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EVALUATION OF COPROEXAMINATION AS A DIAGNOSTIC TEST FOR AVIAN BOTULISM

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Abstract: Fecal extracts and blood sera from 113 ducks showing clinical signs of botulism were examined for *Clostridium botulinum* type C toxin by means of the mouse toxicity test to evaluate coproexamination as a diagnostic procedure, as compared with demonstration of toxin in serum. When death of test mice unprotected with type specific antitoxin (while protected controls survived) was the criterion, 78.8% of the sera and 5.3% of the fecal extracts were positive. When characteristic signs of intoxication in the unprotected mice was included as evidence of toxin in the specimens, these percentages increased to 86.7 and 6.2, respectively.

Fecal specimens were collected hourly for the first 6 h after peroral dosing of eight mallards *(Anas platyrhynchos)* with 1.0 LD_{50} of type C toxin and at 24, 48, and 72 h from birds surviving that long. From 2 to 4 toxin-positive specimens were passed by all eight ducks during the first 6 h, five specimens were positive at 24 h, and three were positive at 48 h. Only three specimens were collected at 72 h, all of which were negative. These findings suggest that attempts to detect toxin in the feces of wild ducks might have been more successful had the birds been captured earlier in the course of the disease.

INTRODUCTION

Although *Clostridium botulinum* was isolated from stools of human botulism patients in the 1920's,[4,8] the presence of botulinum toxins in feces of intoxicated patients was not reported until 1970.[2,5] Since 1972, coproexamination has become a routine diagnostic test in suspected botulism cases at the U.S. Public Health Service's Center for Disease Control, along with culturing feces for *C. botulinum* and demonstration of toxin in blood serum by mouse injection.[3]

In 1932, Hobmaier[6] demonstrated botulinal toxemia in ducks by injecting their whole blood into mice; and Quortrup *et al.*,[10] more than a decade later, measured the duration of toxemia in experimentally intoxicated ducks.

Investigators at the Bear River Research Station had detected toxin in the feces — or, more properly, excreta, since they are a mixture of urine and feces — of ducks after peroral administration of crude cultures of *C. botulinum* in 1974 (unpubl.), but they did not include the detection of either the bacterium or its toxin in feces among our diagnostic procedures until the summer of 1979.

The primary purpose of the study reported here was to evaluate coproexamination for toxin as a diagnostic test (supplementary to demonstration of serum toxin) for avian botulism. An ancillary purpose (not reviewed in this report) was to give antitoxin therapy to the ducks, after serum and fecal samples had been collected for toxicity tests in mice.

MATERIALS AND METHODS

Field Cases

Between 19 July and 29 August, 1979, 113 sick ducks [82 pintails *(Anas acuta)*; 6 green-winged teal *(Anas crecca caroli-*

nensis); 17 mallards *(Anas platyrhynchos);* 3 redheads *(Aythya americana);* 2 northern shovelers *(Anas clypeata);* 2 gadwall *(Anas strepera);* and 1 blue-winged teal *(Anas discors)*] were collected on the marshes of the Bear River Migratory Bird Refuge 24 km west of Brigham City, Utah, USA, for toxicological examinations. All were considered by the collectors to be showing one or more of the characteristic signs of botulism: bright green diarrhea; paralysis of the nictitating membrane; inability to walk, while retaining the ability to propel themselves by wing action; immobility with heads held erect; prostration. Paralysis is progressive and, of course, there was overlapping of one stage and the next. Mildly affected birds — those still able to walk or propel themselves, even for short distances — were not collected, because earlier experience had shown that more than 90% so affected would recover without antitoxin therapy (unpubl.).

Upon arrival at the laboratory, each duck was placed in a cage, the floor of which had been covered with plastic-coated paper. As soon as feces were passed, the entire volume was usually transferred from the paper to a 12 ml graduated centrifuge tube. Gentle digital pressure on the abdomen of the duck was sometimes required to hasten the expulsion of feces, so that antitoxin could be administered as early as possible. A few ducks died before passing fecal samples, and the entire intestinal contents were collected at necropsy. When an intestinal tract was empty at that point, the duck was eliminated from the study. When, uncommonly, a large volume of feces was collected, it was mixed thoroughly, and a 5.0 ml portion was retained for examination.

The procedure for examining feces for toxin was similar to that described by Dowell *et al.*[3] Each specimen was diluted with an equal volume of gelatin-phosphate buffer (pH 6.2), mixed thoroughly, and stored at 4.5 C for 16 to 20 h to allow time for the elution of toxin from the solid material. Specimens were warmed to 37 C, resuspended, and centrifuged at low speed until particles too large to pass through a 25 gauge needle had settled.

Toxicity tests on fecal extracts were performed by injecting 0.25 ml intraperitoneally (i.p.) into each of two laboratory mice, one of which had received a 0.1 ml i.p. dose (5 International Units) of type C specific antitoxin 15 to 30 min earlier. Volume of extract injected and number of test mice used were limited by the volume of feces collected, which was usually scanty. Antibiotics were not used to reduce the possibility of infections by fecal bacteria, because earlier experience had shown that such protection was usually unnecessary (unpubl.).

Mice were observed closely for 3 days (and once daily until the 7th day after injection) for death or signs of botulism. Death of unprotected mice, while those protected with antiserum remain normal, is the generally accepted endpoint of a positive mouse toxicity test. However, if unprotected mice developed characteristic signs of botulinal intoxication (labored breathing; "wasp waist," due to paralysis of the phrenic nerve) but did not die, and protected ones were unaffected, the result was recorded as "probably botulism." The diagnostician commonly receives only one or two bird carcasses or blood samples for examination, and clinical signs exhibited by the test animals (without death) may be the only evidence available to him as to the nature of the disease.

Blood samples were taken by cardiopuncture. The procedure for toxicity tests on serum was the same as for fecal extract, except that samples were not refrigerated overnight, and 0.5 ml was injected into each mouse.

Feces were not examined for *C. botulinum,* as is done in the diagnosis of

human botulism,[3] because the presence of the bacterium in the digestive tracts of birds inhabiting a marsh where the disease has been endemic for many years would have little diagnostic significance.

Experimental Intoxications

Mallards were supplied by a commercial breeder.

The X220B2 strain of *C. botulinum* type C was cultured in LYA broth[1] by the method of Sterne and Wentzel.[12]

Over a period of 2 years, each of eight mallards was given a single oral dose of X220B2 crude culture containing 1.0×10^5 mouse i.p. LD_{50}, estimated to be 1.0 mallard LD_{50} by the method of Reed and Muench.[11] Ducks were caged individually, with access to water and mixed grains. The entire volume of feces, if any had been passed, was collected from all ducks hourly for the first 6 h and at 24 and 48 h from those surviving the intoxication up to that time. Paper on the floor of all cages was changed after each collection. Because of the small volume of feces usually eliminated and the infrequency of elimination by the second day after toxin was administered, samples were collected only occasionally after that time. All ducks were held captive until death or recovery occurred.

The procedure for demonstrating toxin in the feces of experimentally intoxicated ducks was the one used in field investigations.

Serum toxicity tests were not run in conjunction with fecal toxicity tests. Many previous observations here (unpubl.) had suggested that toxemia in mallards was a virtually certain consequence of dosing them perorally with type C toxin (at least in doses close to their LD_{50}), and that the duration of toxemia was usually 2 to 3 days, a range agreeing closely with that reported earlier for ducks by Quortrup *et al.*[10]

RESULTS

Type C toxin was demonstrated in the serum in 78.8% of the field cases of suspected botulism in ducks (Table 1), if death of the unprotected mouse is an immutable endpoint. If development of characteristic signs of botulinal intoxication, without death, in the unprotected mouse is acceptable evidence of the presence of toxin, the incidence of positive tests rises by nearly 8%. The comparable incidences of positive fecal specimens, however, were only 5.3 and 6.2%.

Toxin was demonstrated in 3 to 6 of the fecal specimens from all eight of the experimentally intoxicated ducks within the first 48 h (Table 2).

Only one mouse injected with fecal extract died from infection. Death occurred on the 4th day, by which time the mouse had shown no signs of botulism.

Duck No. 2 (Table 2) was of particular interest. Paralyzed to the point of immobility within 24 h after being dosed with toxin, it was still unable to walk by the 7th day, although it occasionally drank water and ate a small amount of grain. No toxin was detected in the feces on the 7th day. The paralysis gradually diminished in severity and, by the 10th day, it could walk without great difficulty. However, the diarrhea persisted. On the 11th day, both blood and feces were positive for type C toxin. Although the unprotected test mice did not die, they showed marked signs of botulinal intoxication.

DISCUSSION

The results summarized in Table 1 indicate that the fecal toxin test has little diagnostic value, at least in ducks that are intoxicated to the point of immobility. This is particularly evident because in every instance where toxin was detected

[1] Lactalysate (Baltimore Biological Laboratories), 3.0%; yeast autolysate (Albimi Laboratories), 2.0%; glucose, 0.5%; sodium citrate, 0.35%; pH 7.0 (after autoclaving).

in a bird's feces, it was also detected in its blood serum.

These figures differ considerably from those reported by Dowell *et al.*[3] for human botulism. Whereas they detected toxin in feces of 33.9% of 60 patients with clinical botulism, we did so in only 6.2% of such cases in ducks. Moreover, in 10.4% of 48 of their cases, toxin was detected in the feces when the serum was negative. Our success in detecting toxin in serum, however, was considerably higher than theirs: 86.7% in ducks as compared to 33.3% in humans.

TABLE 1. Success rates in detecting *C. botulinum* type C toxin in the blood serum and feces of 113 ducks with clinical botulism by means of the mouse toxicity test, when death of unprotected[a] mice is the only endpoint and when signs of intoxication (SI) are included.

	Death (%)	SI (%)	Total (%)
Serum	89 (78.8)	9 (8.0)	98 (86.7)
Feces	6 (5.3)	1 (.9)	7 (6.2)

[a]All control mice protected with type specific antitoxin survived.

The time lapse between the ducks' consumption of toxin and the collection of fecal specimens was very likely important among the factors determining our success rate in detecting toxin. Early in the course of the disease, intoxicated birds refuse food and water;[7] and since diarrhea is one of the most common signs of avian botulism, there is often little material left in the digestive tract after 24 to 48 hours. Moreover, excreta examined in this study often were largely mucus and uric acid (often with bile), rather than feces.

The results of fecal examinations of experimentally intoxicated ducks (Table 2) suggest that the test might be more useful early in the course of the disease. In the very early hours, when chances of detecting toxin would be greatest, however, wild birds usually would be successful in resisting capture. Even when the ability to fly is lost, ducks can often flap across the water's surface at a rate that makes collection impractical.

TABLE 2. Mouse toxicity tests on the feces of mallards within 48 hours of peroral administration of 1 mallard LD_{50} of *C. botulinum* type C toxin.

Hours after dosing	Duck Number 1	2	3	4	5	6	7	8
1	-[1]	-	-	-	U[2]	-	-	0[3]
2	-	+[4]	+	-	+	-	+	-
3	+	0	+	+	+	-	+	+
4	+	0	0	+	0	+	+	+
5	+	+	+	+	+	+	+	0
6	+	0	+	0	+	0	-	+
24	+	+	-	+	+	+	-	-
48	+	+	-	-	-	+	-	-
Other	D(52)[5]	+[6]	-(72)	D(61)	-(72)	D(76)	-(72)	

[1]- = No toxin detected.
[2]U = Uncertain. Unprotected mouse showed very mild signs of intoxication.
[3]0 =No feces passed.
[4]+ = Toxin demonstrated.
[5]D ( ) = Dead (number of hours after inoculation).
[6]Fecal specimen collected at 7 days was negative; specimen collected at 11 days was positive for type C toxin.
[7]72 hour specimen negative.

There is one circumstance in which coproexamination of wild birds might be of greater value: the diagnosis of suspected botulism in captive birds, when fecal specimens could be collected early in the course of the outbreak, but facilities for drawing blood were not available.

Most ducks recovering from experimental botulism will have regained some use of their legs by the 4th day or earlier, even though paralysis is still clearly evident. With two exceptions, however — one of which was mallard No. 2 in this study — we have never detected toxin in blood serum later than 3 days after oral inoculation (unpubl.), nor have other investigators.[10]

The protracted course of intoxication in mallard No. 2 may have been evidence of a toxico-infection similar to that occurring in infant botulism.[1] Such cases result from toxin production by *C. botulinum* within the infant's gut rather than from its consumption of preformed toxin. Minervin[9] reported on patients who were discharged from the hospital recuperating from botulism and readmitted within a few days with "grave symptoms" of the disease. He believed such cases to be toxico-infections. Hypothetically, an outbreak of botulism could be initiated among waterbirds by their ingestion of *C. botulinum* spores while feeding in a contaminated marsh, but there is little evidence to support such a thesis.

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