BioOne COMPLETE

COMPARISON OF METHODS TO DETECT PASTEURELLA MULTOCIDA IN CARRIER WATERFOWL

Authors: Samuel, Michael D., Shadduck, Daniel J., Goldberg, Diana R., and Johnson, William P.

Source: Journal of Wildlife Diseases, 39(1) : 125-135

Published By: Wildlife Disease Association

URL: https://doi.org/10.7589/0090-3558-39.1.125

BioOne Complete (complete.BioOne.org) is a full-text database of 200 subscribed and open-access titles in the biological, ecological, and environmental sciences published by nonprofit societies, associations, museums, institutions, and presses.

Your use of this PDF, the BioOne Complete website, and all posted and associated content indicates your acceptance of BioOne's Terms of Use, available at www.bioone.org/terms-of-use.

Usage of BioOne Complete content is strictly limited to personal, educational, and non - commercial use. Commercial inquiries or rights and permissions requests should be directed to the individual publisher as copyright holder.

BioOne sees sustainable scholarly publishing as an inherently collaborative enterprise connecting authors, nonprofit publishers, academic institutions, research libraries, and research funders in the common goal of maximizing access to critical research.

Downloaded From: https://complete.bioone.org/journals/Journal-of-Wildlife-Diseases on 28 Mar 2024
Terms of Use: https://complete.bioone.org/terms-of-use

Journal of Wildlife Diseases, 39(1), 2003, pp. 125–135
© Wildlife Disease Association 2003

COMPARISON OF METHODS TO DETECT *PASTEURELLA MULTOCIDA* IN CARRIER WATERFOWL

Michael D. Samuel,[1,3] Daniel J. Shadduck,[1] Diana R. Goldberg,[1] and William P. Johnson[2]

[1] US Geological Survey, Biological Resources Division, National Wildlife Health Center, 6006 Schroeder Road, Madison, Wisconsin 53711, USA

[2] Texas Parks and Wildlife Department, 5900 W. Cemetery Road, Canyon, Texas 79015, USA

[3] Corresponding author (email: Michael_Samuel@usgs.gov)

ABSTRACT: We conducted laboratory challenge trials using mallard ducks (*Anas platyrhynchos*) to compare methods for detecting carriers of *Pasteurella multocida,* the bacterium that causes avian cholera, in wild birds. Birds that survived the initial infection were euthanized at 2–4 wk intervals up to 14 wk post challenge. Isolates of *P. multocida* were obtained at necropsy from 23% of the birds that survived initial infection. We found that swab samples (oral, cloacal, nasal, eye, and leg joint) were most effective for detecting carrier birds up to 14 wk post infection. No detectable differences in isolation were observed for samples stored in either 10% dimethysulfoxide or brain heart infusion broth. The frequency of detecting carriers in our challenge trials appeared to be related to mortality rates observed during the trial, but was not related to a number of other factors including time after challenge, time delays in collecting tissues postmortem, and route of infection. In our trials, there was little association between antibody levels and carrier status. We concluded that swabs samples collected from recently dead birds, stored in liquid nitrogen, and processed using selective broth provide a feasible field method for detecting *P. multocida* carriers in wild waterfowl.

Key words: *Anas platyrhynchos,* avian cholera, carrier, isolation, mallard, *Pasteurella multocida.*

INTRODUCTION

Avian cholera, caused by the bacterium *Pasteurella multocida,* is the most important infectious disease affecting North American waterfowl (Friend, 1999). The disease occurs almost annually as an acute epizootic at waterfowl concentration areas in the Central Valley of California, the Rainwater Basin of Nebraska, areas of Texas and Minnesota, and western Canada (Wobeser et al., 1982; Botzler, 1991). During avian cholera epizootics it is not uncommon for thousands of waterfowl to die (Brand, 1984; Botzler, 1991), with mortality from severe epizootics exceeding 20,000 birds (Brand, 1984; Friend, 1999; Samuel et al., 1999a).

Although there are 16 different serotypes of *P. multocida* (Rimler et al., 1984), type 1 is principally responsible for waterfowl mortality throughout the Pacific, Central, and Mississippi flyways of North America (Brogden and Rhoades, 1983; Windingstad et al., 1983; Hirsh et al., 1990; Wilson et al., 1995). White-fronted geese (*Anser albifrons*) and lesser snow geese (*Chen chen caerulescens*) appear to be disproportionately represented in outbreaks, but other species such as northern pintail (*Anas acuta*) and mallards (*Anas platyrhynchos*) are recovered regularly during epizootics (Samuel, unpubl. data). Two hypotheses have been proposed to explain the recurrence of avian cholera epizootics: 1) *P. multocida* persists year-round in the water, soil, or other reservoirs at specific wetlands and 2) carrier birds reintroduce the disease at migratory bird staging and wintering areas. Previous wetland studies have been inconclusive, but generally do not provide strong support for the hypothesis that wetlands serve as the reservoir for avian cholera epizootics (Botzler, 1991). In contrast, researchers have argued that carrier birds (apparently healthy birds with virulent *P. multocida*) may serve as reservoirs for the bacteria and initiate epizootics by infecting other birds as they congregate on breeding and wintering grounds. Following epizootics in breeding snow geese on Banks Island, approximately 8% of the birds sampled had

Downloaded From: https://complete.bioone.org/journals/Journal-of-Wildlife-Diseases on 28 Mar 2024
Terms of Use: https://complete.bioone.org/terms-of-use

detectable antibody to *P. multocida* serotype 1, indicating recent infection (Samuel et al., 1999a), and an estimated 50% of these infected birds survived the disease (Samuel et al., 1999b). However, serologic techniques may be useful for measuring infection from *P. multocida,* but are inadequate to detect carriers because birds may develop antibodies without becoming carriers (Rimler and Rhoades, 1989; Donnio et al., 1994) and antibodies may only remain elevated for a few months following infection (Samuel et al., 1999b). Thus, determining prevalence of carriers using isolation techniques, the relationship between antibody detection and carrier status, and the role of carriers in avian cholera epizootics remain essential steps in determining how avian cholera is spread and understanding the ecology of this disease (Samuel et al., 1999a, b).

Previous researchers have recovered *P. multocida* from a number of tissues of apparently healthy birds (see Botzler, 1991). Oral and nasal swabs have been used to detect carriers among domestic fowl (Pritchett and Hughes, 1932; Iliev et al., 1965; Heddleston, 1975). In addition, Heddleston (1975) recommended the use of cotton swabs under the eyelid as a technique to find *P. multocida* in carrier birds. In a study of domestic ducks and geese, Muhairwa et al. (2000) found birds had *P. multocida* in their pharynx and cloaca. Among wild birds, the bacterium has been isolated from livers and spleens of American coots (*Fulica americana*) from Missouri (Vaught et al., 1967), the oropharynx of one of 357 nesting common eiders (*Somateria mollissima*) from Maine (Korschgen et al., 1978), lungs of 16 of 172 wild waterfowl and 15 of 37 California gulls (*Larus californicus*) (Titche, 1979), and the pharyngeal swabs of arctic nesting lesser snow geese (Samuel et al., 1999b). However, most of the isolates recovered were not serotyped or tested for pathogenicity, therefore little is known about which tissues are most likely to harbor pathogenic *P. multocida* serotype 1 in apparently healthy wild birds. As a result, appropriate methods for determining the carrier status of birds, especially waterfowl, have been a substantial barrier in validating the carrier hypothesis and subsequently understanding the role of carrier birds in this disease.

The objective of our study was to evaluate different tissue samples and isolation methods for detecting *P. multocida* in carrier waterfowl. We also evaluated different preservation methods, time delays in processing tissues postmortem, time of sample collection post-infection, and route of infection to compare their effect on detecting low densities of *P. multocida* likely to occur in carrier birds. Our goal was to optimize our ability to recover the bacteria from field samples collected from wild birds in future studies.

MATERIALS AND METHODS

One hundred and twenty 6–8 wk old male mallards obtained from Whistling Wings, Inc. (Hanover, Illinois, USA) were banded and separated into four groups of 30 birds and housed in separate isolation rooms (approximately 22 m^2) at the National Wildlife Health Center (NWHC). Birds were habituated to the isolation rooms for 1–3 wk prior to challenge. In the experimental design for our challenge study, each isolation room represented a separate trial. For each of our four trials, two groups of 10 birds were injected subcutaneously in the dorsal caudal region of the neck with 0.2 ml of a logarithmic growth phase culture of *P. multocida* serotype 1. The eight isolates (two per isolation room) used in the challenge trials were recovered from sediment and water samples from different wetlands during previous avian cholera outbreaks (Table 1). Ten birds in each room (nine in Room 3) served as unchallenged, contact controls to determine if *P. multocida* was transmitted among birds. To promote higher survivorship in challenged ducks and increase the likelihood of carrier birds, isolates used in the challenge study were of lower virulence (killed 0–50% of birds in previous challenge trials) than most of our field isolates (Samuel, unpubl. data). The four trials were conducted sequentially, with approximately 1 wk separation, except for the last two trials in Rooms 3 and 4, which began simultaneously.

One third of the ducks were randomly se-

Downloaded From: https://complete.bioone.org/journals/Journal-of-Wildlife-Diseases on 28 Mar 2024
Terms of Use: https://complete.bioone.org/terms-of-use

Downloaded From: https://complete.bioone.org/journals/Journal-of-Wildlife-Diseases on 28 Mar 2024
Terms of Use: https://complete.bioone.org/terms-of-use

TABLE 1. Source of isolate, challenge dose, challenge date, mortality, isolate recovery, and antibody response of surviving mallard ducks experimentally challenged with *Pasteurella multocida*.

Room	Group	Isolate source	Challenge titer ($\times 10^4$ colony forming units)	Challenge date	Mortality[a]	*P. multocida*[b]		ELISA[c]	
						n	Positive	*n*	Positive
6	1	Los Banos WMA[d], Unit GW-3, California	110.00	08/22/2000	40%	6	1	6	6
	2	Colusa NWR[e], California	118.00	08/22/2000	50%	5	2	5	5
	Control 6	NA[f]	NA	NA	10%	9	2	9	8
5	3	Kesterson NWR, California	4.42	08/29/2000	20%	8	3	8	8
	4	Merced NWR, California	11.40	08/29/2000	30%	7	1	7	6
	Control 5	NA	NA	NA	20%	9[g]	1	9[g]	5
3	5	Rainwater Basin, Nebraska	1.86	09/05/2000	20%	7[h]	0	7	4
	6	West Bear Creek NWR, California	2.04	09/05/2000	20%	8	0	8	6
	Control 3	NA	NA	NA	0%	9[i]	0	9	1
4	7	San Luis NWR, California	4.86	09/05/2000	20%	8	2	8	8
	8	Los Banos WMA, Unit GW-1, California	5.78	09/05/2000	10%	9	1	9	9
	Control 4	NA	NA	NA	50%	5	0	5	4

[a] 10 birds in each group except Control 3 where nine birds were tested.
[b] Number of surviving birds where *Pasteurella multocida* was recovered from nasal, oral, and/or cloacal swabs collected from live birds.
[c] Seroconversion based on enzyme-linked immunosorbent assay (ELISA) value % of serum collected at 2, 9, or 10 wk post-challenge or at necropsy.
[d] WMA = Waterfowl Management Area.
[e] NWR = National Wildlife Refuge.
[f] NA = not applicable.
[g] One bird died a few days following sampling at 2 wk.
[h] One bird was found sick with signs other than avian cholera; it was subsequently euthanized and removed from the study.
[i] One bird died before challenge was initiated.

lected and serologically tested for serum antibodies to *P. multocida* prior to challenge trials to confirm there was no previous infection of the flock with the bacterium. Surviving birds were also tested 2 wk post-challenge to determine if seroconversion occurred, indicating they had been infected with *P. multocida.* Blood samples were divided into two equal volumes and one aliquot was centrifuged (450×G) within 2 hr following collection. For half of the birds, the remaining aliquot was centrifuged at 8 hr, and for the other half at 12 hr following collection, to determine if serum antibody titer deteriorated postmortem. These blood samples were refrigerated at approximately 5 C until centrifugation, then serum was removed and frozen (−20 C) until an enzyme-linked immunoassay (ELISA) was conducted to measure antibodies.

Serum antibody against *P. multocida* serotype 1 was measured using the ELISA procedure described by Samuel et al. (1999b) with the following modifications. The *P. multocida* cells used to prepare a new lot of antigen were disrupted using a French press and the disrupted cells were passed through the French press a second time producing a concentration of 432 μg/ml as determined by the Bradford/BioRad micro protein assay (Bradford, 1976). The ELISA procedure was optimized following the methods described by Crowther (2001), providing serum dilutions of 1:100, conjugate dilution of 1:300, and substrate incubation time of 45 min. The 96-well Falcon 3915 Pro-Bind assay microtitration plate was replaced by F96 Polysorp Nunc-immuno plate (Nalge Nunc International, Rochester, New York, USA). The final concentration of antigen for the microtitration plate was 8.3 ng per well. Absorbances (optical density) were obtained on a Biokinetics reader, model EL800-PC, (Bio-Tek instruments, Inc., Winooski, Vermont, USA). Sera from captive snow geese with negative and low positive antibody pools used to standardize the ELISA were increased from four to 10 birds. The negative control pool consisted of pre-vaccination sera and the low positive control pool consisted of sera taken 8 wk post-vaccination (Samuel et al., 1999b). Several minor procedural changes were also implemented in the ELISA to reduce within plate and between plate variations. Due to these modifications in the ELISA methods and the use of new reagents, we compared the results obtained by Samuel et al. (1999b) for Bank's Island snow geese to the present ELISA. We selected 96 snow goose sera with ELISA value percent (EV%) levels ranging above and below the cut-off for indicating a positive level of *P. multocida* antibody.

For each of the four challenge trials, swab samples (nasal, oral, cloacal) were collected periodically (approximately 2–3 wk intervals) from surviving birds for 9–12 wk post-challenge to determine if shedding of *P. multocida* occurred. Beginning 4 wk post-challenge, and at 1–4 week intervals, approximately 25% of the surviving birds in each trial (approximately five birds per room, including contact control birds) were euthanized and tissues were collected to test for the presence of *P. multocida.* All birds were euthanized by cervical dislocation. Carcasses were refrigerated (approximately 5 C) postmortem, until dissection. Birds were dissected and tissues collected at one of three time intervals (2–4 hr, 4–8 hr, or 8–12 hr) postmortem, to determine whether a delay in processing carcasses affected successful isolation of *P. multocida* from carrier birds. Immediately prior to tissue sampling, blood was collected by heart puncture and centrifuged as soon as possible (within 2–6 hr after collection) and the serum frozen (-20 C) until antibody testing by ELISA was conducted. Initially, tissue samples of the liver, spleen, lung, trachea, bone marrow, appendix, Meckel's diverticulum, brain, and swabs of the leg joint, cloaca, surface of the eye, and nasal cleft were collected from each carcass for *P. multocida* isolation. Beginning at 6 and 10 wk post-challenge respectively, swabs of the oral cavity and cecal tonsil tissues were also collected. Ten wk after the start of the trials, we decided to discontinue collection and processing of fresh tissues of the liver, spleen, lung, bone marrow, trachea, appendix, and Meckel's diverticulum. Small sections of each tissue (approximately 0.1 g) were collected and put into cryovials containing 1.25 ml of either brain heart infusion broth (BHI) or 10% dimethylsulfoxide (DMSO) and pulverized using the blunt end of two plastic handled cotton swabs. Two swabs from each of the leg joint, nasal cleft, cloaca, oral cavity, and surface of eye were collected and stored in BHI (1–2 ml) and DMSO (1–2 ml) by snapping the tip of the swab off into cryovials containing each media. Each of the tissue and swab samples were processed within 2–4 hr after necropsy (fresh samples) by removing some of the sample liquid from the vial with a cotton swab, swabbing 1/5 of a blood agar plate (BAP), and streaking for bacterial isolation. After incubating for 20–24 hr at 37 C with 5–10% CO_2 the BAP were examined for colonies similar in appearance to *P. multocida.* Suspect *P. multocida* colonies were identified based on colony morphology and further examined by Gram stain. *Pasteurella multocida*-like, Gram-negative isolates were serotyped using the agarose gel precipitin (AGP) test (Heddleston et al., 1972), and identified

Downloaded From: https://complete.bioone.org/journals/Journal-of-Wildlife-Diseases on 28 Mar 2024
Terms of Use: https://complete.bioone.org/terms-of-use

using the analytical profile index (API) 20E identification system (bioMerieux, St. Louis, Missouri, USA) or confirmed based on the typical fermentation pattern of six sugars (dextrose +, lactose −, maltose ±, sucrose +, mannitol +, and dulcitol −). Vials containing the remaining swabs or tissues were stored in liquid nitrogen (-210 C) until processing (frozen samples). A comparison of isolations obtained from fresh and frozen samples was used to determine what effect liquid nitrogen storage and selective enrichment had on *P. multocida* isolation in our tissue and swab samples.

Frozen samples were thawed at approximately 20 C and the full contents (1.25 ml media plus sample volume) in each vial were emptied into a culture tube containing 5 ml of BHI broth. Each tube was incubated for 2 hr at 37 C with shaking (100 revolutions per minute) for pre-enrichment. Following incubation, 2 ml of the broth was transferred to a tube containing 5 ml of *Pasteurella multocida* selective media (PMSB) (Moore et al., 1994) and incubated for 12–16 hr at 37 C with 5–10% CO_2. Following this second incubation, the tubes were thoroughly mixed and 1/5 of a BAP was swabbed and streaked for bacterial isolation, incubated, and suspect *P. multocida* colonies were identified by Gram stain and API 20E, as indicated above for fresh samples.

We used logistic regression in LogXact (Mehta and Patel, 1996) to analyze the proportion of euthanized mallard ducks that had *P. multocida* recovered from swabs or tissues (carriers). Analyses were conducted using covariates to determine the effects that different experimental variables might have on the proportion of ducks determined to be carriers. These covariates included: challenged vs. contact control birds, different trials (isolation rooms), weeks since challenge, postmortem time until dissection, and seroconversion at time of necropsy. We used kappa statistics (Agresti, 1996) to assess the degree of agreement between carrier and seroconversion status of ducks at necropsy. A paired *t*-test (Zar, 1984) was used to compare ELISA titers of the initial sample to sera processed at 8 and 12 hr following collection from birds 2 wk post-challenge. We used Pearsons's correlation and kappa statistics (Agresti, 1996) to assess the similarity of antibody titers from Banks Island snow geese reported by Samuel et al. (1999b) with results obtained from our revised ELISA procedures.

RESULTS

Of the 120 mallards obtained for the laboratory challenge study, three birds with health problems or mortality unrelated to avian cholera were excluded from our analyses. One additional mallard that showed no obvious signs of illness was euthanized on its assigned schedule (5 wk post-challenge); numerous lesions were found and the bird was diagnosed with amyloidosis. This bird yielded isolates of *P. multocida* from numerous tissues, so it was not excluded from our analyses.

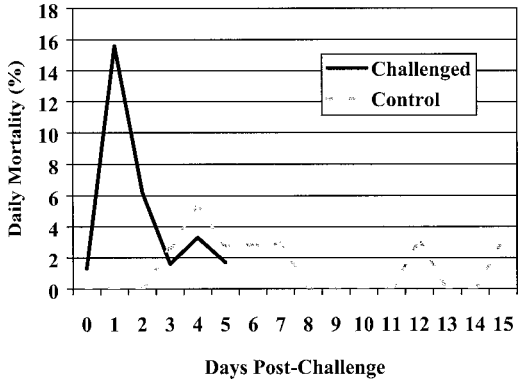


FIGURE 1. Daily mortality (%) of mallard ducks challenged with *Pasteurella multocida* and contact control birds, 0–15 days post-challenge.

Mortality in the challenged groups occurred rapidly, most birds died within 36 hr after inoculation, and all deaths occurred within 5 days post-challenge. By contrast, birds in the contact control groups were unaffected for 2–4 days post-challenge and had a more prolonged period of mortality (3–15 days post-challenge), with most deaths occurring 3–7 days after challenge (Fig. 1). Mortality of ducks challenged with *P. multocida* isolates varied from 10–50% and contact control groups experienced mortality ranging from 0–50% (Table 1). In Room 4, a higher proportion of ducks in the contact control group died compared to challenged ducks. The total mortality was slightly higher for challenged birds (26%) than contact control birds (21%). *Pasteurella multocida* serotype 1 was isolated from all the livers of ducks that died, except the bird diagnosed with amyloidosis. Isolates were confirmed with either API 20E or based on the typical sugar fermentation pattern.

We recovered *P. multocida* serotype 1

Downloaded From: https://complete.bioone.org/journals/Journal-of-Wildlife-Diseases on 28 Mar 2024
Terms of Use: https://complete.bioone.org/terms-of-use

TABLE 2. Recovery of *Pasteurella multocida* from challenged and contact control mallard ducks dissected at different elapsed times following euthanasia.

	Challenged ducks		Control ducks		Total	
Elapsed time	*n*	Positive	*n*	Positive	*n*	Positive
2–4 hr	20	25%	8	38%	28	29%
4–8 hr	25	20%	17	18%	42	19%
8–12 hr	10	10%	6	50%	16	25%
Unknown	2	0%	0	0%	2	0%
Total	57	19%	31	29%	88	23%

using swabs from 13 (14%) of 90 live mallards (Table 1) sampled periodically after challenge. Most isolates were obtained from swab samples collected 2 wk post-challenge, our first sampling period. One bird also was culture positive at 4 wk post-challenge, with isolates from oral and nasal swabs at 2 and 4 wk sample periods. Overall, 16 isolates were recovered from swab samples of live birds: six oral swabs, three nasal swabs, and seven cloacal swabs. Isolates were recovered from most of the treatment groups, except those in Room 3 and the contact control group in Room 4. The prevalence of *P. multocida* shedders was higher in the challenged birds (17%) than the contact control birds (9%). Isolates from these 13 birds were also confirmed using sugar fermentation patterns.

We recovered *P. multocida* serotype 1 (confirmed with API 20E) from 23% of the euthanized ducks that survived challenge (Tables 2, 3). We isolated *P. multocida* from 19% of the challenged birds and 29% of the contact control birds. Birds dissected at 2–4 hr, 4–8 hr, or 8–12 hr postmortem had similar rates of *P. multocida* recovery (Table 2). The proportion of euthanized ducks with *P. multocida* isolates was fairly evenly distributed post-challenge (Table 3). Logistic regression indicated that isolation room ($P=0.04$) was the only covariate that explained differences in the frequency of ducks where *P. multocida* were isolated at necropsy. The bacterium was recovered from 0 (0%) of 24 birds in Room 3, 2 (9%) of 23 birds in Room 5, 8 (40%) of 20 birds in Room 6, and 10 (48%) of 21 birds in Room 4. None of the remaining covariates explained differences in the proportion of carriers detected: challenged vs. contact control birds ($P=0.30$), weeks since challenge ($P=0.88$), postmortem time until dissection ($P=0.67$), or seroconversion ($P=0.72$).

Oral swabs frozen in liquid nitrogen and processed in PMSB accounted for 16 (80%) of the 20 culture positive birds (Table 4) euthanized post-challenge, with isolates from swabs stored in BHI (12 of 82 birds) recovered as frequently as those preserved in DMSO (14 of 83 birds). We also isolated *P. multocida* from the brain and several swabs (cloaca, nares, eye, and leg joint) of one of these 16 ducks. From the other four positive birds, isolates were

TABLE 3. Proportion of challenged and contact control mallard ducks with *Pasteurella multocida* isolated from euthanized birds 4–14 wk post-challenge.

Weeks post-challenge	Challenged ducks		Control ducks		Total	
	n	Positive	*n*	Positive	*n*	Positive
4–6	15	27%	10	30%	25	28%
9–11	20	14%	9	33%	29	21%
12–14	22	18%	12	25%	34	21%
Total	57	19%	31	29%	88	23%

Downloaded From: https://complete.bioone.org/journals/Journal-of-Wildlife-Diseases on 28 Mar 2024
Terms of Use: https://complete.bioone.org/terms-of-use

TABLE 4. *Pasteurella multocida* isolates from euthanized mallard ducks. Samples were preserved in either brain heart infusion broth (BHI) or 10% dimethylsulfoxide (DMSO) and processed either fresh or frozen.

Sample type	Processing and preservation methods			
	Fresh BHI	Fresh DMSO	Frozen BHI	Frozen DMSO
Leg joint swab	88[a] (1)[b]	88 (0)	88 (1)	88 (0)
Cloacal swab	88 (2)	88 (1)	88 (2)	88 (3)
Eye swab	87 (1)	87 (1)	87 (1)	87 (1)
Oral swab	82 (0)	83 (0)	82 (12)	83 (14)
Nasal swab	88 (1)	88 (1)	88 (2)	88 (2)
Liver tissue	25 (0)	25 (0)	88 (0)	88 (0)
Spleen tissue	24 (0)	24 (0)	87 (0)	87 (0)
Lung tissue	25 (0)	25 (0)	88 (1)	88 (0)
Tracheal tissue	25 (0)	25 (0)	88 (0)	87 (0)
Bone marrow tissue	25 (1)	25 (0)	88 (0)	88 (0)
Appendix tissue	25 (0)	25 (0)	88 (0)	88 (0)
Meckel's diverticulum tissue	25 (1)	25 (1)	88 (1)	88 (1)
Brain tissue	88 (0)	88 (1)	88 (2)	88 (2)
Cecal tonsil tissue	63 (0)	63 (0)	63 (0)	63 (0)
Total	758 (7)	759 (5)	1199 (22)	1198 (23)

[a] Number of swabs or tissues processed.
[b] Number of swabs or tissues yielding *P. multocida* isolates.

obtained from the cloacal swab of one bird, the nasal swabs of two birds, and numerous tissues (lung, Meckel's diverticulum, brain, and cloacal swab) of the bird that was diagnosed with amyloidosis. *Pasteurella multocida* was not isolated from any of the other tissues collected (liver, spleen, appendix, and cecal tonsil). There appeared to be little association between culture results from swabs collected from live birds and samples from subsequently euthanized birds. Four of the 13 live birds that were culture positive from swabs at 2 wk post-challenge were also positive for *P. multocida* at necropsy, 4–9 wk post-challenge. At necropsy, *P. multocida* were recovered from numerous tissues of two of these birds. Isolates from the other two birds at necropsy were from oral swabs; however, bacteria were isolated from other swab samples at 2 wk post-challenge. We were unable to isolate the bacteria from the remaining nine live sample culture positive birds at the time of necropsy, 5–14 wk post-challenge. Similarly, we were unable to isolate bacteria from swab samples at 2 wk from 16 of the live ducks that were culture positive at necropsy.

Processing of fresh tissues and swabs was used to determine what effect liquid nitrogen storage had on *P. multocida* isolation in our tissue and swab samples. With the exception of the bird diagnosed with amyloidosis, which produced *P. multocida* isolates from fresh samples of bone marrow, Meckel's diverticulum, and brain tissues, we were unable to isolate *P. multocida* from fresh tissues. Comparison between different methods (fresh vs. frozen samples) indicated that processing frozen samples using PMSB selective enrichment was more effective than processing fresh samples without PMSB for recovering *P. multocida* isolates from carcasses (Table 4). This was especially true for frozen oral swabs, which had the highest recovery rate of *P. multocida* isolates.

We found significant correlation ($r=0.85$, $n=96$, $P<0.0001$) between our previous antibody titer levels measured for the 96 Bank's Island snow geese (Samuel et al., 1999b) and antibody titers from the ELISA we used for mallards. Using a threshold ELISA of 17.5 EV% to indicate seroconversion provided good agreement ($\kappa=0.83$) between the two ELISA procedures. This threshold produced a slightly lower seropositive rate, 47% positive for

Downloaded From: https://complete.bioone.org/journals/Journal-of-Wildlife-Diseases on 28 Mar 2024
Terms of Use: https://complete.bioone.org/terms-of-use

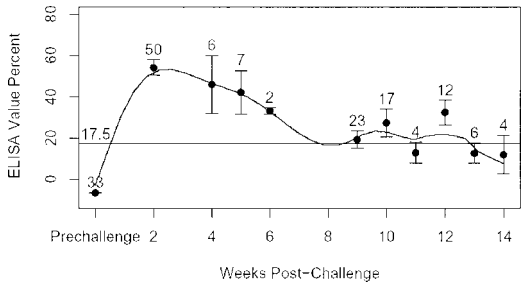


FIGURE 2. Antibody titer as ELISA value percent (EV%) for challenged mallard ducks pre- and post-challenge. Circles indicate mean estimate and sample size is indicated on top±1 standard error bars. Spline fit to the EV% data and positive threshold level (17.5 EV%).

the new ELISA compared with 49% for the original ELISA for snow geese. The paired *t*-test on ELISA results for sera processed at different times following collection from euthanized birds showed that initial EV% levels were significantly (P=0.001, n=22) lower by 1.6 EV% than those processed after 8 hr. However, there was no difference (P=0.91, n=21) between the initial EV% levels and sera that were held for 12 hr.

Pre-challenge antibody titers were negative for all 40 birds sampled (range −7.9 EV% to 0.6 EV%). In challenged birds, antibody titers increased dramatically from pre-challenge levels to 2 wk post-challenge (54 EV%) and slowly decreased during the remainder of the study (Fig. 2). In contrast, the antibody response of the contact control birds was lower at 2 wk post-challenge (22 EV%), slower to peak (64 EV% at 4 wk), and was more variable at the end of the study than the antibody response in challenged birds. By 13 to 14 wk post-challenge antibody response in many of the challenged birds was lower than our positive threshold level of 17.5 EV%. Higher titers in some contact control birds occurred later in the study and likely reflected later infection of susceptible birds. Antibody titers of mallards surviving challenge indicated that challenged birds seroconverted with greater frequency (52 positive of 58 sampled, 90%) than the contact control birds (18 of 32 positive, 56%) over the course of the experiment (Table 1). Four (8%) of 50 challenged birds had not seroconverted after 2 weeks, and two (3.8%) of these were still seronegative at necropsy. In contrast, 14 (47%) of 30 contact control birds were seronegative 2 wk post-challenge, and 10 of these birds (33%) remained seronegative at necropsy. In Room 3 only one of nine contact control birds seroconverted during the study. We found little association between seroconversion and detection of carriers for birds at necropsy (κ=0.12). We detected *P. multocida* in 14 (29%) of the 49 birds that were seropositive and in 6 (15%) of the 39 birds that were seronegative at necropsy. Of the 39 birds that were seronegative at necropsy, 20 (61%) of 33 sampled 2 wk post-challenge were seropositive, but antibody levels had declined below our positive threshold by the time of necropsy.

DISCUSSION

Using field isolates of *P. multocida* with relatively low virulence and low concentrations of bacteria in our inoculation doses, we successfully challenged mallard ducks without causing overwhelming mortality. These birds transmitted bacteria to contact control mallards housed in the same isolation room. Swab samples from challenged and contact control birds indicated that transmission probably occurred by both oral and cloacal shedding. Mortality in contact control birds began within 3 days following inoculation of challenged birds, indicating that transmission occurred rapidly post-challenge. Contact control birds died over a more prolonged period than challenged birds, indicating either infection with a smaller quantity of the *P. multocida* bacteria and/or that transmission of bacteria to contact control birds occurred over an extended period of time. Some of the contact control birds seroconverted late in the study, especially in Room 5, indicating extended transmission of bacteria. Many of the contact control birds that survived remained seronegative

Downloaded From: https://complete.bioone.org/journals/Journal-of-Wildlife-Diseases on 28 Mar 2024
Terms of Use: https://complete.bioone.org/terms-of-use

throughout the study (44%), indicating they may not have been infected with *P. multocida.* In contrast, ≥90% of the challenged birds became seropositive during the trial.

We isolated *P. multocida* from both challenged and contact control birds from 4–14 wk post-challenge providing evidence that birds can be carriers of the agent that causes avian cholera. Because our study was terminated at 14 weeks, we are uncertain how long birds can remain carriers of *P. multocida.* Previous authors (Pritchett et al., 1930a, b; Pritchett and Hughes, 1932; Wobeser, 1997) have suggested that birds can remain carriers for their lifetime. We detected carriers in approximately 20% of our challenged and contact control birds using a variety of tissue and swab samples and several different preservation and culture methods. We found little association between detection of carriers and serconversion of birds at necropsy, thus we suspect that antibody levels are not a reliable indicator of carrier status in wild birds. Antibody levels declined within 3–4 mo following infection; however, we found no indication that the proportion of carriers declined during this time period. Antibody levels may be useful to determine the incidence of infection in wild birds, but this does not appear to be a valid measure of carrier prevalence.

Logistic regression analysis showed that difference among isolation rooms was the only significant factor affecting our ability to recover the bacteria, and recovery was not substantially affected by challenge or control status, the number of weeks post-challenge that birds were tested, or delays of <12 hr in conducting dissections of euthanized birds. Some of these findings were contrary to our expectations. We predicted detection of carrier birds would decline with the amount of time after infection. However, it may be that the length of our study was too short for a substantial reduction in the prevalence of carrier birds or that sample sizes used in our study were insufficient to document a minor reduction in prevalence of carriers. We also predicted that detection of carriers might be affected by delays in dissecting birds. We were encouraged to find that delays of 8–12 hr did not adversely affect our ability to detect carriers. This result should facilitate obtaining suitable samples for detecting *P. multocida* carriers from birds collected under field conditions. We hoped that detection of carriers would be similar between challenged and contact control birds because this would indicate that birds challenged by injection were similar to birds infected by "natural" transmission. While this prediction was supported, interpretation of the results may be complex because a large portion of the contact control birds never seroconverted, and therefore may never have become infected with *P. multocida.* Despite the similarity in detection rates between challenged and contact control birds, it should be recognized that we used an unnatural route of infection that may have affected our results.

Finally, we expected the frequency of carriers would be related to the virulence of isolates used in the challenge trial. In particular, previous authors have speculated that less virulent isolates, which may occur near the end of outbreaks, would produce more sick birds and birds which survive to become carriers (Rosen and Bischoff, 1949; Rosen and Morse, 1959; Botzler, 1991). We found significant differences in detection of carriers among the trials conducted in different isolation rooms. Trials with the highest rates of mortality from *P. multocida* had the highest prevalence of carriers. In contrast, trials with the lowest rates of mortality had the lowest prevalence of carriers. Perhaps birds are able to completely clear isolates of lower virulence from their tissues or more virulent isolates create a higher frequency of carriers among birds that survive infection. These results appear contrary to the prediction that less virulent isolates produce more carriers and we believe further research is needed on this subject. However, two cautions should be considered in in-

Downloaded From: https://complete.bioone.org/journals/Journal-of-Wildlife-Diseases on 28 Mar 2024
Terms of Use: https://complete.bioone.org/terms-of-use

terpreting our results: 1) different challenge doses used in our trials (especially Room 6) make direct comparison of virulence difficult and 2) we purposely selected *P. multocida* isolates of lower virulence.

We found that swab samples (oral, nasal, cloacal, eye, and leg joint) preserved in liquid nitrogen using either BHI or DMSO and cultured in PMSB provided a successful method for recovering *P. multocida* isolates. This result was auspicious because swab samples are much easier to collect from wild birds than tissue samples. Swab samples are also easier to preserve and culture in the laboratory than tissue samples. In addition, the minor delays (<8–12 hr) required in collecting samples from dead birds in the field did not appear to adversely affect our ability to detect carriers. We suspect the difficulty we had in isolating *P. multocida* from fresh tissues and swab samples occurred because we did not use PMSB to enrich these samples. Some delay in processing blood samples from carcasses may provide a decline in measured antibody titer, but the magnitude of this change appeared of little consequence unless birds had antibody titers near our positive threshold level. To maximize the opportunity to detect carriers in wild birds, we recommend that tissue and swab samples are collected and preserved as soon as possible after mortality and delays of >8 hr in processing should be avoided.

Although *P. multocida* isolates have been recovered from wild waterfowl (see Botzler, 1991; Wobeser, 1992), only Samuel et al. (1997) determined the serotypes of the isolates and demonstrated that the isolates were virulent. Samuel et al. (1997) reported recovering only a single *P. multocida* serotype 1 isolate from >3,400 adult snow geese (Samuel et al., 1999b). Based on results presented in this study, we suspect the sampling and preservation methods used by Samuel et al. (1997) were insufficient to insure adequate isolation of *P. multocida* in carrier birds. We suspect the sampling and preservation methods developed in this study will fail to detect all birds that may be carriers. For example, we isolated *P. multocida* from approximately 20% of the ducks that survived challenge. At present we are uncertain whether many survivors were able to clear all the bacteria from their tissues or whether we were simply unable to detect the bacterium using our sampling, preservation, and culturing methods.

ACKNOWLEDGMENTS

We appreciate the dedicated assistance of many individuals who contributed to this project. J. Hayden, L. Baeten, E. Murphy, and R. La Rose assisted with the laboratory challenge trials. Funding for this study was provided by the US Fish and Wildlife Service, the Playa Lakes Joint Venture, Texas Parks and Wildlife, and the US Geological Survey. J. Ray played an important role in initiating the study. D. Joly, R. Botzler, and M. Lehr provided comments on the draft manuscript.

LITERATURE CITED

AGRESTI, A. 1996. An introduction to categorical data analysis. John Wiley & Sons, Inc., New York, New York, 290 pp.

BOTZLER, R. G. 1991. Epizootiology of avian cholera in waterfowl. Journal of Wildlife Diseases 27: 367–395.

BRADFORD, M. M. 1976. A rapid and sensitive method for quantitation of microgram quantities of protein using the principal of dye binding. Annals of Biochemistry 72: 248–254.

BRAND, C. J. 1984. Avian cholera in the central and Mississippi flyways during 1979–80. Journal of Wildlife Management 48: 399–406.

BROGDEN, K. A., AND K. R. RHOADES. 1983. Prevalence of serologic types of *Pasteurella multocida* from 57 species of birds and mammals in the United States. Journal of Wildlife Diseases 19: 315–320.

CROWTHER, J. R. 2001. Methods in molecular biology, Vol. 149. The ELISA guidebook. Humana Press, Totowa, New Jersey, 421 pp.

DONNIO, P. Y., C. L. GOFF, J. L. AVRIL, P. POUEDRAS, AND S. GRAS-ROUZET. 1994. *Pasteurella multocida*: Oropharyngeal carriage and antibody response in breeders. Veterinary Research 25: 8–15.

FRIEND, M. 1999. Avian cholera *In* Field manual of wildlife diseases, general field procedures and diseases of migratory birds, M. Friend and J. C. Franson (eds.). US Geological Survey, Information and Technology Report 1999–001, pp. 74–92.

Downloaded From: https://complete.bioone.org/journals/Journal-of-Wildlife-Diseases on 28 Mar 2024
Terms of Use: https://complete.bioone.org/terms-of-use

HEDDLESTON, K. L. 1975. Pasteurellosis *In* Isolation and identification of avian pathogens. S. B. Hitchner, C. H. Domermuth, H. G. Purchase and J. E. Williams (eds.). American Association of Avian Pathologists, Arnold Printing Corporation, Ithaca, New York, pp. 38–51.

———, J. E. GALLAGER, AND P. A. REBERS. 1972. Fowl cholera: Gel diffusion precipitin test for serotyping *Pasteurella multocida* from avian species. Avian Diseases 16: 925–936.

HIRSH, D. C., D. A. JESSUP, K. P. SNIPES, T. E. CARPENTER, D. W. HIRD, AND R. H. MCCAPES. 1990. Characteristics of *Pasteurella multocida* isolated from waterfowl and associated avian species in California. Journal of Wildlife Diseases 26: 204–209.

ILIEV, T., R. ARSOV, E. IOVCHEV, G. GIRGINOV, AND I. DIMOV. 1965. The carrier state in fowl infected with nonpathogenic *Pasteurella* strains. Nauchni Trudove Vissh Veterinarnomeditsinski Institute "Prof. D-r-G Pavlov" 14: 19–22. [In Bulgarian with German summary.]

KORSCHGEN, C. E., H. C. GIBBS, AND H. L. MENDALL. 1978. Avian cholera in eider ducks in Maine. Journal of Wildlife Diseases 14: 254–258.

MEHTA, C., AND N. PATEL. 1996. LogXact for Windows. Cytel Software Corporation, Cambridge, Massachusetts.

MOORE, M. K., L. CICNJAK-CHUBBS, AND R. J. GATES. 1994. A new selective enrichment procedure for isolating *Pasteurella multocida* from avian and environmental samples. Avian Diseases 38: 317–324.

MUHAIRWA, A. P., J. P. CHRISTENSEN, AND M. BISGAARD. 2000. Investigations on the carrier rate of *Pasteurella multocida* in healthy commercial poultry flocks and flocks affected by fowl cholera. Avian Pathology 29: 133–142.

PRITCHETT, I. W., AND T. P. HUGHES. 1932. The epidemiology of fowl cholera. VI. The spread of epidemic and endemic strains of *Pasteurella avicida* in laboratory populations of normal fowl. Journal of Experimental Medicine 55: 71–78.

———, F. R. BEAUDETTE, AND T. P. HUGHES. 1930a. The epidemiology of fowl cholera IV. Field observations of the "spontaneous" disease. Journal of Experimental Medicine 51: 249–258.

———, ———, AND ———. 1930b. The epidemiology of fowl cholera V. Further field observations of the spontaneous disease. Journal of Experimental Medicine 51: 259–274.

RIMLER, R. B., AND K. R. RHOADES. 1989. *Pasteurella multocida. In* Pasteurella and pasteurellosis, C. Adlam and J. M. Rutters (eds.). Academic Press, London, UK, pp. 37–73.

———, P. A. REBERS, AND M. PHILLIPS. 1984. Lipopolysaccharides of Heddleston serotypes of *Pasteurella multocida.* American Journal of Veterinary Research 45: 759–763.

ROSEN, M. N., AND A. I. BISCHOFF. 1949. The 1948–1949 outbreak of fowl cholera in birds in the San Francisco Bay area and surrounding counties. California Fish and Game 35: 185–192.

———, AND E. E. MORSE. 1959. An interspecies chain in a fowl cholera epizootic. California Fish and Game 45: 51–56.

SAMUEL, M. D., D. R. GOLDBERG, D. J. SHADDUCK, J. I. PRICE, AND E. G. COOCH. 1997. *Pasteurella multocida* serotype 1 isolated from a lesser snow goose: Evidence of a carrier state. Journal of Wildlife Diseases 33: 332–335.

———, J. Y. TAKEKAWA, G. SAMELIUS, AND D. R. GOLDBERG. 1999a. Avian cholera mortality in lesser snow geese nesting on Banks Island, Northwest Territories. Wildlife Society Bulletin 27: 780–787.

———, D. J. SHADDUCK, D. R. GOLDBERG, V. BARANYUK, L. SILEO, AND J. I. PRICE. 1999b. Antibodies against *Pasteurella multocida* in snow geese in the western arctic. Journal of Wildlife Diseases 35: 440–449.

TITCHE, A. 1979. Avian cholera in California. Wildlife Management Branch Administrative Report 79-2. California Department of Fish and Game, Sacramento, California, 49 pp.

VAUGHT, R. W., H. C. MCDOUGLE, AND H. H. BURGESS. 1967. Fowl cholera in waterfowl at Squaw Creek National Wildlife Refuge, Missouri. The Journal of Wildlife Management 31: 248–253.

WILSON, M. A., R. M. DUNCAN, G. E. NORDHOLM, AND B. M. BERLOWSKI. 1995. *Pasteurella multocida* isolated from wild birds of North America: A serotype and DNA fingerprint study of isolates from 1978 to 1993. Avian Diseases 39: 587–593.

WINDINGSTAD, R. M., R. M. DUNCAN, AND D. THORNBURG. 1983. Outbreak of avian cholera on the wintering grounds of the Mississippi Valley Canada goose flock. Journal of Wildlife Diseases 19: 95–97.

WOBESER, G. 1992. Avian cholera and waterfowl biology. Journal of Wildlife Diseases 28: 674–682.

———. 1997. Diseases of wild waterfowl, 2nd Edition. Plenum Press, New York, New York, 324 pp.

———, F. A. LEIGHTON, A. D. OSBORNE, D. J. NIEMAN, AND J. R. SMITH. 1982. Avian cholera in waterfowl in western Canada, 1978–1981. Canadian Field-Naturalist 96: 317–322.

ZAR, J. H. 1984. Biostatistical analysis. Prentice-Hall, Inc., Engelwood Cliffs, New Jersey, 718 pp.

Received for publication 15 April 2002.

Downloaded From: https://complete.bioone.org/journals/Journal-of-Wildlife-Diseases on 28 Mar 2024
Terms of Use: https://complete.bioone.org/terms-of-use