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PERSISTENCE OF *PASTEURELLA MULTOCIDA* IN NEBRASKA WETLANDS UNDER EPIZOOTIC CONDITIONS

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ABSTRACT: Gleason Basin, a marsh located in the western part of the Rainwater Basin in Nebraska, was selected during the 1980 spring waterfowl migration as a study site to determine the presence and persistence of virulent *Pasteurella multocida*. Avian cholera mortality in migratory waterfowl using the Basin increased during a 2-wk period of a die-off beginning the first week of March when 2,409 carcasses were collected from the marsh. Study sites within the marsh were established for sampling water associated with and not associated with intact and scavenged carcasses. Isolations of virulent *P. multocida* were made from five of six study sites associated with either intact or scavenged carcasses for 3 days and from three of five non-carcass-associated study sites for 2 days. Recovery of these bacteria from this environment suggested a possible source of infection for susceptible waterfowl using the contaminated site.

INTRODUCTION

Contaminated environments are important in transmission and maintenance of avian cholera in domestic poultry (Bendheim and Even-Shoshan, 1975), but the epizootiology of avian cholera in wild waterfowl is not fully understood. This study describes persistence of *Pasteurella multocida* in a marsh environment during an epizootic of avian cholera that occurred in Nebraska's Rainwater Basin. Estimated losses ranged between 70,000 and 100,000 waterfowl establishing this die-off as the largest ever recorded (Friend, 1981). This 1980 die-off began during the first week of March and ended during the third week of April when waterfowl moved out of the area to continue their northward migration. Gleason Basin (Fig. 1) was selected because of heavy mortality at that site. A total of 4,145 waterfowl carcasses were collected from that marsh during the die-off. Of these, 2,409 were picked up during the first 2 wk. Study sites within the marsh were established for sampling water associated with and not associated with either intact or scavenged carcasses.

MATERIALS AND METHODS

Beginning 2 April 1980, the first samples of water were collected from Gleason Basin using 1-liter glass jars filled 0.2 m below the water surface. Collections were made at sites either in association with carcasses or not associated with carcasses. Sample size was 1,000 ml.

Carcass-associated collections: Six sites (designated by letters a–f in Fig. 1) representing three scavenged carcasses, one Canada goose (*Branta canadensis*) (a) and two whitefronted geese (*Anser albifrons*) (b, c), and three intact carcasses, one green-winged teal (*Anas carolinensis*) (d) and two mallards (*Anas platyrhynchos*) (e, f), were sampled on days 2, 3 (morning), 3 (afternoon), 4, 8, 15, and 21. Water was collected at distances from 0.1 to 1 m from the birds. Carcasses were either left at the site (a, d, f) for the duration of the study or removed (b, c, e) after the first water sample was taken.

Non-carcass-associated collections: Five randomly selected water collection sites not associated with carcasses (1–5, Fig. 1) were sampled on days 1, 2, 3, 8, and 15. Collections were made 0.1 m from wooden markers driven firmly into the Basin bottom. Sludge samples not associated with carcasses were collected on days 1, 2, 3, and 15. These samples were collected in 1-liter jars, shaken vigorously and allowed to settle (2–4 min).

Processing sites for P. multocida isolation: Water samples were processed on day 1 at the Basin. Mice were taken to the site and injected immediately after the water was collected. Samples on days 2, 3, and 4 were processed within 2 hr of collection time at a temporary

laboratory set up at Kearney State College, 24 km from Gleason Basin. Samples collected on days 8, 15, and 21 were shipped by air to Madison, Wisconsin, on wet ice and were processed within 24 hr of collection at the National Wildlife Health Laboratory (NWHL).

Isolation and characterization of P. multocida: Hsd: (CF-1) BR white male mice (Harlan Sprague-Dawley Farms, 3134 Seminole Highway, Madison, Wisconsin 53711, USA) 6–10 wk of age, weighing about 23 g, were used for this study. Inocula consisted of either undiluted test water or test water diluted through three 10-fold serial dilutions. All inoculations were made by injecting 0.2 ml inoculum into each mouse intraperitoneally (i.p.) using one to four mice/dilution. Only mice that died within 48 hr post-inoculation were considered presumptively positive for *P. multocida.* Liver and lung impressions of these mice were made onto microscope slides and stained with Harleco Diff-Quik blood stain (American Scientific Products, 1210 Waukegan Road, McGaw Park, Illinois 60085, USA). Stained impressions were viewed microscopically (970×) for evidence of rods or coccobacilli with the bipolar staining characteristics of avian cholera organisms. Subsequently, the lungs and livers were cultured on 5% sheep blood agar and Rosen's dextrose starch agar plates (Rosen, 1972). Typical *P. multocida* colonies were selected and characterized biochemically (Heddleston et al., 1972b). The agar gel diffusion test (Heddleston et al., 1972a) was used with reference sera (National Animal Disease Center, Ames, Iowa 50010, USA) for serotyping these isolates.

Control samples: Three carcass-associated samples were prepared on day 1 as positive controls. Nasal discharge from mallard and whitefronted goose carcasses was sampled with dacron swabs. A tracheal swab was also collected from the goose carcass. A fourth sample was collected on day 2 by swabbing the trachea of a moribund mallard. Each swab was mixed vigorously in 10 ml sterile physiological saline. A 0.2-ml portion of each suspension was injected i.p. into each of two to four mice/sample. Two additional mice were sham-inoculated i.p. with sterile saline as negative controls.

RESULTS

The results of the water sampling studies are summarized in Table 1. Ninety-eight (46.8%) of 209 mice injected with water from test sites not associated with
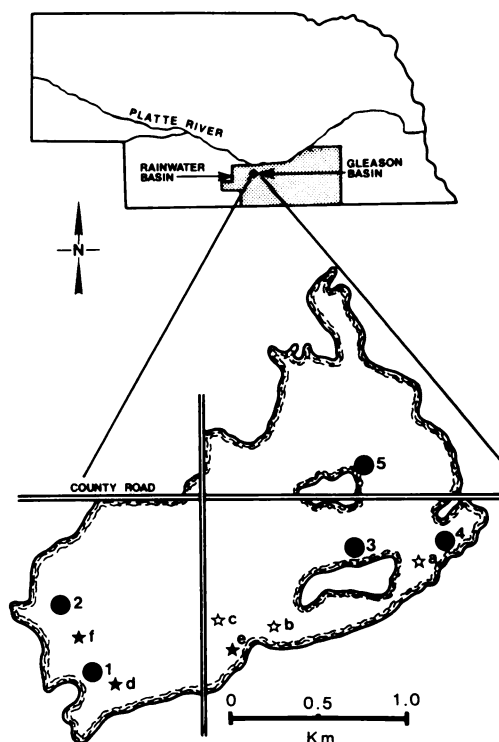

FIGURE 1.   Distribution of collection sites in Gleason Basin, Nebraska associated with carcasses (☆—scavenged: a, Canada goose; b and c, whitefronted geese. ★—intact: d, green-winged teal; e and f, mallards), and water collection sites not associated with carcasses (●—1, 2, 3, 4, 5).

carcasses became clinically ill but did not die within the 48-hr observation period. Twelve (5.7%) of these mice inoculated with water from sites 1, 2, and 3 (Fig. 1) died within 48 hr without appearing ill during the first 2 days of sampling. At site 1 deaths occurred in three of four mice inoculated with undiluted Basin water from day 1; at site 2 all four mice receiving undiluted water died as did all four mice receiving a 10^{-1} dilution of that water from day 1; at site 3, one of two mice inoculated with undiluted water from day 2 died. In addition, the single mouse inoculated with undiluted Basin sludge on day 1 died. None of the other

TABLE 1.   Recovery of *P. multocida* from mice injected with undiluted water samples associated with carcasses.

Test site	Condi-tions[*]	Collection time (day)						
		2	3 a.m.	3 p.m.	4	8	15	21
Scavenged								
a Canada goose	A/L	2/2[b]	2/2	2/2	2/2	NS[c]	0/2	NS
	B	2/2	1/2	NS	2/2	NS	NS	NS
b Whitefronted goose	A/R	2/2	0/2	0/2	0/2	NS	NS	NS
	B	0/2	NS	NS	NS	NS	NS	NS
c Whitefronted goose	A/R	2/2	0/2	0/2	0/2	0/2	NS	NS
	B	2/2	NS	NS	NS	NS	NS	NS
Intact								
d Green-winged teal	A/L	NS	2/2	2/2	2/2	0/2	0/2	0/2
	B	NS	1/2	1/2	0/2	NS	NS	NS
e Mallard	A/R	2/2	1/2	0/2	1/2	NS	NS	NS
	B	2/2	NS	NS	NS	NS	NS	NS
f Mallard	A/L	NS	0/2	0/2	0/2	0/2	0/2	NS
	B	NS	0/2	0/2	0/2	NS	NS	NS
Controls—Positive								
Dead								
Whitefronted goose—D[d]		4/4						
Mallard—D		1/2						
Whitefronted goose—T[d]		1/2						
Live								
Mallard—T		NS	2/2					

[*] A = Sample taken adjacent to carcass, B = 0.1-1 m from carcass, L = carcass left at site, R = carcass removed from site.
[b] No. dead mice/no. tested.
[c] No sample tested.
[d] D = nasal discharge cultured, T = trachea cultured.

mice inoculated with water or sludge samples from days 1, 2, 3, 8, or 15 at undiluted or 10^{-1}, 10^{-2}, or 10^{-3} dilutions died. Only undiluted sludge material was inoculated.

Nine (11%) of the 82 mice injected with carcass-associated water were sick but did not die. Although 33 (40%) mice (Table 1) died within 48 hr without any sign of clinical illness, the rest of the inoculates in that group appeared to be healthy. No sick mice were noted in either group after the morning collections on day 3; however, all ensuing mortality occurred within 48 hr. When sick mice were killed and cultured, no *P. multocida* organisms were isolated.

All remaining unaffected mice in each group were killed 48 hr post-inoculation.

Liver and lung tissue impressions made from sick mice had few free bacterial cells. Most of the bacteria present in two of these mice were in clumps and appeared to be engulfed by phagocytes. In contrast, however, myriads of extracellular, unclumped-, typical bipolar cells characteristic of *P. multocida* were observed in tissue impressions made from all of the mice that died. These mice were cultured, bacteria were isolated, tested biochemically, and positively identified as *P. multocida*, serotype 1.

Avian cholera organisms were re-

covered for 3 days from carcass-associated sites a and e (Fig. 1, Table 1). Thirteen (81%) of the 16 mice injected with water associated with the scavenged Canada goose carcass (a) were positive. Water samples taken from the site from which the scavenged whitefronted geese (b, c) were removed were positive only on day 2 (Table 1). Water samples associated with the intact green-winged teal (d) were positive on days 3 and 4, but not thereafter. Samples taken from the site from which the mallard (e) had been removed were positive on days 2, 3, and 4. In contrast, no *P. multocida* were isolated from water samples taken at the site of the intact mallard carcass (f). Skeletonized remains of this carcass were removed 2 wk later (about 1 mo after the bird had died), and *P. multocida* were isolated from the bone marrow. Upon completion of the water survival tests, the remainder of the Canada goose carcass, the two whitefronted goose and mallard carcasses that were removed from the test sites, and the positive control carcasses were necropsied. Liver, brain, or bone marrow were cultured and *P. multocida* serotype 1 was recovered from each bird.

DISCUSSION

The period during which avian cholera organisms could be recovered from water was shorter in our study than that reported in California where water collected from the shoreline of a small pond was infectious for mice for 3 wk after 100 snow goose carcasses had been removed (Rosen, 1969). Later, Titche (1979) reported recovery of *P. multocida* by mouse inoculation for 23 days after marsh water had been seeded with broth cultures of these bacteria and for 27–30 days from marsh water associated with either intact or scavenged carcasses.

The first samples in our study were collected during a snowstorm when air and water temperatures averaged 0 and 4 C,

respectively. During the next 2 days, air temperatures rose to 15 C, melting the 10–18 cm of snow that had accumulated during the storm, resulting in an estimated 25% dilution of water in the Basin.

The absence of *P. multocida* in water samples collected at Gleason Basin coincided with the subsidence of the die-off. Daily carcass collection from the Basin dropped from over 400 per week before water testing began to less than 50 per week immediately afterwards. As other wetlands became flooded from the melting snow, additional ponds appeared and fewer birds were seen using the Basin. The absence of *P. multocida* may have been caused by differences in weather conditions or melting snow may have diluted the water in Gleason Basin to such an extent that it was no longer possible to recover *P. multocida* by the sampling method used.

Shipment of samples is not considered to be a factor regarding isolation of *P. multocida* in this study. Samples collected on days 2, 3, and 4 were inoculated into mice within 2 hr of collection at a local site; no isolations were made from samples collected on days 3 or 4. Shipment of samples to Madison did not occur until day 8.

These observations suggested that a number of factors may contribute to the appearance and survival of avian cholera organisms in the environment. In our study, *P. multocida* were recovered from water collected from five of six sites directly associated with either scavenged or intact carcasses and from skeletal remains. Positive isolations were made from three of five non-carcass-associated sites. The number of positive isolations might have been greater for a longer period of time had a different method been used for collecting the water samples. Results from this study suggested that continued presence of infected carcasses or appearance and subsequent removal of infected carcasses from pond water assures survival of

virulent avian cholera organisms outside of the host. These findings emphasize the necessity for immediate carcass removal from die-off areas.

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BOOK REVIEW . . .

Manual of Procedures for the Salvage and Necropsy of Carcasses of the West Indian Manatee (*Trichechus manatus*), Robert K. Bonde, Thomas J. O'Shea, and Cathy A. Beck. Sirenia Project, U.S. Fish and Wildlife Service, Denver Wildlife Research Center, 412 N.E. 16th Ave., Room 250, Gainesville, Florida 32601, USA. Available from: National Technical Information Service, Springfield, Virginia 22161, USA. NTIS Document Number: PB83-255273. 1983. 175 pp., 3 tables, 23 figs., 6 appendices. Paper $17.50 US, Microfiche $4.50 US.

This is a well written, nicely illustrated and carefully edited paperback manual. It consists of six sections: 1. agency responsibilities, 2. carcass retrieval, 3. record keeping, 4. necropsy, 5. determining cause of death and 6. disposition of specimens. Even though only Section 4 deals with the actual necropsy procedure the others are important for completeness. The authors should be commended for their efforts to produce a manual that unfortunately will have very limited circulation. There are a few instances where a better selection of words or better arrangement of words would have enhanced the clarity of the manual. The first encounter with an unexplained abbreviation was annoying, for example, when AFA (Alcohol-Formalin-Acetic acid) was first encountered on page 19 and when GAA was encountered on page 26. The reviewer has never seen Glacial Acetic Acid referred to as GAA. There are other minor inconsistencies however they do not detract from the manual's worth. Appendix V is a very worthwhile addition.

In spite of a few "picky" criticisms the manual will be of tremendous help to anyone working or contemplating working with this or other sirenians.

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