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Avian Botulism in a Mixed Population of Resident Ducks in an Urban River Setting

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ABSTRACT

An outbreak of fatal duck sickness among a resident flock of mixed mallard, Peking white, and mallard-Peking white crossed ducks was investigated and proved to be caused by botulism type C intoxication. The incident was deemed unusual because it occurred on a flowing river in the apparent absence of the usual conditions associated with avian botulism. Furthermore, *Clostridium botulinum* could not be demonstrated in bottom samples from the shallow water at the site of the outbreak. Although a comprehensive scheme was followed for detection and isolation of *C. botulinum* type C, and ample evidence of the toxic anaerobe in enrichment cultures was obtained, examination of approximately 125 isolates failed to yield the toxic anaerobe in pure culture. A possible association between this outbreak of avian botulism and an alteration in the aquatic environment occasioned by the building of a high dam, with the attendant rise in water levels and decreased river flow rate, is suggested but can not be definitely proved. Interested workers should be alert to the possibility of botulism in unusual or recently altered environments and the attractive hazard to migratory fowl posed by afflicted resident waterfowl.

INTRODUCTION

Botulism, or "duck sickness," among aquatic wild birds is widely recognized as a serious and destructive epizootic disease,[10,12,14] usually caused by ingestion of the toxin produced by Clostridium botulinum type C. Most episodes have occured along the shores of lakes, ponds, and flooded mud flats, particularly in alkaline waters and waters in which oxygen has been depleted by layers of pond weed, algae, and rotting vegetation.[14] Attracted to such waters, the feeding shore birds and waterfowl in large numbers often ingest fatal quantities of the toxin formed during growth and metabolism of the toxic anaerobe. Recently type E botulinum toxin, heretofore not associated with large episodes of avian botulism, has been implicated in many deaths of gulls, loons, grebes, and mergansers on the Great Lakes.[5,6,13]

The present report calls attention to an episode of avian botulism in an environment that is not commonly reported as a setting for this disease. The outbreak occurred on the shores of a flowing river in the absence of the common conditions of stagnation and unusually high vegetative growth. Moreover, the ducks involved were not entirely dependent on wild feed but received heavy additions of dried corn, which conceivably limited the amount of toxin they might have ingested had they been entirely dependent on wild food.

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On August 28, 1964, two of us (Crisley and Angelotti) were asked by the proprietor of the Newport Yacht Club located on the Kentucky shore of the Ohio River opposite Cincinnati, Ohio, for an opinion on the cause of illness among a flock of resident ducks that he had attracted to the site and kept there by feeding them for the previous two years. The flock of approximately 40 mallards, white Pekings, and crosses between the two types had been decimated by the loss of over 30 birds. The symptoms he described in various birds and those we observed at 3:00 p.m. the same day coincided almost exactly with those previously described,[1,2] including extreme muscle weakness (inability to fly or walk), wing droop, loss of control of neck muscles (limberneck), diarrhea, and in some birds, obstruction of the vent by hardened renal matter.

We collected four ducks for examination: duck D-1, an adult mallard-white Peking cross that died at about 7:00 a.m. that morning and had been immediately refrigerated; duck D-2, an adult mallard that had died between 11:00 a.m. and about 2:00 p.m. when it was retrieved and refrigerated; duck D-3, a dead adult mallard; and duck D-4, a dead mallard duckling. Ducks D-3 and D-4 had been taken from the river at the same time as duck D-2. We also took samples of sand found in the shallows where the ducks habitually fed and probed and wet mud from the zone between high and low water. From submerged surfaces of the dock, moored boats, and sunken debris, we also collected quantities of blue-green and green algae on which the ducks were seen to feed sporadically. Considerable anaerobic microbial activity was present in the bottom sand as evidenced by rather active gas generation and bubbling when the sand was disturbed. A deposit of fine, black sediment, probably coal dust, was noticed in the sand particularly at the fringes of wave action. All samples were returned to the laboratory and immediately examined, except for D-3 and D-4, which were stored in the deep freeze.

MATERIALS AND METHODS

Dissection of D-1 and D-2 revealed almost empty alimentary tracts (another common sign of avian botulism), the gizzards containing only gravel and a few shreds of algae.

Toxicity Tests

Tissue, mud, sand, and algae samples were extracted with 0.1 M phosphate buffer, pH 6.5, containing 0.2% gelatin. Mouse toxicity texts were performed as previously described,[3] except that no 10^{-1} dilution was made, since no putrefaction was found in the tissue and we wished to enhance our chances of detecting possible toxin by using the concentrated extract. Anti-toxin used for the tests had been previously purchased from the National Communicable Disease Center, Atlanta, Georgia. Also, to detect low levels of the toxin in the sand (which possibly could have cumulatively poisoned the feeding birds), several samples of wet sand, 600 ml/sample, were extracted with 130 ml of buffer, and the extracts were dialyzed in the cold against 30% (w/v) polyvinylpyrrolidone (Plasdone C)[1] [2] to concentrate the extracts to 17.0 ml. The results of these tests are recorded in Table 1.

Seven days after our first visit, we returned to the site to obtain a specimen of blood from a sick white Peking female (duck D-5) that had been confined by the yacht club proprietor to an abandoned boat with a supply of clean food and water. This bird appeared to be recovering. The results of a mouse protection test on blood plasma from duck D-5 are recorded in Table 1.

Culture Methods

Specimens from eviscerated ducks D-1 and D-2, and sand, mud, and algae samples (Table 1) were examined essentially by an abbreviated version of the enrichment method outlined on the flow sheet in Fig. 1. Two versions of modfiied Noyes medium in screw-capped 16 mm tubes were used, one adjusted to approximately pH 7.0 to 7.2 and the other to pH 8.0 with a small button of powdered $CaCO_3$ on the bottom to neutralize any acid that might develop.

[1] General Aniline and Film Corp., New York, N. Y.
[2] Mention of commercial products does not imply endorsement by the Public Health Service.

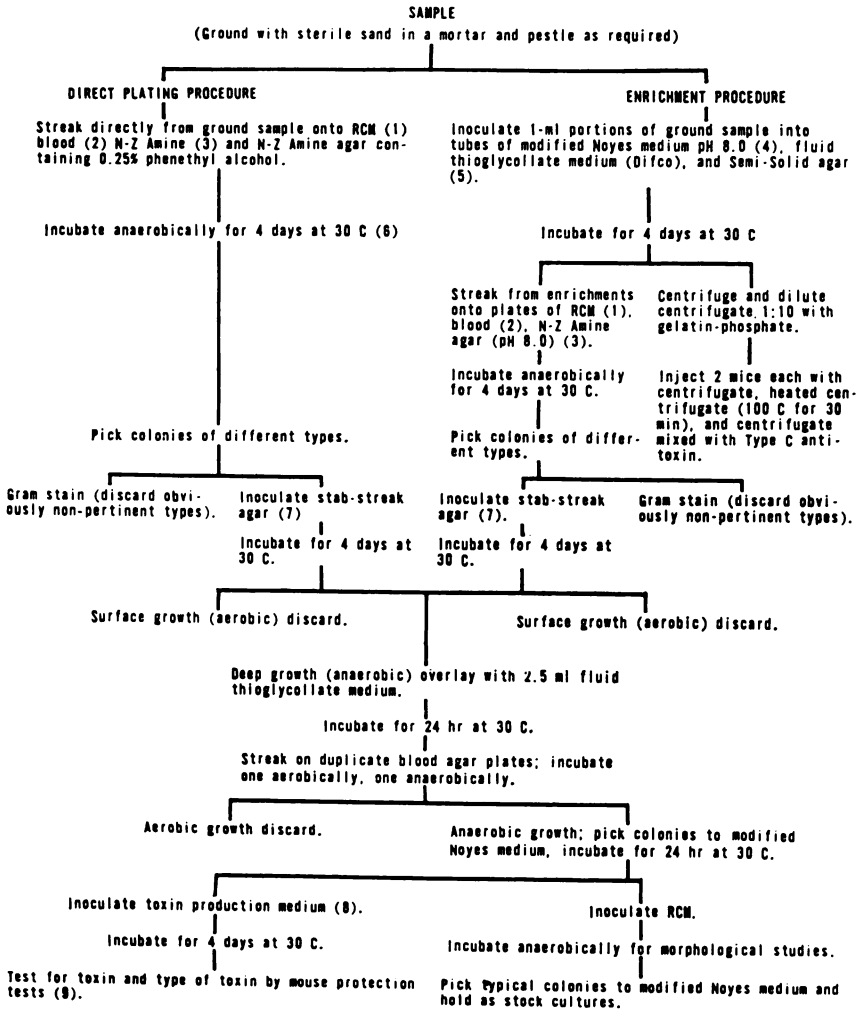
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Table 1. *Toxicity of intraperitoneally injected heated and unheated extracts from tissue, blood, and environmental samples on unprotected mice and mice protected by addition of* Clostridium botulinum *antisera types A, B, C, D, and E.*

[1] D = dead N = normal

Extract	Mouse No.	Effect[1] on Mice receiving 0.5 ml. extract plus 0.20 ml. of						Effect on mice receiving heated extract + 0.25 ml. saline
		Antiserum A	Antiserum B	Antiserum C	Antiserum D	Antiserum E	0.85% saline	
D-1 Crop 1:1 buffer	1	D-18 hr	D-18 hr	N-5 days	D-40 hr	D-18 hr	D-18 hr	N-5 days
	2	D-24 hr	D-40 hr	N-5 days	D-40 hr	D-24 hr	D-24 hr	N-5 days
D-1 Congealed blood plus heart tissue 1:1 buffer	1	D-18 hr	D-18 hr	N-5 days	D-18 hr	D-18 hr	D-18 hr	N-5 days
	2	N-5 days	N-5 days	N-5 days	D-18 hr	D-18 hr	D-18 hr	N-5 days
D-2 Congealed blood 1:1 buffer	1	D-18 hr	D-18 hr	N-5 days	N-5 days	D-18 hr	D-18 hr	N-5 days
	2	D-18 hr	D-40 hr	N-5 days	N-5 days	N-5 days	N-5 days	N-5 days
Algae 1:2 buffer	1	N-5 days	—	N-5 days	—	N-5 days	N-5 days	N-5 days
	2	N-5 days	—	N-5 days	—	N-5 days	N-5 days	N-5 days
Bottom Sand	1	—	—	N-5 days	—	—	N-5 days	N-5 days
	2	—	—	N-5 days	—	—	N-5 days	N-5 days
Mud, 1:2 buffer	1	—	—	N-5 days	—	—	N-5 days	N-5 days
	2	—	—	N-5 days	—	—	N-5 days	N-5 days
Bottom sand concentration by dialysis	1	N-5 days	—	N-5 days	—	N-5 days	N-5 days	N-5 days
	2	N-5 days	—	N-5 days	—	N-5 days	N-5 days	N-5 days
D-5 blood plasma	1	D-24 hr	D-24 hr	N-5 days	D-42 hr	D-24 hr	D-16 hr	Not heated because of plasma coagulation
	2	D-42 hr	D-24 hr	N-5 days	D-48 hr	D-24 hr	D-42 hr	
	3	D-42 hr	D-24 hr	N-5 days	D-48 hr	D-48 hr	D-69 hr	

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LEGEND Fig. 1. *Scheme used for the determination and isolation of* Clostridium botulinum *type C from tissues of sick ducks and from bottom sand, mud, and algae samples*: (1) RCM is Reinforced Clostridial Medium (Oxoid). (2) Blood agar was made by adding 5% sheep blood to sterilized and cooled (45° C) Blood-Agar Base (BBL). (3) N-Z Amine agar is composed of N-Z Amine type B (Sheffield Chemical Co., Norwich, N.Y.) 2%, autolyzed yeast extract (Vico type 75) (Vico Products Co., Chicago, Ill.) 0.5%, and glucose (CP) 0.5%. (4) Modified Noyes medium is the veal broth medium of Angelotti et al.,[1] with the addition of 0.2% soluble starch and 0.5% glucose (CP) before autoclaving. (5) Semi-Solid agar is composed of: peptone (Oxoid), 5%; Bacto yeast extract, 0.5%; Bacto agar, 0.3%; pH 7.0-7.2. After the agar is autoclaved, sterile 10% sodium thioglycollate, sterile 20% glucose, and sterile 10% sodium bicarbonate are added to give final concentrations of 0.1%, 0.5% and 0.14% respectively. A Millipore-filtered 1:10,000 solution of crystal violet is added to give a final concentration of 1:800,000. (6) The anaerobic method employed with most plates was that

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of Angelotti et al.[1] The method of Parker [16] was also used successfully. (1) Stab-streak agar is Semi-Solid agar without crystal violet and containing 1.5% agar. It was tubed with very deep butts and short slants. It was inoculated by thrusting the needle straight to the bottom of the tube and streaking over the slant on withdrawal. (8) Toxin production medium is Semi-Solid agar with the crystal violet omitted. It is melted, cooled to 45-50° C, inoculated, cooled to solidify agar and overlaid with 1.5 ml. of sterile 1.5% Bacto agar (melted and cooled to 45-50° C). After incubation medium is broken up with sterile glass rods and centrifuged at 3,000 rpm to obtain a clear centrifugate for toxin tests. (9) Mouse protection tests were carried out as described by Crisley[3].

The media were steamed to drive off dissolved oxygen; half of the tubes were allowed to cool, while the remaining tubes were equlilibrated at 80° C. All tubes were inoculated with fragments of sample. The tubes inoculated at 80° C were left in the water bath for 20 min. and were then rapidly cooled in a container of tap water. Cultures were incubated at 30° C for 96 hours, and small samples were withdrawn for toxicity tests at 48 and 96 hours. Streak cultures were made from the enrichments to RCM and N-Z Amine agar (pH 8.0) at 36, 48, and 96 hours in an attempt to isolate the organisms. Suspicious colonies were picked and inoculated into steamed and cooled unbuffered N-Z Amine B-yeast extract broth[2] and modified Noyes medium. Tubes showing growth after 96 hour incubation at 30° C were examined for toxicity. Toxicity tests on both enrichment and isolated colony cultures were accomplished by centrifuging portions of the culture fluid at approximately 4,000 rpm in a refrigerated centrifuge at 4° C and making 1:10 dilution of the clear centrifugate in gelatin-phosphate buffer, pH 6.5.[3] Mouse protection tests on the diluted centrifugate were then done exactly as on the extracts from duck tissues.

Later culture work on samples D-3, D-4, and blood and heart tissues of D-5 was carried out according to the detailed scheme presented in Fig. 1.

RESULTS AND DISCUSSION

Table 1 indicates that type C toxin was present in duck D-1 and in congealed blood from duck D-2, although the pattern of survival of mice receiving extracts of the latter sample suggests that less toxin may have been present in D-2. Survival of the antiserum-D-treated pair may possibly be attributed to the combined manifestation of low toxin levels and the cross protection afforded by a reported serological relation between types C and D toxin.[18] Certainly no sign of type D toxicity was encountered in further tests on tissue samples or enrichment cultures. Because the carcasses of D-1 and D-2 had not undergone putrefactive changes that might have resulted in post-morten toxin formation by resident type C organisms, the presence of toxin was considered to have fulfilled the minimal requirement of evidence for the incrimination of *C. botulinum* type C as the cause of the intoxication of the ducks. This evidence was strengthened by the demonstration of type C toxin in the blood from specimen D-5.

Table 1 also shows that pre-formed toxin was not present in bottom sand, mud, and algae samples from the area commonly frequented by the birds. Neither was toxin demonstrated in the sample prepared by concentration dialysis. We believe, however, that concentration dialysis with polyvinylpyrrollidone or carbowax might be a fruitful approach in testing for toxin in submerged soils, since it may well reveal low levels of toxin undetectable by routine methods provided it is not destroyed by factors in the environment. Such low levels might be harmless to ducks receiving only a single dose or a few ingested doses, but could be quite lethal if accumulated by feeding over a short period. Kalmbach and Gunderson[12] suggested the hypothesis that land appearing dry on the surface during periods of hot weather may occasionally be moist and sufficiently organic and anaerobic to encourage formation of toxin that is later released by flooding. They observed severe outbreaks in puddling ducks on newly flooded land and surmised that these outbreaks may have been caused by consumption

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of such preformed toxin. The concentrations of toxin need not necessarily be high to be lethal, if the possibility of ingestion of cumulative doses is considered.

Type C toxin, indicating the presence of *Clostridium botulinum* type C, was demonstrated in the enrichment cultures of intestinal sections and contents, and crop and gizzard fragments of ducks D-1, D-2, and D-5. Although repeated enrichment cultures of the samples of algae, sand, and mud were made, and the anaerobic microbial activity was very pronounced, no botulinus toxin of any type could be detected, which indicated that the organism either was not present or was present in such small numbers that it was overgrown by competing microorganisms in the samples.

Streak cultures from the toxic enrichments of duck viscera yielded a number of colonies with characteristics similar to those described and photographed by Dolman.[4] When these were picked from plates and seeded into modified Noyes medium and toxin production broth (pH 7.0 and 8.0) some of these cultures failed to grow, and those that produced growth showed no evidence of toxicity. Therefore, either we failed to isolate the type C organisms that were present in the enrichment cultures (as indicated by the presence of type C toxin), or our isolates become atoxic in the course of the study.

The pressure of other work forced us to forego temporarily attempts to isolate the type C toxic anaerobe in pure culture from the remaining samples in frozen storage. This was done reluctantly because of the possibility that a study of the characteristics of the organisms in pure culture might reveal more information about the outbreak. During the summer of 1966, however, V. R. Dowell attempted to isolate *C. botulinum* type C from the gizzard, crop, and intestinal tract of frozen D-3 and D-4, and frozen blood and heart tissues of sample D-5 according to the comprehensive scheme presented in Fig. 1. Enrichment preparations of D-3 viscera in both semisolid agar and in modified Noyes medium yielded toxin in the former but not in the latter medium. Sample D-4 revealed toxin in enrichments in both media; D-5 heart and blood enrichments yielded no toxin-producing growth, although they originally were shown to be toxic. The latter result is not surprising because the cells of *C. botulinum* do not normally invade these tissues although the pre-formed toxin is often found in the blood stream. Altogether 91 isolated organisms from direct plating of the samples and from streak cultures from enrichments were examined. Of these, 8 cultures showed presumptive toxicity that could have been caused by *C. botulinum* toxin, but which, on further testing, proved to be *Clostridium sordelli, C. bifermentans, C. tetani* and an unidentified *Clostridium* species. If the initial studies are included, then the total number of isolates examined in this study without a successful isolation of *C. botulinum* is approximately 125; the evidence for its presence and its implication in the botulism episode, however, is conclusive. Difficulties in isolating and subculturing single colonies of the type C anaerobe were recognized by early workers and have been experienced by many workers to the present time. Better methods are greatly needed.

An attempt to explain the cause of this episode of avian botulism is hampered by our failure to demonstrate the causative organism and/or its toxin in the immediate environment. The death of the duckling, D-4, would tend to place the source of the toxin at the site; the bird appeared to be fully feathered except for muture wing feathers, the lack of which precluded its flying far from the site. The proprietor of the yacht club established that, at the height of the episode (prior to our visit), many decaying duck carcasses on shore were washed away by a rise in the water. The affected birds could have acquired toxin by consuming insects that had fed on the decaying carcasses. Bengtson (Meyer[14]) investigated an outbreak in barnyard fowl caused by ingestion of larvae of *Lucilia caesar* that had infested infected carrion.

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Demonstrations of toxin in carcasses of invertebrates[9,11] and also in living sarcophagid larvae[12] have adequately confirmed invertebrates as sources of type C toxin. If carrion were in fact the source of the present episode, initially the death of a bird or other animal harboring the organism, from avian botulism or other causes, would have been necessary, followed by development of toxin in the carcass. The latter event is quite possible if we consider the ubiquitous nature of this toxic anaerobe and the ability of adult birds to fly from the site.

The possibility that the present episode originated in an initial case (or cases) of avian botulism among the flock is predicated on the existence of botulinogenic material somewhere on the feeding ground. Were aquatic invertebrates responsible for harboring the organism, as is strongly suggested by Jensen and Allen,[11] then we might have expected the presence of the organism in enrichment cultures of the bottom samples containing remains of such aquatic forms. Either toxic aquatic invertebrates harboring *C. botulinum* type C were not present or our method was not effective enough to detect the toxic anaerobe. Since the usual conditions of marked stagnation and decaying vegetation were not apparent, it is also possible that the outbreak resulted from a pocket of botulinogenic material at a spot or spots on the feeding ground (missed by our sampling), which sometimes occurs in intermittent flooding.[12]

The only possible ecological change in the Cincinnati pool of the Ohio River that might conceivably have contributed to the development of such botulinogenic pockets and prevented their subsequent rapid removal by rapidly flowing water was the closing of the new high-level Markland dam. A recent report in preparation for publication[7] shows that this dam, fully operational in January 1963, has increased the river depth by 13.9 feet, decreased the mean velocity to 0.61 of its value (now 10,000 cfs) before construction, and decreased the dissolved oxygen content of the water at any given flow rate. Of less significance may be the slight increase in the mean pH levels of the water from July through the first week of September in 1963 (pH 7.4) and in 1964 (pH 7.5), as compared with the values for 1962 (pH 7.1) prior to closing and for 1965 and 1966 (pH 7.3)[15] since the botulism episode. It is not unusual for the water pH to reach slightly alkaline levels during this period of the year (readings of pH 7.8 and pH 7.5 were noted in 1960 and 1961, respectively). In 1963 and 1964, however, which were the first two years following closing of the dam, the high pH level occurred in the period of hot weather and low flow, together with altered water level, reduced flow, and low dissolved oxygen content. Although alkalinity is not essential for toxin development, the growth of *C. botulinum* type C appears to be favored by pH conditions slightly above neutrality.[12] Alteration of the water level with better stabilization of the shoreline in itself may also have been responsible. From experimental floodings of stable shorelines Sperry[17] concluded that avian botulism increased as soon as the water level was raised enough to make the former stable feather edge a feeding ground for waterfowl. At the time of our investigation the water was gradually receding from a higher level that had evidently submerged an increased area of shallows along the shore.

Whether or not these conditions actually interacted to activate growth and development of toxin in pockets of organic matter along the shoreline that was submerged during 1963 and 1964 is largely conjecture, since we have no proof. We feel, however, that officials concerned with game management should at least be alerted to the possibility that such changes may have occurred, particularly along flyways, irrespective of the absence of the obvious type of botulinogenic conditions, now so well recognized. The present episode, confined as it was to resident waterfowl, did not involve a crippling loss of waterfowl. The presence of such resident populations

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along a flyway, however, might well attract migratory fowl to a potentially hazardous site and result in more serious losses.

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