The Inhibition of Clostridium Botulinum Type C by Other Bacteria in Wetland Sediments

Authors: Renee J. Sandler, Tonie E. Rocke, and Thomas M. Yuill
Source: Journal of Wildlife Diseases, 34(4) : 830-833
Published By: Wildlife Disease Association
URL: https://doi.org/10.7589/0090-3558-34.4.830
The Inhibition of *Clostridium Botulinum* Type C by Other Bacteria in Wetland Sediments

Renee J. Sandler, Tonie E. Rocke, and Thomas M. Yulli
Department of Veterinary Science, University of Wisconsin, 1655 Linden Dr., Madison, Wisconsin 53705, USA; National Wildlife Health Center, 6006 Schroeder Road, Madison, Wisconsin 53711, USA; Present address: 1622 Jefferson Street, Madison, Wisconsin 53711, USA; and Present address: Institute for Environmental Studies, Room 30B Science Hall, Madison, Wisconsin 53706, USA.

ABSTRACT: Bacteria with inhibitory activity against *Clostridium botulinum* type C were isolated from 32% of sediment samples (n = 1600) collected from 10 marshes in a northern California wetland over a 12 mo period. Aerobic and anaerobic bacteria with inhibitory activity were isolated from 12% and 23% of the samples, respectively. Bacteria with inhibitory activity were isolated from all 10 study sites and throughout the year. This study demonstrates that bacteria with inhibitory activity against *C. botulinum* type C occur naturally in wetland sediments.

Key words: Avian botulism, *Clostridium botulinum* type C, growth inhibition, survey, wetlands.

Avian botulism, which kills thousands of wild birds annually (National Wildlife Health Center, unpublished data), is caused by ingestion of toxin produced by *Clostridium botulinum* type C. It is generally believed that this anaerobic bacterium can persist in wetland sediments in a spore state for years (Smith et al., 1982) until environmental conditions allow the spores to germinate into vegetative cells, propagate, and synthesize toxin. Waterbirds inadvertently ingest the preformed toxin as they feed.

The diverse microbial community co-existing with *C. botulinum* type C in wetland sediments may influence one or more steps in this progression of the bacteria from resting spore to toxin-secreting cell. Previous studies have demonstrated inhibition of *C. botulinum* type A (Hall A) toxin production by aerobic soil bacteria (Hall and Peterson, 1922) and bacteriostatic and bacteriolytic effects on *C. botulinum* type E by closely related bacteria (Kautter et al., 1966). Graham (1978) added *C. botulinum* type C to 105 mud samples and failed to reisolate the bacteria in over 50% of the samples after a 28 day incubation; from these apparently inhibitory samples, he isolated several *Bacillus* spp. that produced peptide antibodies active *in vitro* against *C. botulinum* type C.

The initial colonization of *C. botulinum* type C in a wetland and its subsequent population dynamics might be influenced by the presence of inhibitory organisms (Moulton et al., 1976). Our objectives were to determine if bacteria with the ability to inhibit the growth of *C. botulinum* type C exist naturally in wetland sediments, to determine the prevalence of these bacteria, and to investigate their temporal and spatial distribution. We also compared the prevalence of these sediment bacteria to that of *C. botulinum* type C.

Sediment samples were collected from 10 marshes within the Sacramento National Wildlife Refuge (SNWR) in Willows, California (USA; 122°20'N, 39°20'W) as previously described (Sandler et al., 1993). Samples collected in January through December 1987 were used for this study. Briefly, 10 sampling locations in each marsh were randomly selected and changed for each sampling period. A core sampler was used to collect samples from the top 6 to 7 cm of sediment and was rinsed between samples to minimize cross-contamination. Samples were placed in sterile plastic cups and kept on ice until frozen at -70 C. The water levels in these marshes were independently regulated. Four marshes were completely drained from approximately April to September of each year and were designated as seasonal marshes; six were flooded year-round and designated as permanent marshes. As reported previously, botulism outbreaks oc-
occurred in waterbirds in four of 10 marshes sampled in 1987 (Sandler et al., 1993). *Clostridium botulinum* type C was demonstrated throughout the year in all 10 marshes sampled, and its prevalence was similar in marshes with and without botulism outbreaks (Sandler et al., 1993).

An inhibition-zone assay was used to detect and isolate both aerobic and anaerobic bacteria from SNWR sediment samples with inhibitory activity against *C. botulinum* type C. Spore suspensions of *C. botulinum* strain 468C, originally isolated from a carcass (Segner et al., 1971), were prepared initially by inoculating bottles of fortified egg meat medium (Segner et al., 1971). When a much less expensive, alternate sporulation medium (Ito et al., 1966) proved to be equally productive and did not require separation of spores from meat particles, we used it to produce all subsequent spore suspensions. Wirtz-Conklin malachite green spore stains (Hendrickson, 1985) were performed daily on inoculated growth media to determine the optimal time for spore harvesting. Peak spore density occurred after 6 to 10 days of incubation at 35 C. Spores were washed 5 times with sterile distilled water and held at 4 C until use.

A total of 1,600 sediment samples were examined for the presence of bacteria with inhibitory activity. Sediments were diluted 1:10 (w/v) in either phosphate buffered saline (PBS; 0.01M phosphate) to isolate aerobic bacteria with inhibitory activity or anaerobic dilution blank solution (ADBS; Holdeman et al., 1977) to isolate strictly anaerobic and facultative bacteria with inhibitory activity. Suspensions were mixed and serially diluted. Aliquots of 100 µl of each PBS sediment suspension were plated on trypticase soy agar (TSA; Difco Laboratories, Detroit, Michigan, USA) and incubated aerobically. Similarly, dilutions of the ADBS suspensions were plated on reinforced clostridial agar (RCA; Difco Laboratories) and incubated within an anaerobic chamber (Forma Scientific; Marietta, Ohio, USA) filled with N₂, CO₂, and H₂ gases (85:10:5). All plates were incubated for 3 days at 35 C. One TSA and one RCA plate to represent each sediment sample were selected on the basis of good colony separation. These plates were overlaid with 10 ml molten RCA containing approximately 1 × 10⁷ spores of *C. botulinum* type C, incubated anaerobically at 35 C for 3 days, and examined for zones of inhibition in the layer of *C. botulinum*.

Representative colony types that produced zones of inhibition were saved for stock culture by inserting a sterile Pasteur pipette through the upper agar layer to the desired colony and streaking the isolate on another plate of the same medium. Aerobically-isolated, growth-inhibiting bacteria were then streaked on TSA slants, and anaerobically-isolated, growth-inhibiting bacteria were inoculated into tubes of Pre-dused Chopped Meat Glucose media (Gibco Division, BBL Microbiology, Systems; Madison, Wisconsin, USA). These stock cultures were characterized using the 20E API and 20A API systems (Analytab Products, Plainview, New York, USA) and other standard biochemical tests (Lemnette et al., 1985).

The prevalence of growth-inhibiting bacteria in each study site was calculated as the proportion of the 10 samples/marsh/sampling period that contained any zone-producing colonies; aerobically and anaerobically-isolated bacteria with inhibitory activity were evaluated separately. We found bacteria with inhibitory activity against *C. botulinum* type C in all 10 marshes sampled throughout the study period and in 32% of the 1,600 sediment samples collected. We isolated aerobic bacteria with inhibitory activity from an average of 12% (7–18%) of samples collected in each marsh and anaerobic/facultative inhibitors from an average of 23% (19–33%). Bacteria with inhibitory activity to *C. botulinum* type C that have been tentatively identified include *Bacillus licheniformis, B. macerans/polymyxa, B. mycoides/cereus,* an actinomycete, several
Streptococcus spp., and several Clostridium spp.

Using repeated measures analysis of variance (ANOVA; Milliken and Johnson, 1984), we determined if the prevalence of growth-inhibiting bacteria (aerobes, anaerobes, and total) was related to the prevalence of C. botulinum type C reported previously (Sandler et al., 1993). In these analyses, we used the proportions of growth-inhibiting bacteria from each sampling period within a marsh as the repeated measurements. Similarly, we compared the prevalence of growth-inhibiting bacteria between seasonally and permanently flooded marshes, between marshes with and without botulism mortalities during the study, and between marshes with high and low waterfowl losses from botulism in the previous 5 yr. A marsh was considered to have high losses from avian botulism mortality if epizootics occurred at that site in 3 of the 5 yr preceding the study (Sandler et al., 1993). Marshes with less than three epizootics were considered to have low avian botulism losses. Levels of significance for \( P \) were set at \( P \leq 0.05 \).

Temporal trends in the prevalence of C. botulinum growth-inhibiting bacteria were evaluated by contrasting specific intervals (groups of successive sampling periods) using a randomized block ANOVA (Kirk, 1982); marshes were used as blocks, and different intervals were considered to be treatments. With this approach, we compared the prevalence of growth-inhibiting bacteria between wet and dry intervals in seasonally flooded marshes. The wet interval included samples collected from January to March 1987 and October to December 1987, and the dry interval included samples collected from May 1987 to September 1987. Data collected in April 1987 was eliminated because it was the transition (draw-down) period for the seasonal marshes. We also compared the prevalence of growth-inhibiting bacteria between time intervals with and without botulism epizootics. Levels of significance for \( P \) were set at \( P \leq 0.05 \).

The prevalence of growth-inhibiting bacteria (aerobes, anaerobes, and total) did not correlate significantly with the prevalence of C. botulinum type C in marsh sediments. Also, no differences were detected in the prevalence of growth-inhibiting bacteria in seasonal versus permanent marshes, in marshes with mortality during the study period versus those without, and in marshes with higher previous losses from avian botulism versus those with lower losses. Furthermore, no differences in the prevalence of growth-inhibiting bacteria were detected between wet and dry intervals in seasonally flooded marshes and between intervals with and without botulism mortality.

This study is the first to demonstrate that growth-inhibiting microorganisms co-exist naturally with C. botulinum type C in marsh sediments. In addition, we found a much greater diversity of species inhibitory to C. botulinum type C than previously reported. The vast majority (81\%) of Graham’s (1978) isolates were Bacillus cereus-like or B. pumilis-like bacteria, and all were aerobes. We have found other Bacillus species with inhibitory activity against C. botulinum type C and also have isolated strict anaerobes with inhibitory activity. Inhibition by anaerobes against any bacteria has rarely been reported and, in non-C strains of C. botulinum, has been limited to inhibition by other C. botulinum strains or other Clostridium spp. (Kautter et al., 1966; Smith, 1975).

In this study, we did not find evidence to support the hypothesis that growth-inhibiting bacteria in wetland sediments at SNWR significantly influenced populations of C. botulinum type C. Perhaps this was because we could not directly quantify the bacteria with inhibitory activity. Discerning separate zones of inhibition in the spore layer required the overlay of plates with <30–300 colonies considered statistically-optimal for the plate count method. Also, our assay quantified a highly diverse group of bacteria related only by their inhibitory activity. Interactions among these
bacteria as they colonized the agar plate may have interfered with the counts from our serial dilutions. Often when one bacteria was diluted out, another could flourish on the agar. These limitations made it difficult to monitor seasonal trends in the prevalence of C. botulinum inhibitors and their relationship to the occurrence of botulism in specific marshes. Selective culture procedures or molecular detection methods specific to particular growth-inhibiting species would permit better quantification.

Although it is unlikely that growth-inhibiting bacteria alone regulate the timing, location and severity of avian botulism outbreaks, they may act in concert with such factors as soil and water temperature, pH, redox potential, and other environmental conditions to limit the colonization and propagation of C. botulinum type C in wetlands. We do not know if the growth-inhibiting bacteria we isolated in this study secrete a bacteriocin with bacteriostatic or bactericidal effects (Tagg et al., 1976) or induce a change in their microenvironment that precludes the growth of C. botulinum type C. With further research, it may be possible to take advantage of the presence of these growth-inhibiting bacteria by encouraging their growth in wetlands at high risk for botulism outbreaks or by artificially reproducing inhibitory conditions created by antagonistic species.

This study was generously supported by the U.S. Fish and Wildlife Service (USFWS), National Wildlife Health Research Center under the USFWS Cooperative Unit Agreement No. 14-16-0009-1511, Research Work Order No. 23. The authors acknowledge the support services of P. Slota, S. Smith, and M. Samuel. We also appreciate the cooperation of the Sacramento National Wildlife Refuge staff and the advice of J. Ensign and E. Johnson.

LITERATURE CITED


Received for publication 12 November 1997.