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IMMUNIZATION OF DUCKS FOR TYPE C BOTULISM

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ABSTRACT: A single subcutaneous immunization with a vaccine used for protecting ranch mink (*Mustela vison*) against type C botulism reduced morbidity and mortality in mallard (*Anas platyrhynchos*) and northern pintail (*Anas acuta*) ducks challenged with approximately 4.5×10^4 and 2.25×10^4 mouse lethal doses (MLD_{50}), respectively, of botulinum toxin at 10 and 15 days post-immunization (pi). There was no significant protection at 5 days pi. Protection persisted in mallards for 90 days pi. To simulate use of vaccine as a part of treatment of sick birds in the field, mallards were exposed to toxin and, when clinical signs were evident, each bird was treated by intraperitoneal injection of type C botulinum antitoxin and one-half of the birds were immunized. Immunization had no significant effect on recovery from intoxication. At 10 days post-treatment, all birds were challenged with toxin. Clinical signs and mortality were significantly less frequent among immunized birds than among non-immunized birds after the second exposure. Immunization might be useful as part of the treatment regimen in botulism outbreaks.

Key words: *Anas acuta*, *Anas platyrhynchos*, mallard, northern pintail, treatment, type C botulism, vaccine, waterfowl.

INTRODUCTION

Botulism, caused by *Clostridium botulinum* type C, is one of the most important diseases of wild waterfowl. During outbreaks, management usually consists of collection and disposal of sick and dead birds to reduce the amount of carcass material that can act as substrate for further toxin production. Approximately 10 to 20% of birds collected during outbreaks are alive, with varying degrees of paralysis. In most outbreaks, sick birds are killed rather than treated, although 75 to 90% of these would recover if treated (Locke and Friend, 1987). Treatment is simple and consists of provision of fresh water, shade, protection from predators and, if available, injection with antitoxin (Wobeser, 1987). Because treatment is usually done near the outbreak site, recovered birds are at risk of reexposure to toxin. Birds that recover from botulism are thought not to acquire protective immunity (Haagsma, 1987), so that such birds may ingest further toxin and die, negating the treatment effort.

Immunization might be used to reduce the risk of reintoxication in treated waterfowl. Immunization has been used to protect pheasants (*Phasianus colchicus*), broiler chickens, and other birds against botulism (Boroff and Reilly, 1962; Dohms et al., 1982; Shimizu and Kondo, 1987). Usually two doses of vaccine, administered about 14 days apart, have been used. However, under field conditions in which treated birds often are held in open top pens and release themselves when they are able to fly, holding ducks to administer a "booster" dose of vaccine would be impractical. Schwartz and Smart (1963) reported that a single dose of botulinum toxoid protected ducks for several months under field exposure to toxin-laden maggots. Cambre and Kenny (1993) suggested that one dose of a vaccine intended for use in ranch mink (*Mustela vison*) protected waterfowl in a zoological collection; however, they did not measure the degree, onset or duration of protection. A single dose of bivalent (types C and D) botulinum toxoid has been used to immunize cattle in Australia (Gregory et al., 1994).

The objectives of this study were to determine the protection afforded by a single dose of a commercially available vaccine given to ducks under experimental conditions, and to test the effectiveness of si-

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multaneous administration of antitoxin and vaccine to intoxicated birds, as might be done under field conditions.

MATERIALS AND METHODS

Three trials were conducted. Experimental birds were held indoors in the Laboratory Animal Care Unit (Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, Saskatchewan, Canada). The study was approved by the University of Saskatchewan Committee on Animal Care and Supply (protocol 960115). Control and immunized birds in each trial were held together in a single room. Each room was equipped with a child's wading pool with running water for bathing and had a light-dark cycle typical of midsummer. The birds had unlimited access to commercial duck ration (Federated Co-operatives Ltd., Saskatoon, Saskatchewan) and fresh water. The vaccine used was a bacterin-toxoid vaccine prepared from a pure culture of *C. botulinum* type C, inactivated with formalin and combined with an aluminium adjuvant (Botumink, United Vaccines Inc., Madison, Wisconsin, USA). This was the same vaccine used by Cambre and Kenny (1993).

In Trial I, 60 adult male mallards (*Anas platyrhynchos*) from a commercial supplier (Whistling Wings, Hanover, Illinois, USA) were divided randomly on the basis of leg band numbers into two groups of 30. Each bird in the immunized group was injected subcutaneously on the dorsum of the lower neck with 1 ml of vaccine. The 30 control birds were injected with 1 ml of sterile 0.85% saline at the same site. Birds in each group were then subdivided by drawing band numbers into three groups of 10 birds each and marked with distinctive colored leg bands. On days 5, 10 and 15 post-immunization (pi), a sub-group of 10 immunized birds and a subgroup of 10 control birds were moved to a separate room and each bird was given approximately 4.5×10^4 mouse lethal doses (MLD_{50}) of type C botulinum toxin (see below) by gastric intubation. The birds were then observed several times each day. Clinical signs of botulism were recorded as Stage I (bird is able to walk but has paresis or ataxia), Stage II (bird has difficulty walking, often using the wings to assist, but is able to evade capture and can reach food and water), or Stage III (bird is prostrate and paralyzed). Birds in Stage III were euthanised by overexposure to anaesthetic (Halothane®, Halocarbon Laboratories, River Edge, New Jersey, USA) and a necropsy was performed. It was assumed that birds with Stage III botulism would die in a natural situation. Birds in Stages I and II were observed until they had recovered completely. Immunized birds that survived were retained and 10 of these birds were challenged with the same dose of toxin at 60 days pi, and another 10 birds were challenged at 90 days pi. No control birds were challenged at 60 days pi. Three adult male mallards that had not been immunized or received toxin were challenged at 90 days pi as controls.

Trial II was similar to Trial I. Northern pintails (*Anas acuta*), hereafter referred to as pintails, were used rather than mallards. These were surplus adult birds that had been used as decoys for trapping wild ducks in other studies and were destined for euthanasia. The method of assignment to groups was similar to Trial I except that, because of the birds available, each sub-group contained seven male and three female birds. The dose and method of administration of vaccine or saline was as in Trial I. The challenge dose contained approximately 2.25×10^4 MLD_{50} of type C botulinum toxin (see below) and was administered to subgroups of birds on days 5, 10, and 15 pi, as in Trial I.

Trial III was intended to mimic a field situation in which birds with clinical signs of botulism, collected during an outbreak, would be treated and immunized simultaneously. Adult male mallards from the same source as in Trial I were used. In each of two replicates (a and b), 20 ducks were given approximately 1.0×10^5 MLD_{50} of type C botulinum toxin (see below) by gastric intubation. As birds developed clinical signs of botulism, they were assigned alternately to either a control or an immunized subgroup. Sick birds in both groups were given 0.5 ml of type C botulism antitoxin (serial 86-7, subserial 2-A, #172, National Wildlife Health Centre, Madison, Wisconsin, USA) by intraperitoneal injection. This antitoxin contained approximately 100 IU/ml at the time of preparation (T. Rocke, pers. comm.). Birds in the immunized group also received vaccine, as in Trials I and II, at the time of treatment. The birds were monitored and any that were unable to reach water were given water by gastric intubation as necessary. Birds that became moribund were euthanised. On day 10 after treatment, birds in both the control and immunized groups were given 1.0×10^5 MLD_{50} of toxin to mimic a situation in which birds that had recovered after treatment were re-exposed to toxin, as might occur if they returned to an outbreak site.

The toxic material used to challenge birds in Trials I and II was a suspension of fly maggots collected in July 1997 from duck carcasses at Eyebrow Lake, Saskatchewan (50°55′N, 106° 08′W), an enzootic site for botulism. Maggots collected from 10 carcasses were pooled and

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ground in a commercial food blender with five volumes of sterile water. The resulting suspension was mixed thoroughly, divided in aliquots, and held frozen at −20 C until required. A different toxin source was used in Trial III. Tissue, primarily collagen strands, from within the body cavity of eight desiccated wild duck carcasses, collected from the shore of Old Wives Lake, Saskatchewan (50°08′N, 105°55′W) in September 1997 following a severe botulism epizootic, was minced with scissors, pooled, and placed in approximately five volumes of sterile water. The material was shaken thoroughly and then 3 ml of fluid was used as an inoculum that was introduced into bacteriologic medium in 500 ml flasks. The medium in each flask contained 18.75 g cooked meat, 0.75 g calcium carbonate, 1.5 g yeast extract, 1.5 g ammonium sulfate, 1.2 g glucose, 0.75 g soluble starch, 0.15 g L-cystine hydrochloride, and 150 ml water, all adjusted to pH 7.6. Each inoculated flask was placed in a 2.5 L anaerobic jar, together with a moistened anaerobic strip (Anaerocult A, Merck KGa, Darmstalt, Germany) and an anaerobic indicator strip (Anaerotest, Merck KGa, Darmstalt, Germany), and incubated at 37 C for 3 days. The content of the flasks was pooled, mixed, divided into aliquots and held frozen at −20 C until required.

Both toxin sources were tested after freezing for the presence of type C toxin by inoculation of dilutions into mice, some of which had previously received type C antitoxin. Paralysis and death of mice that had not received antitoxin together with survival of mice that had received antitoxin was regarded as proof of the presence of type C toxin. The concentration of toxin was then determined by sequential dilution and inoculation into groups of two mice. The MLD_{50} was the highest doubling dilution that resulted in death of at least one of the mice exposed to that dose. The appropriate dosage for ducks was determined by gastric intubation of groups of four ducks with graded amounts of toxin to find a dosage that resulted in production of clinical signs in almost all the exposed birds and that produced Stage III botulism in approximately 50% of exposed birds. The dose of the maggot suspension used in Trials I and II for mallards and pintails contained approximately 4.5×10^4 and 2.25×10^4 MLD_{50} of toxin, respectively. The dose of the bacteriologic media used in Trial III contained approximately 1.0×10^5 MLD_{50}. The amount of toxin used in the trials was within the range of toxic oral doses (in MLD_{50}) reported previously for mallards: 4.5 to 8.0×10^4 (Hunter et al., 1970); $>3.6 \times 10^5$ (Haagsma, 1973); 2.0 to 8.0×10^4 (Duncan and Jensen, 1976); and for pintails: 1.6 to 7.6×10^4 (Hunter et al., 1970).

Blood collected from five control mallards with Stage III botulism (two birds from each of Trials I and III, and one bird challenged together with birds that had been immunized 90 days earlier) was used in a mouse toxicity test (Wobeser, 1997) to confirm that the clinical signs seen resulted from botulism. Serum (0.5 ml) extracted from the blood of each duck, was injected intraperitoneally into each of four mice, two of which had received 0.1 ml of type C antitoxin 30 min earlier.

In all trials, differences in the number of birds in the control and immunized groups that developed clinical signs of botulism and in the number of birds that were euthanized in Stage III in the same groups were determined using a two-tailed Fisher's Exact test (Zar, 1984). Significance was inferred at $P \leq 0.05$.

RESULTS

All 30 control mallards in Trial I developed clinical botulism and 12/30 (40%) of these birds were euthanized after becoming paralyzed (Stage III). Unprotected mice injected with serum from two birds with Stage III botulism died in <24 hr, while mice that had received antitoxin remained clinically normal after receiving the same amount of serum. There was no significant difference in the proportion of birds in the immunized and control groups that developed botulism when challenged on day 5 pi; however, significantly fewer birds in the immunized groups developed botulism when challenged on days 10 and 15 pi (Table 1). Two immunized birds developed Stage III botulism after challenge on day 10; none of the immunized birds developed Stage III botulism when challenged on day 15 pi. The number of immunized birds that developed botulism after challenge at 10 and 15 days pi was not significantly different ($P = 0.444$). One of 10 immunized birds rechallenged at 60 days pi developed mild (Stage I) signs of botulism that persisted for <48 hr. One of 10 immunized birds challenged at day 90 developed slight ataxia that persisted for <33 hr. All three control birds challenged at day 90 developed botulism. Two had Stage I botulism that persisted for >50 and >67 hr, respectively, and one developed Stage III botulism. An unprotected

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TABLE 1. Number of ducks that developed clinical signs of botulism when challenged with botulinum toxin at 5, 10 and 15 days after immunization. Immunized birds received 1.0 ml of bacterin-toxoid vaccine by subcutaneous injection; control birds were injected with 1.0 ml of saline. Immunized and control groups, on each day, contained 10 birds.

	Day 5		Day 10		Day 15	
	Normal[a]	Botulism[b]	Normal	Botulism	Normal	Botulism
Trial I (Mallards)						
Immunized	3	7(5)[c]	8	2(2)	9	1
Control	0	10(4)	0	10(4)	0	10(4)
P value[d]		0.2105		0.0007		0.0001
Trial II (Northern pintails)						
Immunized	0	10(8)	9	1	8	2
Control	3	7(7)	0	10(5)	0	10(5)
P value		0.2105		0.0001		0.0007

[a] Bird had no clinical signs of botulism.
[b] Bird had clinical signs compatible with botulism.
[c] Number in brackets indicates the number of birds with Stage III clinical signs of botulism.
[d] Fisher's Exact Test.

mouse injected with serum from the latter bird died in <17 hr, while a mouse that received both serum and antitoxin remained clinically normal.

Of 30 control pintails in Trial II, 27 developed clinical signs of botulism and 17 (57%) were euthanized after becoming paralyzed (Stage III). There was no significant difference in the proportion of birds in the immunized and control groups that developed botulism when challenged on day 5 pi; however, as in trial I, significantly fewer birds in the immunized groups developed botulism when challenged on days 10 and 15 pi (Table 1). No immunized bird developed Stage III botulism when challenged on days 10 and 15. The number of immunized birds that developed botulism after challenge at 10 and 15 days pi was not significantly different ($P = 0.444$).

All birds in trial IIIa developed clinical signs of botulism (Class I or II) and were treated between 10.8 and 18.8 hr ($\bar{x} \pm SD = 16.7 \pm 2.4$) after receiving the initial dose of toxin. One bird in each of the immunized and control groups subsequently became moribund and was euthanized. The remaining birds had signs ranging from severe ataxia to partial paralysis but all recovered and by 71 hr after dosing with toxin only very slight ataxia was evident in a few birds. All nine surviving control birds developed botulism following exposure to toxin on day 10 after treatment; six became paralyzed and were euthanized. Unprotected mice that received serum from two birds with Stage III botulism died in <24 hr, while mice that received antitoxin remained clinically normal. Fewer of the immunized birds developed botulism after challenge (Table 2); however, the difference between immunized and control groups was not significant ($P = 0.0824$). Two immunized birds developed Stage III botulism.

All birds in trial IIIb developed clinical signs of botulism and were treated between 11 and 34 hr ($\bar{x} \pm SD = 22.6 \pm 8.3$) after the initial dose of toxin. No birds became moribund and by 73 hr all appeared normal. All 10 control birds developed botulism following exposure to toxin on day 10 after treatment; six became paralyzed and were euthanized. Significantly fewer of the immunized birds developed botulism after challenge (Table 2). One immunized bird became paralyzed and was euthanized. If the results of Trials III a and b are combined, 9 of 19 immunized birds remained normal after challenge, while all non-immunized birds developed signs of botulism; 16% (3/19) of immu-

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TABLE 2. Number of mallards that developed clinical signs of botulism when challenged with toxin 10 days after being treated for clinical signs of botulism. Immunized birds received 0.5 ml antitoxin by intraperitoneal injection and 1.0 ml vaccine by subcutaneous injection at the time of treatment. Control birds received 0.5 ml antitoxin by intraperitoneal injection at the time of treatment.

	Remained normal	Clinical signs of botulism
Trial IIIa		
Immunized $n = 9$	4	5(2)[a]
Control $n = 9$	0	9(6)
P value[b]	0.0824	
Trial IIIb		
Immunized $n = 10$	5	5(1)
Control $n = 10$	0	10(6)
P value	0.0325	

[a] Number in brackets indicates the number of birds with Stage III botulism.
[b] Fisher's Exact Test.

nized birds developed Stage III botulism compared to 63% (12/19) of non-immunized birds.

DISCUSSION

The results of Trials I and II confirm earlier reports (Schwartz and Smart, 1963; Cambre and Kenny, 1993) that a single dose of toxoid vaccine will provide significant protection to ducks against botulism. Protection was not evident at 5 days pi but developed by 10 days pi and appeared to persist for 90 days. Approximately 85% of the immunized mallards and pintails did not have any clinical signs of botulism when challenged at 10 and 15 days pi, while all of the control birds had clinical botulism. Only 3% (2/60) of immunized mallards or pintails challenged at those times developed Stage III signs, compared with 48% (29/60) of control birds. Most outbreaks in northern locations in North America occur during mid to late summer, so that immunity that persists for 3 months is likely sufficient to protect treated birds until the onset of cool weather when most outbreaks cease. The amount of toxin used in these experiments was approximately an LD_{50} dose for the two duck species tested. The amount of toxin ducks ingest during outbreaks is likely highly variable. In a field trial in which mallards were exposed to maggots from carcasses of birds that died of botulism, Schwartz and Smart (1963) reported that all 80 mallard immunized with a single dose of toxoid survived, while all 20 non-immunized control birds died.

The results of Trial III confirm that birds which recover from botulism remain susceptible to intoxication; all non-immunized birds developed clinical botulism following re-exposure to toxin. To our knowledge, no one has measured antibodies in waterfowl exposed to botulinum toxin. Ring-billed gulls (*Larus delawarensis*) fed sublethal amounts of type E botulism toxin over a 3 week period tolerated amounts of toxin that were lethal for control birds, suggesting that protective immunity may follow oral exposure to toxin (Kaufman and Crecelius, 1967). Cattle exposed to sublethal levels of type C toxin develop detectable antibodies (Gregory et al., 1994) but it is not clear that the antibody levels are protective against subsequent challenge.

Simultaneous administration of vaccine with antitoxin did not interfere with recovery after treatment and reduced morbidity and the severity of clinical disease when birds were re-exposed to toxin 10 days later. The presence of either residual toxin or residual injury as a result of cleavage of syntaxin, a protein in the presynaptic membrane of neurons that is cleaved by type C neurotoxin (Schiavo and Montecucco, 1997), may have reduced the effectiveness of immunization in Trial III compared with Trials I and II.

This was a pilot study in which birds were treated in the early stages of botulism. Immunization did not interfere with treatment and did provide a measure of protection against reintoxication. The vaccine cost approximately $0.11 US per dose, so that it would not contribute materially to the costs involved in treating sick birds. The next step will be to deter-

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mine if immunization, used as a part of the treatment for sick birds under field conditions, will improve post-release survival. If it is effective for that purpose, immunization during treatment might be particularly valuable for species whose population numbers are low, such as the pintail, and for threatened species that may be involved in botulism outbreaks. Valuable birds might be held for 10 days after treatment to ensure development of adequate immunity; if that was done, administration of a booster dose of vaccine prior to release should be considered.

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