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SEASONAL PREVALENCE OF *CLOSTRIDIUM BOTULINUM* TYPE C IN SEDIMENTS OF A NORTHERN CALIFORNIA WETLAND

Renee J. Sandler,[1,2] Tonie E. Rocke,[3] Michael D. Samuel,[3] and Thomas M. Yuill[1,4]

[1] Department of Veterinary Science, University of Wisconsin, 1655 Linden Dr., Madison, Wisconsin 53706, USA
[2] Present Address: Department of Comparative Biosciences, School of Veterinary Medicine, University of Wisconsin, 2015 Linden Dr. West, Madison, Wisconsin 53706, USA
[3] U.S. Fish and Wildlife Service, National Wildlife Health Research Center, 6006 Schroeder Road, Madison, Wisconsin 53711, USA
[4] Pathobiological Sciences, School of Veterinary Medicine, University of Wisconsin, 2015 Linden Dr. West, Madison, Wisconsin 53706, USA

ABSTRACT: The prevalence of *Clostridium botulinum* type C (% of positive sediment samples) was determined in 10 marshes at Sacramento National Wildlife Refuge (SNWR), located in the Central Valley of California (USA), where avian botulism epizootics occur regularly. Fifty-two percent of 2,200 sediment samples collected over an 18-mo period contained *C. botulinum* type C (both neurotoxic and aneurotoxic) which was present throughout the year in all 10 marshes. The prevalence of *C. botulinum* type C was similar in marshes with either high or low botulism losses in the previous 5 yr. Marshes with avian botulism mortality during the study had similar prevalences as marshes with no mortality. However, the prevalence of *C. botulinum* type C was higher in marshes that remained flooded all year (permanent) compared with marshes that were drained in the spring and reflooded in the fall (seasonal). The prevalence of *C. botulinum* type C declined in seasonal marshes during the dry period. Similar declines did not occur in the permanently flooded marshes.

Key words: Avian botulism, C_1 toxin, C_2 toxin, *Clostridium botulinum* type C, microbial ecology, waterfowl disease.

INTRODUCTION

Since 1930, when toxin produced by the bacterium *Clostridium botulinum* type C was confirmed as the cause of avian botulism (Giltner and Couch, 1930), millions of waterfowl throughout the world have reportedly died from this disease. In North America, botulism epizootics typically occur in late summer or autumn and often recur at the same locations year after year. The principal factors believed to precipitate these epizootics include low dissolved oxygen, high temperatures, and shallow water (Bell et al., 1955). Birds often die from botulism in one wetland but not in nearby wetlands that appear ecologically similar; thus, unknown wetland characteristics play an important role in epizootics. Despite numerous research efforts to identify wetland factors associated with avian botulism (Kalmbach and Gunderson, 1934; Coburn and Quortrup, 1938; Jensen and Allen, 1960; Graham, 1978; Smith, 1979; Marion et al., 1983), current knowledge is inadequate to successfully prevent epizootics.

Further research is needed to assess whether changes in the abundance of *C. botulinum* type C in wetland sediments are related to botulism epizootics. Spores of *C. botulinum* type C can persist in soils for years (Smith et al., 1982), and a high prevalence has been demonstrated in soils during avian botulism epizootics (Haagsma, 1973; Borland et al., 1977). Because of the seasonal occurrence of this disease, changes in temperature and other environmental conditions have been assumed to stimulate spore germination and bacterial multiplication (Bell et al., 1955). However, the seasonal abundance of *C. botulinum* type C has received little study (Serikawa et al., 1977; Marion et al., 1983). In the United States, marshes often are drained in the spring and then flooded in late summer and early fall to provide resting and feeding areas for migrating waterfowl. The effect of this water manage-

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TABLE 1. Prevalence of total and neurotoxic *C. botulinum* type C in six permanently flooded and four seasonally flooded marshes at the Sacramento National Wildlife Refuge, Willows, California, during July 1986 to December 1987, related to avian botulism mortality during the preceding five years.

Marshes	Past botulism mortality	Epizootic during study		Number of sampling periods	Mean prevalence (%) of *C. botulinum* type C	
		1986	1987		Total (SD[a])	Neurotoxic (SD[a])
Permanent						
Tract F	High	–	–	23	69 (12)	60 (17)
Pool 1	High	–	–	23	73 (14)	57 (16)
Pool 8	High	+	+	23	62 (17)	55 (19)
Pool 10	High	–	+	23	81 (13)	64 (23)
Tract 19	Low	–	–	23	30 (11)	24 (12)
Pool 2	Low	+	+	23	77 (21)	64 (23)
Seasonal						
Pool 3	High	–	–	21	51 (26)	41 (26)
Pool 1A	High	–	–	20	22 (20)	18 (18)
Pool 5	Low	–	+	21	36 (25)	30 (19)
Tract E	Low	–	–	20	12 (10)	10 (09)

[a] Calculation of standard deviation (SD) based on number of sampling periods.
[b] –, no epizootic occurred; +, epizootic occurred at that site and year.

ment regime on the prevalence of *C. botulinum* type C is unknown.

Because botulism in waterfowl most frequently results from the ingestion of C_1 botulinum neurotoxin, previous workers have considered only neurotoxic strains of *C. botulinum* type C. However, not all strains of *C. botulinum* type C produce C_1 toxin. These aneurotoxic strains often produce C_2 toxin (Eklund et al., 1987), which is not a neurotoxin. After activation with trypsin, C_2 toxin is lethal when injected into laboratory mice. Eklund et al. (1971) demonstrated that aneurotoxic strains can be converted to neurotoxic strains (C_1 producers) in the laboratory by infection with specific bacteriophages. The extent of bacterial conversion in nature is unknown, but resulting increases in the neurotoxic *C. botulinum* population in a wetland may be related to the occurrence of epizootics (Eklund et al., 1987).

Our objectives were to determine the prevalence of *C. botulinum* type C in the sediments of several managed marshes at Sacramento National Wildlife Refuge (SNWR) throughout the year; evaluate the changes in prevalence among different marshes within the wetland complex; and relate prevalence to ongoing botulism epizootics, water management regime, and previous history of mortality from botulism.

MATERIALS AND METHODS

Our study was conducted at the SNWR near Willows, California (USA; 122°20'N, 39°20'W), a 40-km² wetland complex that provides wintering habitat for waterfowl of the Pacific flyway. The refuge is divided into a series of marshes supplied by common water sources, but the water level in each marsh is individually managed. Some marshes are flooded permanently; others are flooded seasonally from approximately September until April. Seasonally flooded marshes dry completely between flooding periods. Botulism epizootics occur frequently at SNWR and often result in the loss of thousands of waterfowl over several weeks. Mortality from botulism often recurs on certain marshes in the refuge.

We selected 10 marshes to represent both high and low levels of recent botulism mortality and both seasonal and permanent water management regimes (Table 1). A 16,000-m² fenced enclosure was built by the National Wildlife Health Research Center (NWHRC) within each marsh to assess site-specific mortality of wing-clipped sentinel mallards (*Anas platyrhynchos*). These enclosures served as our experimental units. Sediment samples were collected approximately bi-weekly starting in July (permanently flooded) or September (seasonally flooded) 1986 to December 1987, except during the winter (January to May 1987), when samples were taken

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monthly. Ten sediment samples were collected from each enclosure over a 2 to 3-day period. Sampling locations within each enclosure were randomly selected using a random number generator and grid maps, and changed for each sampling period. A core sampler was used to obtain 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. A total of 2,200 samples were collected and evaluated over 24 sampling periods.

Because *C. botulinum* type C could not be selectively grown on plates for direct counts, we determined the proportion of sediment samples that contained type C toxin-producing bacteria by the following procedure. Sediment samples were thawed. Each sample was stirred, and 0.5-g wet weight or 0.35-g dry weight (wet weight/1.4 = dry weight equivalent; Sandler, 1990) was inoculated into a tube of chopped meat glucose medium (Gibco Division of BBL Microbiology Systems, Cockeysville, Maryland, USA), incubated for 5 days at 35 C, and stored at 4 C. After 5 to 10 days of refrigeration, each sample was centrifuged at 350 × *g* and the presence of C_1 toxin was determined by the mouse protection test (Quortrup and Sudheimer, 1943) in a pair of 14 to 16 g Swiss-Webster mice (Simonson Laboratories, Gilroy, California, USA). One mouse from each pair was inoculated intraperitoneally (IP) with 0.1 ml of type C specific botulinum antitoxin (NWHRC, Madison, Wisconsin, USA). After 30 min, both mice were inoculated IP with 0.5 ml of supernatant from the culture tubes combined with 0.1 ml of a broad-spectrum antibiotic solution [1,500 units/ml penicillin G, 1,500 μg/ml streptomycin sulfate, 100 μg/ml gentamycin (Sigma Chemical Company, St. Louis, Missouri, USA), 100 units/ml mycostatin (Gibco BRL, Grand Island, New York, USA) in Hanks BSS (Gibco BRL), containing 5% glycerine (Sigma Chemical Company), adjusted to pH 7.6 with $NaHCO_3$ (Sigma Chemical Company)] to guard against nonspecific mortality. Mice were observed for 5 days; death or clinical signs of botulinum intoxication in the unprotected mouse indicated the presence of viable, neurotoxic *C. botulinum* type C cells or spores that germinated during the course of incubation. If the protected and unprotected mice died, the sample was filtered through a 0.20 micron syringe filter to remove bacteria and retested. To test for type C_2 botulinum toxin, samples that were initially negative for C_1 toxin were similarly filtered. The filtrate was mixed with trypsin (Difco, Detroit, Michigan, USA; 0.25% final concentration; Ohishi et al., 1980), and 0.5 ml injected IP into pairs of mice. One mouse from each pair was protected with antitoxin as described above. If the unprotected mouse died, the sample was considered positive for C_2 toxin and assumed to contain aneurotoxic *C. botulinum* type C.

We estimated the prevalence of *C. botulinum* type C for each sampling period within each marsh with two proportions; the total proportion of samples containing *C. botulinum* type C (neurotoxic or aneurotoxic) and the proportion of samples containing specifically neurotoxic *C. botulinum* type C. These two proportions were converted using an arcsine square-root transformation to normalize the data (Sokal and Rohlf, 1969).

We compared the prevalence of *C. botulinum* type C within three different marsh categories: seasonally and permanently flooded marshes, marshes with low and high waterfowl losses from botulism in the previous 5 yr, and marshes with and without botulism mortalities during the study. A marsh was considered to have high losses from avian botulism mortality if epizootics occurred at that site in three of the five years preceding the study. Marshes with less than three epizootics were considered to have low avian botulism losses. Comparisons were made using repeated measures analysis of variance (Milliken and Johnson, 1984). In these analyses, we used the proportions of *C. botulinum* type C from each sampling period within a marsh as the repeated measurements. We also contrasted the prevalence of *C. botulinum* type C between wet and dry periods in seasonally flooded marshes and before and after botulism epizootics. These data were analyzed with a randomized block analysis of variance test (ANOVA) (Kirk, 1982); marshes were used as blocks, and different seasons (groups of successive sampling periods) were considered to be treatments.

RESULTS

Clostridium botulinum type C was demonstrated throughout the year (Fig. 1) in all 10 marshes sampled at SNWR (Table 1). Fifty-two percent of our 2,200 sediment samples contained *C. botulinum* type C (C_1 or C_2 toxin producers), detectable by the mouse protection test; 43% of the sediment samples contained sufficient neurotoxic *C. botulinum* type C spores or cells to produce a detectable amount of C_1 botulinum toxin. Therefore, by subtraction, 9% of the sediment samples contained only aneurotoxic *C. botulinum* type C (C_2 producers). Because our assay could not detect C_2 toxin in the presence of C_1 toxin, we

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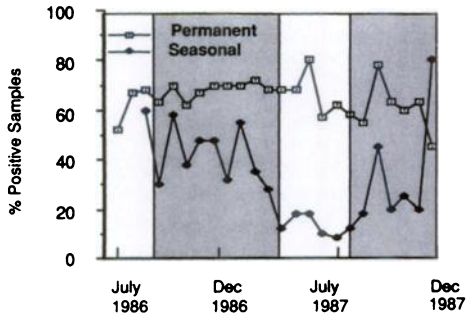


FIGURE 1. Prevalence of *C. botulinum* type C in seasonally and permanently flooded marshes, Sacramento National Wildlife Refuge, Willows, California, from July 1986 to December 1987. Each point represents a sampling period. Shaded areas indicate when seasonal marshes were flooded.

assume some of the samples that contained neurotoxic bacteria also contained aneurotoxic bacteria. Nonspecific mortality of mice (death from causes other than botulism) occurred in approximately 1% of our tests.

The prevalence (Table 1) of total *C. botulinum* type C in permanently flooded marshes (62%) was higher ($P = 0.01$, $F_{1,8} = 8.82$) than in seasonally flooded marshes (31%), as was the prevalence of neurotoxic *C. botulinum* type C (54 vs. 24%; $P = 0.01$, $F_{1,8} = 9.78$). We also found a significant interaction ($P = 0.0003$, $F_{21,166} = 2.64$; $P = 0.005$, $F_{21,166} = 2.11$) for total and neurotoxic strains, respectively, between sampling period and water management regime, which we interpret to be due to a differential time effect for seasonally and permanently flooded marshes. To further investigate this interaction in the seasonally flooded marshes, we compared prevalences measured during the first flooded interval (September 1986 through March 1987) and the dry interval (May 1987 through September 1987). The prevalence of both total and neurotoxic *C. botulinum* type C was higher ($P = 0.0001$, $F_{1,45} = 33.19$, and $P = 0.0001$, $F_{1,45} = 23.0$, respectively) when the marshes were first flooded ($\bar{x} = 41\%$ and 31%, respectively) than after they were drained ($\bar{x} = 15.8\%$ and 11.2%, respectively). Also, prevalence increased when the seasonal marshes were reflooded in September 1987 (Fig. 1). In contrast, the prevalence of total and neurotoxic *C. botulinum* type C in permanent marshes did not vary ($P = 0.87$, $F_{1,75} = 0.03$ and $P = 0.66$, $F_{1,75} = 0.20$, respectively) during these time intervals (Fig. 1).

Marshes with high losses from botulism in the previous 5 yr did not differ in prevalence (Table 1) of total ($\bar{x} = 61$ vs. 40%; $P = 0.26$, $F_{1,8} = 1.45$) or neurotoxic ($\bar{x} = 50$ vs. 33%; $P = 0.24$, $F_{1,8} = 1.64$) *C. botulinum* type C strains compared with those having low losses. There was no interaction between sampling periods and levels of past botulism losses for either measurement ($P = 0.73$, $F_{23,164} = 0.80$ and $P = 0.41$, $F_{23,164} = 1.04$). Likewise, prevalence of total and neurotoxic *C. botulinum* type C did not differ between marshes with epizootics and marshes with no epizootics in 1986 ($P = 0.57$, $F_{1,8} = 0.35$ and $P = 0.58$, $F_{1,8} = 0.33$, respectively) or in 1987 ($P = 0.19$, $F_{1,8} = 2.031$ and $P = 0.18$, $F_{1,8} = 2.11$, respectively).

In all of our analyses, trends in the neurotoxic portion of the *C. botulinum* type C population paralleled those of the total *C. botulinum* type C population, and the results were not altered by the arcsine square-root transformation. The proportion of the total *C. botulinum* type C positive samples that contained neurotoxic *C. botulinum* type C (C_1 producers) in all marshes was 83% (43% neurotoxic/52% total). In marshes with major epizootics (pool 2 and pool 8), this proportion did not change ($P > 0.2$) between time periods with botulism mortality and without mortality.

DISCUSSION

Neurotoxic *Clostridium botulinum* type C was ubiquitous at SNWR, persisted throughout the year in all 10 marshes, and occurred at considerably higher prevalences in permanent marshes than in seasonal marshes. In the permanent marshes, prevalence of *C. botulinum* type C remained consistent throughout the year.

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Serikawa et al. (1977) also demonstrated the year-round presence of type C spores in a permanent lake in Japan, but found that prevalence appeared to be greater in the fall. In a study of botulism in phosphate-mine settling ponds in Florida (USA), Marion et al. (1983) found detectable type C spores only in samples collected during summer (April to October); however, the prevalence of neurotoxic *C. botulinum* in their study was low (6%) compared with the prevalence (43%) obtained in our study. High prevalences of *C. botulinum* type C similar to our study have been reported in wetlands of Saskatchewan (38%; Wobeser et al., 1987), in shallow lakes of the Norfolk Broads (51%; Borland et al., 1977), and in several lakes and rivers in Japan (Azuma and Itoh, 1987). Our study differs from previous investigations because we sampled more frequently throughout all seasons, compared prevalence between permanent and seasonal marshes, and tested for both neurotoxic and aneurotoxic *C. botulinum*.

In seasonally flooded marshes at SNWR, prevalence of *C. botulinum* type C was reduced when marshes were drained, but increased after flooding. Because this pattern did not occur in permanently flooded marshes, desiccation is the most likely explanation for this reduction in prevalence. This result was surprising considering the resistance of bacterial spores to desiccation. Watanabe and Furusaka (1980) observed that *Bacillus* and *Clostridium* populations remained nearly constant in rice soils when major portions of these populations were in the spore state; however, considerable fluctuations occurred when vegetative cells predominated. Because we measured the combined spore and vegetative cell population in our assay, and because vegetative cells are more vulnerable to environmental fluctuations, we suspect that a large proportion of the *C. botulinum* type C population existed as vegetative cells, which declined in number when seasonal marshes were drained. However, we did not test this hypothesis directly. More information is needed to determine the significance of the relative proportions of spores and vegetative cells in *C. botulinum* type C populations.

Both neurotoxic (C_1 toxin producers) and aneurotoxic (C_2 toxin producers) *C. botulinum* type C were detected in sediments at SNWR. However, most (83%) of our positive samples contained neurotoxic bacteria. The abundance of both neurotoxic and of total *C. botulinum* type C did not change when botulism occurred in SNWR marshes. Also, the ratio of neurotoxic bacteria to total *C. botulinum* type C did not significantly change in any of the marshes sampled throughout our study. Based on our findings, we believe that changes in the prevalence of neurotoxic bacteria were not related to the occurrence of botulism epizootics in SNWR waterfowl.

Clostridium spores are highly resistant, and spores produced during epizootics can be expected to persist in marsh sediments for long periods of time. Several investigators, including Haagsma (1973), Smith et al. (1978), Borland et al. (1977), and Wobeser et al. (1987), found a higher prevalence of spores in wetlands with documented losses from botulism compared with wetlands having no known history of the disease. However, in our study we did not find a difference in prevalence of *C. botulinum* type C in marshes with high and low losses from avian botulism. The marshes at SNWR may not fit the expected pattern because they are part of a single wetland complex separated by water control dikes, and avian botulism epizootics previously occurred on all but one of the marshes we studied.

Although the prevalence of *C. botulinum* type C in wetlands may provide an indication of previous botulism epizootics, it is not necessarily a predictor of current risk. In our study, the prevalence of *C. botulinum* type C did not differ in marshes with and without epizootics; thus, other wetland factors influenced the probability of botulism epizootics. Predicting the occurrence of avian botulism depends on

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achieving an understanding of numerous biological and environmental factors. Certain environmental conditions may deter spore germination or cell growth or may alter the ability of cells to synthesize and release toxin. Other microbes commonly found in the sediments at SNWR also may influence or inhibit cell growth and toxin production by *C. botulinum* type C (Graham, 1978; Sandler, 1990). Even if toxin is produced in a wetland, the occurrence of botulism requires a mechanism to transfer toxin into the avian food chain, such as invertebrate food items.

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