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EFFICACY OF A TYPE C BOTULISM VACCINE IN GREEN-WINGED TEAL

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ABSTRACT: We tested the efficacy of a single dose of Botumink® toxoid for protecting wild green-winged teal (*Anas crecca*) during botulism epizootics caused by *Clostridium botulinum* type C. We challenged control and immunized ducks with four different doses of type C botulinum toxin to determine the LD_{50} for this species and to evaluate vaccine protection. Fewer immunized ducks were affected with botulism than control ducks, indicating that a single dose of Botumink® toxoid could increase the survival of ducks during epizootics. However, the frequency of immunized ducks with signs of botulism increased with the challenge dose of botulinum toxin. Even at doses of botulinum toxin approximately 2 to 4 green-winged teal LD_{50}, about 50% of the immunized ducks were affected. We believe an improved vaccine or a better delivery system is required to justify immunization of wild birds for experimental survival studies.

Key words: *Anas crecca*, avian botulism, *Clostridium botulinum* type C, green-winged teal, vaccination.

INTRODUCTION

Avian botulism is a paralytic disease caused by ingestion of toxin produced by the bacterium, *Clostridium botulinum* type C. It has been recognized as one of the most important diseases affecting wild waterfowl in North America (Friend, 1985). In some years more than 1,000,000 waterbirds were estimated to have died from botulism, and certain wetlands experience losses almost every year (Locke and Friend, 1987). The large number of mortalities caused by avian botulism have been highly visible to the general public and of considerable concern to waterfowl managers. However, the actual impact of avian botulism on waterfowl populations either locally, regionally, or on a continent-wide basis has been difficult to assess (e.g., Samuel, 1992).

Estimation of waterfowl population losses to disease is usually complicated by the spatial and temporal variation of the disease, the difficulty of studying highly mobile species, and the confounding influences of predation, scavenging, and decomposition on detecting disease mortality. Direct estimates of disease mortality may be conservative, because scavengers dispose of many carcasses or carcasses may be difficult to find in wetland vegetation (Humburg et al., 1983; Stutzenbaker et al., 1986). The experimental use of vaccines provides one method for assessing the impact of disease losses, by comparing the mortality rates between vaccinated and unvaccinated birds (Samuel et al., 1999). However, this approach typically requires that vaccination provide a high degree of protection against the specific disease agent. If vaccination provides only limited protection, impact of the disease on the population would be underestimated (Samuel et al., 1999). Alternatively, these biased mortality estimates can be corrected if vaccination provides a constant and known level of protection under field conditions.

The objective of this study was to evaluate the feasibility of using a single dose of a commercially available toxoid vaccine (Botumink®) to protect wild green-winged teal (*Anas crecca*) from botulism. We expected to determine whether vaccination would provide increased protection of green-winged teal during avian botulism outbreaks and whether this vaccine could provide sufficient protection to warrant ex-

perimental field studies to assess the impact of avian botulism on duck populations. Because of the difficulty of providing a booster vaccination to free-ranging waterfowl 2 to 4 wks after initial vaccination, we specifically evaluated the protection provided by a single dose of toxoid.

MATERIALS AND METHODS

Unidentified maggots were collected in September, 1998 from dead birds at the Bear River National Wildlife Refuge, Brigham City (Utah, USA; 41°23′N, 112°10′W) during a botulism outbreak in waterfowl and shipped frozen to the National Wildlife Health Center (Madison, Wisconsin, USA). After thawing, the maggots were weighed, diluted 1:2 in 0.85% saline (weight per volume), homogenized in a Waring blender, aliquoted in 50 ml quantities, and frozen at −20 C. One aliquot was centrifuged and the supernatant used in a mouse neutralization test to confirm the presence of type C botulinum toxin (Quortrup and Sudheimer, 1943) using type C specific antitoxin. The aliquot was serially diluted and inoculated intraperitoneally into groups of four mice to determine the 50% mouse intraperitoneal lethal dose/ml ($MIPLD_{50}$/ml) using the method of Reed and Muench (1938).

Eighty green-winged teal, primarily males (n = 72) and adults (n = 72), were captured near the Sacramento National Wildlife Refuge (39°30′N, 112°11′W) and Mendota Wildlife Management Area (36°45′N, 120°22′W) in California (USA) using rocket nets. The teal were wing-clipped to prevent flight, marked with numbered legbands, and placed in two large outdoor pens at the California Department of Fish and Game (Wildlife Investigations Lab, Rancho Cordova, California, USA). The birds were provided a large pool of water and fed Purina Game Bird Maintenance diet ad libitum. After an adjustment period of >5 days, birds were randomly divided into two groups of 40 each. One group was immunized with 1 ml of a type C botulinum toxoid vaccine (Botumink®, United Vaccine, Madison, Wisconsin, USA) by subcutaneous injection on the dorsal side of the neck. The vaccine consisted of partially purified, formalin-inactivated toxoid delivered in a medium of 0.1 M citrate buffer, 7.0% aluminum hydroxide, and 0.05% formalin (pH 5.8–6.0). The control group was injected similarly with 1 ml of a placebo that was identical to the delivery medium for the toxoid.

At 28 days post-immunization, the birds were randomly divided into four groups of 20, each group consisting of 10 immunized and 10 control birds. Blood samples (2 ml) were drawn from the jugular veins of one group of 10 immunized and 10 control birds and placed in heparinized tubes. The blood was centrifuged and the plasma harvested and frozen to be tested later for antibodies to type C botulinum toxin. Each group then received by oral intubation 4 ml of maggot suspension diluted in saline to a toxin dose of 200,000, 50,000, 25,000, or 10,000 $MIPLD_{50}$. The birds were observed daily, and the band numbers of sick and dead birds were recorded. Sick birds were classified into three categories: Class I (bird obviously affected but able to walk and evade capture), Class II (bird unable to walk but alert with head held erect) and Class III (bird unable to walk or move and unable to hold head erect). Birds in the Class III category were euthanized to prevent further suffering. We assumed that wild birds with Class II or Class III signs would typically die during a botulism outbreak. Heart blood was collected from 40 dead birds (both controls and immunized birds) to confirm botulism intoxication using either the mouse neutralization test or an ELISA test for type C botulinum toxin (Rocke et al., 1998). Surviving birds were bled as previously described and euthanized 14 days after challenge.

Antibody assays were conducted as previously described (Rocke et al., 1998), although a much lower concentration of toxin was used. Two-fold serial dilutions of the plasma were made in gelatin phosphate buffer and an equal volume of type C botulinum toxin (2.5 ng or 10 $MIPLD_{50}$) were added to each dilution. After 1 hr incubation at room temperature, 0.2 ml volumes of the mixtures were inoculated into each of two mice. Plasma from a hyper-immunized chicken (Rocke, et al. 1998) served as a positive control to ensure that antibody was detectable using this method, and the toxin dose was serially diluted 10-fold and inoculated into mice to ensure that the correct toxin dose was used. The mice were observed for 5 days, and the number of sick and dead mice was recorded. The antibody titer of the plasma was calculated as the reciprocal of the dilution for which 50% of the mice survived the inoculation.

The oral LD_{50} for green-winged teal was calculated by the Reed-Muench method (Reed and Muench, 1938) using data from control groups only. The frequency of affected control and immunized birds was compared using Fisher's exact test (Agresti, 1996) for each challenge dose. An overall test for differences in the frequency of affected control and immunized birds was conducted using the Cochran-Mantel-Haenszel test (Agresti, 1996). A test for trends of increasing frequency of affected birds relative to toxin dose was conducted using the

TABLE 1. Number of green-winged teal affected (Class II or Class III clinical signs or death) when challenged with different doses of botulinum toxin approximately 30 days after immunization with a single dose of Botumink toxoid (immunized) or a placebo (control). Each challenge group consisted of 10 birds.

Toxin dose[a]	Grams of maggots[b]	Control ducks	Immunized ducks
200,000	2.00	10	7
50,000	0.50	9	5
25,000	0.25	10	4
10,000	0.10	3	2

[a] 50% mouse intraperitoneal lethal doses ($MIPLD_{50}$).

[b] Approximate grams of macerated toxic maggots administered to green-winged teal in saline (1:2 weight : volume). Maggots were collected from bird carcasses during avian botulism outbreaks in 1998.

Mantel-Haenszel test (SAS Institute Inc., 1990).

RESULTS

Mortality and signs of botulism were detected in both immunized and control birds challenged with the three highest toxin doses within 24 hr post exposure (PE), and clinical signs persisted in birds that survived challenge for as long as 6 days PE. All of the control birds and 70% of the immunized birds that received 200,000 $MIPLD_{50}$ of botulinum toxin died or developed Class II or III signs (Table 1), while 90% of the control birds and 50% of the immunized birds that received 50,000 $MIPLD_{50}$ of botulinum toxin were similarly affected. No differences were detected in the percentage of affected birds in the immunized and control groups at either of these doses (Fisher's exact test, $P > 0.14$).

At the challenge dose of 25,000 $MIPLD_{50}$ of botulinum toxin, the number of affected birds was significantly higher (Fisher's exact test, $P = 0.011$) for controls (100%) than immunized birds (40%). Within 24 hr PE, three of the control birds receiving 25,000 $MIPLD_{50}$ had died and all others exhibited clinical signs of botulism, four with Class II signs. By 48 hr PE, the remaining three birds either died or developed Class II signs. In contrast, five immunized birds developed Class I signs within 24 hr. One of these birds died on day 3, and three birds developed Class II signs on day 2. Two of these birds improved significantly by the next day and were completely recovered by the fourth day PE. The remaining bird improved, but died on the sixth day PE.

At the lowest challenge dose (10,000 $MIPLD_{50}$ of botulinum toxin), no significant difference was detected in the frequency of affected birds in control and immunized groups (Fisher's exact test, $P = 1.0$). One control bird died 4 days PE and two additional control birds developed Class II signs by day 2. In the immunized group, two birds developed Class II signs within 2 days PE; one subsequently died.

Overall, 32 of 40 (80%) control ducks died or had clinical signs of botulism compared with 18 of 40 (45%) immunized birds (Table 1). Control birds were more likely to contract botulism than immunized birds based on the overall Cochran-Mantel-Haenszel test ($\chi_1^2 = 12.7$, $P = 0.001$). An increase in the frequency of affected birds was also evident as the dose of toxin increased for both control (Mantel-Haenszel $\chi_1^2 = 5.4$, $P = 0.02$) and immunized (Mantel-Haenszel $\chi_1^2 = 4.1$, $P = 0.04$) ducks. The LD_{50} for green-winged teal was estimated at 14,000 $MIPLD_{50}$ of toxin.

Type C botulinum toxin was detected in the heart blood of most (35 of 40) birds that were tested either by mouse test or ELISA. Protective antibody to type C botulinum toxin was not detected in any of the 20 blood samples (from 10 control and 10 immunized birds) collected prior to challenge, although protective antibody was detected in the positive control chicken plasma at a titer > 1:4,000.

DISCUSSION

Previous studies have explored the potential use of botulinum toxoid to protect wild or captive ducks during botulism outbreaks (Schwartz and Smart, 1963; Mears and East, 1969). Our results generally confirm these early studies, the suggestion by

Cambre and Kenny (1993), and work by Martinez and Wobeser (1999) that a single dose of botulinum toxoid (Botumink®) can provide waterfowl with some level of protection from botulism. However, the amount of toxin that ducks ingest during botulism outbreaks can be highly variable. To overcome this problem we used a controlled study to insure that all birds received a known amount of botulinum toxin. Thus, our conclusions about the level of protection provided by immunization may be less affirmative than earlier uncontrolled studies. In addition, current methods of immunization would require inoculation of individual birds with botulinum toxoid and are impractical for protecting wild bird populations from annual botulism losses.

Approximately 55% of the immunized green-winged teal had no significant clinical signs of avian botulism compared with 20% of the control birds. However, we found that the frequency of affected birds in both groups increased as the amount of toxin in the challenge dose increased. At a toxin dose of only 2 to 4 LD_{50} for control green-winged teal, approximately 40–50% of the immunized teal were so impaired by the toxin they were unable to walk or fly and could barely hold their heads up. We believe these birds would probably have died during natural botulism outbreaks, either as a result of botulinum toxin or from increased predation. It is important to note that this toxin dose (25,000–50,000 $MIPLD_{50}$) was equivalent to consuming 0.25–0.5 g of maggots. Although there have been few studies of summer foods consumed by green-winged teal (see Hohman et al., 1992; Krapu and Reinecke, 1992), these birds need approximately 8 g/day of commercial waterfowl food. Thus, $\leq$1 g of maggots represents a potential dose of toxin that could readily be consumed by birds during a botulism outbreak. In addition, none of the immunized birds we tested had detectable levels of antibody 28 days post-immunization, suggesting that the vaccine did not induce measurable levels of antibody in green-winged teal, at least by our measurement of neutralizing antibodies. Perhaps mucosal or cellular immunity to the toxoid played a greater role in protecting immunized birds from intoxication.

Our study also demonstrates that green-winged teal were considerably more sensitive to botulinum toxin than mallards (*Anas platyrhynchos*) or pintails (*Anas acuta*). Martinez and Wobeser (1999) found that doses of approximately 45,000 and 22,500 $MIPLD_{50}$ caused stage III paralysis (our Class II morbidity) or mortality in about 50% of mallards and pintails, respectively. With an estimated LD_{50} of 14,000 $MIPLD_{50}$, green-winged teal in our study were approximately three times more sensitive than mallards. However, on a body weight basis for average sized males (Bellrose, 1976), the sensitivity to the toxin was similar among species (36,000–43,000 $MIPLD_{50}$/kg). Hunter et al. (1970) also found LD_{50} values for mallard, pintail, and green-winged teal which were roughly proportional to body weight and similar to those reported here and in Wobeser (1997: Table 11-1).

At present, we do not believe a single immunization of Botumink® provides sufficient protection for use in experimental field studies designed to measure botulism-specific mortality of marked waterfowl. First, approximately 50% of vaccinated teal were still affected at doses of 4 to 5 LD_{50} for unvaccinated teal. We believe this level of protection is too low to insure that experimental studies do not substantially underestimate botulism impacts. Second, the level of protection provided by Botumink® depended on the dose of toxin consumed by vaccinated birds and probably on the species vaccinated. Thus, it is very unlikely that a single immunization of Botumink® could provide a consistent and predictable level of protection which could be used to determine the true impact of botulism on mortality. In the future, it may be possible to improve the efficacy of Botumink® for birds

using slow release adjuvants. Previous studies in pheasants (Reilly and Boroff, 1961) demonstrated that a single dose of type C botulinum toxoid, mixed with Freund's incomplete adjuvant, provided protection against botulism at toxin doses of 200 LD_{50} by intramuscular challenge, and that two inoculations provided even higher levels of protection. Newer technology, such as biodegradable microspheres that slowly release vaccine over a longer period of time and more effective vaccines, should also be considered.

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