settling ponds in northern Florida. *Clostridium botulinum* type C is prevalent under a wide range of conditions which occur on the settling ponds during the summer, and is fairly well distributed throughout the ponds during the months when it is present. These characteristics of the organisms' distribution make it difficult to predict where future outbreaks may occur.

Compounding the problem of predicting epizootics is the possibility that outbreaks may occur when *C. botulinum* cannot be identified in the substrate. Under this scenario, the organism may be brought into an aquatic system at any time by waterbirds feeding on other contaminated areas or food items. *Clostridium botulinum* is common in decaying carcasses (Hobmaier, 1932; Kalmbach and Gunderson, 1934; Gunderson, 1935; Bell et al., 1955) and several waterbirds have been known to feed on dead animals (Guillory and LeBlanc, 1975). We have found necrophagous soldier-fly larvae (Stratiomyidae) in stomachs of wood ducks (*Aix sponsa*) collected on phosphate-mine settling ponds. Deaths of such birds contaminated with the bacteria concurrent with favorable conditions for toxin production within the carcasses could conceivably lead to an epizootic independent of ecological conditions in the aquatic system and despite the absence of *C. botulinum* type C in the substrate.

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