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CHARACTERIZATION OF *PASTEURELLA MULTOCIDA* ISOLATES FROM WETLAND ECOSYSTEMS DURING 1996 TO 1999

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ABSTRACT: We cultured 126 *Pasteurella multocida* isolates, 92 from water and 34 from sediment samples collected from wetlands in the Pacific and Central flyways of the United States between 1996 and 1999. Most (121) of the isolates were *P. multocida* serotype 1, but serotypes 3, 3/4, 10, and 11 were also found. Many (82) of the isolates were further characterized by DNA fingerprinting procedures and tested in Pekin ducks for virulence. Almost all the serotype 1 isolates we tested caused mortality in Pekin ducks. Serotype 1 isolates varied in virulence, but the most consistent pattern was higher mortality in male ducks than in females. We found no evidence that isolates found in sediment vs. water, between Pacific and Central flyways, or during El Niño years had consistently different virulence. We also found a number of non-serotype 1 isolates that were avirulent in Pekin ducks. Isolates had DNA fingerprint profiles similar to those found in birds that died during avian cholera outbreaks.

Key words: Avian cholera, DNA fingerprinting, *Pasteurella multocida*, serotyping, wetlands.

INTRODUCTION

Pasteurella multocida, the etiologic agent of avian cholera, is responsible for widespread disease that affects more than 100 wild avian species and occurs in all major flyways of North America (Botzler, 1991). Epizootics caused by *P. multocida* occur almost every winter in the Pacific flyway and during winter and early spring in the Central flyway. In addition, summer epizootics also occur on Arctic breeding areas of colonial nesting waterfowl such as lesser snow geese (*Chen caerulescens caerulescens*; Samuel et al., 1999a). *Pasteurella multocida* strains isolated from carcasses during these epizootics are commonly serotype 1 (Hirsh et al., 1990; Wilson et al., 1995; Samuel et al., 1999a). The source of disease agent, the route of transmission, virulence, and other characteristics of the agent may play an important, but not well understood, role in the dynamics of these epizootics. Two major reservoirs have been hypothesized as the source of avian cholera in waterfowl: carrier birds, and wetland sediments and water (Botzler, 1991). Regardless of the source of bacteria, once an epizootic starts, contamination of the environment, especially water, likely facilitates transmission of *P. multocida* among dense populations of waterfowl, either by ingestion and/or the inhalation of aerosols (Botzler, 1991). In addition, bird-to-bird contact may be an important component of transmission (Wobeser, 1992). To better understand the importance of wetlands in avian cholera epizootics, several authors have investigated the survival of *P. multocida* in water and sediment (Botzler, 1991). From these laboratory studies, it appears *P. multocida* can survive for extended periods of time under favorable environmental conditions and survive longer in sediments than in water (Price and Brand, 1984; Backstrand and Botzler, 1986; Botzler, 1991). Rosen and Bischoff (1950) speculated that survival of *P. multocida* in sediment was inversely related to its virulence, but their proposition has not been evaluated.

While previous studies have reported the isolation of *P. multocida* from the environment at epizootic sites, the character-

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ization of those isolates was usually incomplete. Isolates were seldom serotyped or tested for virulence in birds (Botzler, 1991). Not all serotypes of *P. multocida* cause disease in waterfowl, and strains of the same serotype could have less virulent forms that occur in carrier birds (Wobeser, 1997). Most studies involving characterization of *P. multocida* have concentrated on isolates from dead waterfowl during epizootics. One such study was conducted with isolates from wild birds of North America using serotype and DNA fingerprint methods (Wilson et al., 1995), but an equivalent study of wetland isolates is lacking.

The objectives of our study were to characterize *P. multocida* isolated during 1996 to 1999 from wetlands in the Pacific and Central flyways where avian cholera outbreaks occurred, to determine the virulence of these isolates, and to compare these wetland isolates with those previously obtained from wild birds. We characterized the isolates from the water and sediment samples by serological types and DNA fingerprint profiles and determined the virulence characteristics of these isolates in Pekin ducks. We evaluated the relative susceptibility of male and female Pekin ducks to these environmental isolates, whether virulence of the isolates changed among years or across geographic regions, and the relative virulence of isolates obtained from water and sediment. Finally, we compared the serotype and DNA fingerprint profiles for these environmental isolates to isolates obtained from wild birds that died during avian cholera outbreaks occurring from 1978–1993 (Wilson et al., 1995).

MATERIALS AND METHODS

Field collection and laboratory processing

As part of our research investigations on the importance of wetlands as a source of avian cholera and the role of environmental conditions in outbreaks, we sampled 291 wetlands in the western and central United States (west of the Mississippi River) according to one of several different criteria: 1) wetlands that experienced a recent avian cholera outbreak (≥100 dead birds reported) were typically sampled within 2 wk of observed mortality; 2) control wetlands that were in local proximity and sampled concurrently to these outbreak wetlands, but where little or no avian cholera mortality occurred; 3) wetlands with recent outbreaks that were subsequently monitored at 2–4 wk intervals for the persistence of *P. multocida*; and 4) wetlands at Sacramento National Wildlife Refuge (California, USA) that were monitored systematically during two winters (Lehr, 2000). *Pasteurella multocida* isolates obtained from 48 of these wetlands were assessed for serotype, DNA fingerprint profile, and virulence characteristics. For evaluation of virulence, wetlands where avian cholera epizootics occurred during a winter (from categories 1, 3, and 4) were combined into an outbreak category; wetlands where epizootics did not occur (from categories 2 and 4) were combined into a control category.

Regardless of the sampling criteria used, environmental samples were collected and processed using the same procedures. Each wetland <40–80 ha, or a similar size area for wetlands >80 ha, was sampled at 10 sites distributed throughout the wetland to represent the most common types of environments present (e.g., open water, near cattails, near shorelines). Water and sediment samples were collected for *P. multocida* isolation at each of the 10 sites. A 10–15 ml water sample was collected in a sterile centrifuge tube (Corning, Inc., Corning, New York, USA) at the surface of the water and a sediment sample (approximately 30 g) was collected in a sterile polypropylene container with screw-top lid (PGC Scientifics, Gaithersburg, Maryland, USA) from the upper layer (top 10 cm) of sediment using a metal scoop sampler consisting of an open faced collection bowl attached to a extension rod. All samples were stored in a cooler with ice packs until processed in the field laboratory within 24 hr.

At the field laboratory, the 15 ml centrifuge tube containing each water sample was mixed and 4 ml of water for *P. multocida* isolation was removed and transferred to a cryovial containing 1 ml of 50% dimethylsulfoxide (DMSO). Each sediment sample was thoroughly mixed using a sterile wooden applicator to obtain a homogenous mixture. A sterile cotton swab was used to remove approximately 0.5 g of sediment/water from the sample and was immersed and swirled vigorously in a cryovial containing 4 ml of a 10% solution of DMSO. The cryovials containing water and sediment samples for testing were stored frozen in a dry-shipper liquid nitrogen tank (model SC 4/2v, Minnesota Valley Engineering, Inc., Bloomington, Min-

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nesota, USA). As a quality assurance procedure, *P. multocida* seeded water samples were prepared using water from one of the sampled wetlands and three different concentrations of a stock *P. multocida* culture according to the procedures used for wetland water samples. These seeded samples were also stored in the dry-shipper liquid nitrogen tank with the water and sediment samples and shipped to the National Wildlife Health Center (NWHC; Madison, Wisconsin, USA) for subsequent *P. multocida* isolation. One shipment thawed during transport and bacteria could not be isolated from the seeded water samples included, so we rejected the samples from the three wetlands shipped in that container and they were not used in our evaluation. Competing bacteria in seeded water samples from three additional wetlands interfered with isolation of *P. multocida*, but these wetland samples were included in the study.

At NWHC, the cryovials containing the water and sediment samples were processed for *P. multocida* isolation following the procedure described by Moore et al. (1998). After 20–24 hr of incubation, suspect *P. multocida* colonies (Rimler and Rhoades, 1989) were selected. In addition to colony types described by Rimler and Rhoades (1989), we also found some *P. multocida* isolates that produced colonies as small as 0.5 mm, had a more convex or slightly domed appearance, and on a blood agar (BA; Becton, Dickinson and Company, Sparks, Maryland) plate exhibited a slight darkening of the media surrounding the colony resembling a weak alpha hemolysis. After re-isolation, suspect colonies were Gram stained and the API 20E or API NE identification system (bioMerieux, Inc., Hazelwood, Missouri, USA) was used to identify *P. multocida* isolates. All *P. multocida* isolates were then serotyped using the agarose gel precipitin (AGP) test (Heddleston et al., 1972) and stored at −80 C until virulence testing could be completed.

Pasteurella multocida isolates were DNA fingerprinted at the National Veterinary Services Laboratories (NVSL; Ames, Iowa, USA) using the method described by (Wilson et al., 1992). Briefly, a rapid organic extraction technique was used to harvest DNA from each isolate. DNA from the isolates was digested with *Hha*I restriction endonuclease for comparison with *P. multocida* somatic reference serotype strains at NVSL and further differentiated by digestion with *Hpa*II restriction endonuclease. The DNA fragments were electrophoresed in a horizontal electrophoresis system. *Hha*I profiles 1 to 16 correspond with *P. multocida* somatic serotypes 1 to 16, respectively.

Isolate virulence

Production of challenge inocula: All wetlands with *P. multocida* isolates were represented in the virulence testing. When >1 *P. multocida* serotype 1 isolate was obtained from a wetland, isolates were arbitrarily selected for virulence testing in Pekin ducks. When recovered, we tested at least one isolate from both water and sediment samples. In addition, all non-serotype 1 isolates were tested for virulence.

Each *P. multocida* isolate was thawed and streaked on dextrose starch agar (Difco Laboratories, Detroit, Michigan, USA) with mycoplasma supplement (Difco Laboratories) (DSA-MS) and incubated 18–24 hr at 37 C with 5% CO_2. Each plate was checked for a mixture of blue, gray, and fluorescent colony types. If a mixture was observed, a fluorescent colony was selected and used to inoculate a tube containing 5 ml of brain heart infusion (BHI) broth (Difco Laboratories). Each tube was incubated in a shaker incubator (G24 Environmental Incubator Shaker, New Brunswick Scientific Co., Inc., New Brunswick, New Jersey, USA) at 37 C for 5–7 hr with shaking. An initial dilution of each isolate was prepared from this 5–7 hr culture by adding drop wise into 3–5 ml of BHI broth with 5% chicken serum (Sigma Chemical Co., St. Louis, Missouri) to obtain standardized cultures containing 10^6–10^7 colony forming units (CFU)/ml, estimated using a McFarland nephelometer standard #0.5. The logarithmic growth phase culture was then diluted 1:10 using BHI broth containing 5% chicken serum to obtain a final dilution for the test inoculum. Our target challenge dose was 100 Peking duck 50% lethal dose (LD_{50}; 6.2×10^5 CFU) in 0.2 ml (El Tayeb, 1993). Each culture was titrated at the time the dilutions were made using a standard plate count to determine the actual challenge dose.

Virulence testing: Pekin ducks used for virulence testing were obtained from Maple Leaf Farms (Racine, Wisconsin, USA) as 2–4 day old ducklings and reared in isolation facilities at NWHC to ensure no exposure to *P. multocida* occurred. Birds were housed in brooders with a heat source for the first 3 wk, then moved to an isolation room (approximately 22 m^2) with a 1.3 cm × 2.5 cm gauge diamond shaped Tenderfoot® (Tandem Products, Inc., Minneapolis, Minnesota) covered floor. Waterfowl starter feed (Purina Mills, Inc., St. Louis, Missouri) and water were provided ad libitum. At 6 wk of age, the ducks were transferred to individual stainless steel rabbit cages (76 cm × 61 cm × 41 cm) (Lab Products, Inc., Aberdeen, Maryland) prior to challenge at 7 wk. Challenge tri-

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als ($n=10$) were conducted sequentially over a 3 yr period, with a range of 3–12 isolates tested during each trial. For each isolate, four birds were injected subcutaneously in the dorsal caudal region of the neck with 0.2 ml of the challenge inocula. Although this represents an unnatural route of infection, subcutaneous inoculation has been previously used to determine virulence of *P. multocida* isolates (Samuel et al., 1997), was used to ensure infection of individual birds, facilitate comparison among isolates, and has produced similar rates of carrier birds compared with natural transmission (Samuel et al., 2003).

Ducks were monitored daily for morbidity or mortality for 7–9 days, after which, surviving birds were euthanized by cervical dislocation. Following death from challenge, ducks were refrigerated (5 C) and necropsied within 48 hr to confirm lesions associated with avian cholera, collect livers for *P. multocida* re-isolation, and determine sex. An abbreviated necropsy was performed on euthanized ducks, to determine sex, note signs of latent infection, and collect liver samples for *P. multocida* isolation. Sex was not determined in 11 birds from our initial trials.

Following necropsy, the livers from all ducks were cultured to confirm the presence of *P. multocida.* Each liver was flamed and a fresh cut surface was cultured on a BA plate and incubated at 37 C with 5% CO_2 for 20–24 hr. Pure cultures of *P. multocida* were serotyped to confirm isolation of the challenged serotype. A *P. multocida* culture from one of the four necropsied birds per challenge isolate was identified using API 20E and the cultures from the remaining birds were identified based on the typical fermentation pattern of six sugars (dextrose +, lactose −, maltose ±, sucrose +, dulcitol −, and mannitol +) (Holt et al., 1994).

Statistical analyses: We used logistic regression (Woodward, 1999) to evaluate factors associated with virulence (probability of mortality from *P. multocida* challenge). We considered sex of challenged ducks, geographic location of the isolate (Pacific vs. Central flyway), study site type (cholera outbreak vs. control sites), and whether the isolate was collected during an El Niño event (April 1997 to April 1998; Trenbeth, 1997). Logistic regression models were fit using program R, version 1.4.1 (Ihaka and Gentleman, 1996). The effect of each variable in the model was estimated by averaging the model coefficients over all models, weighted by Akaike weight (Burnham and Anderson, 1998). Odds ratios (Woodward, 1999) were estimated using a generalized linear model with a logit link function. This method allows estimation of the effect of each factor on virulence, controlling for other factors and incorporating uncertainty in selection among alternative models. Ducks of undetermined sex were excluded from our analysis. We conducted a second analysis to investigate factors affecting mortality of challenged ducks based on acute death following inoculation. In this analysis we evaluated whether sex, flyway, type of study site, or an El Niño event affected probability of dying within 24 hr post-inoculation. Finally, we used conditional logistic regression to stratify challenge data by individual wetlands and examine whether virulence differed among isolates obtained from water vs. sediment. This analysis was conducted on the subset of wetlands with both sediment and water isolates, and sex was included as a covariate in this analysis.

RESULTS

We cultured 126 *P. multocida* isolates from 48 (Table 1) of the 291 wetlands sampled from 1995 to 1999. These isolates were identified as serotypes 1 ($n=121$), 3 ($n=2$), 3/4 ($n=1$), 10 ($n=1$), and 11 ($n=1$). Multiple serotypes were found at three wetlands in Nebraska (USA): Hansen in 1998, Harvard in 1997, and Sandpiper in 1996. Isolates from water comprised 74% (93 isolates) and isolates from sediment were 26% (33 isolates) of our isolates. We obtained 97 and 29 isolates from the Pacific and Central flyways, respectively. Within each flyway, isolates were obtained from water and sediments with similar frequency: Pacific flyway, 70 from water (72%) and 27 from sediment (28%); Central flyway, 23 from water (79%) and six from sediment (21%). In the winters of 1995–96, 1996–97, 1997–98 we found one, one, and 65 isolates, respectively, from the Pacific flyway and seven, 13, and nine isolates, respectively, from the Central flyway. During the winter of 1998–99 there were 30 isolates obtained from the Pacific flyway and no sampling was done in the Central flyway.

We tested 87 wetland isolates for virulence (62 water isolates and 25 sediment isolates) in Pekin ducks: 82 of these isolates were serotype 1 and five were other serotypes. None of the 20 (five groups of four) ducks exposed to non-serotype 1 iso-

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TABLE 1. Location, date collected, number isolated, serotype, number tested in Pekin ducks and virulence of *Pasteurella multocida* isolated from 48 of 291 wetlands in the western and central United States from 1996–99.

State	Wetland	Lat.	Long.	Date	Ser[a]	Total isolates		Isolates tested[c]		Male mort.[d]		Female mort.[d]	
						W[b]	S[b]	W	S	W	S	W	S
CA	Colusa NWR[e], T13A	39°09′N	122°03′W	01/08/1998	1	3	0	3	0	6/7	—	3/5	—
CA	Colusa NWR, T13A	39°09′N	122°03′W	01/15/1999	1	0	1	0	1	—	—	—	2/4
CA	Colusa NWR, T13A	39°09′N	122°03′W	02/04/1999	1	2	0	1	0	1/2	—	1/2	—
CA	Colusa NWR, T15A	39°09′N	122°03′W	02/15/1996	1	1	0	1	0	2/4[f]	—	—	—
CA	Colusa NWR, T15A	39°09′N	122°03′W	11/25/1997	1	1	0	1	0	0/2	—	0/2	—
CA	Colusa NWR, T15A	39°09′N	122°03′W	01/08/1998	1	2	0	1	0	3/3	—	1/1	—
CA	Delevan NWR, T11	39°19′N	122°07′W	01/15/1998	1	1	0	1	0	2/4	—	—	—
CA	Delevan NWR, T15	39°19′N	122°05′W	01/30/1998	1	1	0	1	0	1/1	—	2/3	—
CA	Delevan NWR, T16	39°19′N	122°07′W	01/23/1998	1	2	0	1	0	3/3	—	0/1	—
CA	Delevan NWR, T2-1	39°20′N	122°06′W	12/03/1997	1	1	0	1	0	0/1	—	2/3	—
CA	Delevan NWR, T6	39°20′N	122°07′W	01/15/1998	1	5	1	2	1	2/2	2/2	6/6	0/2
CA	Llano Seco NWR, T10-1	39°35′N	121°56′W	01/13/1998	1	2	2	1	1	1/1	2/2	3/3	1/2
CA	Llano Seco NWR, T6	39°35′N	121°57′W	01/13/1998	1	1	0	1	0	3/3	—	0/1	—
CA	Sacramento NWR, T10-3	39°26′N	122°10′W	02/23/1999	1	3	0	2	0	1/2	—	5/6	—
CA	Sutter NWR, T20-2	39°04′N	121°44′W	03/14/1997	1	1	0	1	0	1/1	—	2/3	—
CA	Yolo Bypass, Unit F	38°31′N	121°36′W	01/07/1999	1	1	2	1	1	3/3	1/1	1/1	3/3
CA	Conaway Ranch, 10-1	38°38′N	121°38′W	01/08/1999	1	1	1	1	1	3/3	1/1	0/1	3/3
CA	Salton Sea, HQ-D	33°11′N	115°37′W	01/13/1998	1	1	0	1	0	2/3	—	0/1	—
CA	Tule Lake, 12C	41°55′N	121°39′W	11/24/1997	1	1	0	1	0	2/2	—	1/2	—
OR	Upper Klamath, Agency Lake	42°31′N	121°57′W	11/25/1997	1	0	1	0	1	—	2/2	—	2/2
OR	Upper Klamath, Howard Bay	42°21′N	121°55′W	11/25/1997	1	2	1	1	1	1/1	—	2/3	4/4
CA	Hayward Park, 2B	37°38′N	122°08′W	01/06/1999	1	2	1	1	1	3/3	2/2	1/1	1/2
CA	Hayward Park, 2B	37°38′N	122°08′W	02/17/1999	1	1	1	1	1	2/3	—	1/1	4/4
CA	Arena Plains, North Lake	37°17′N	120°44′W	02/04/1998	1	3	0	3	0	3/4	—	4/8	—
CA	Kesterson NWR, West Teal	37°17′N	120°57′W	01/30/1999	1	3	0	3	0	6/9	—	3/3	—
CA	W. Bear Creek NWR, Pintail	37°16′N	120°50′W	01/28/1999	1	0	1	0	1	—	—	—	2/4
CA	W. Bear Creek NWR, Pintail	37°16′N	120°50′W	02/19/1999	1	4	3	2	2	1/1	3/3	1/7	4/5
CA	Los Banos WMA[g], Gadwall-1	37°03′N	120°47′W	02/03/1998	1	5	1	3	1	4/8	1/4	3/4	—
CA	Los Banos WMA, Gadwall-2	37°03′N	120°47′W	01/07/1998	1	1	5	1	2	3/3	2/4	1/1	4/4
CA	Los Banos WMA, Gadwall-3	37°02′N	120°46′W	01/07/1998	1	0	1	0	1	—	—	—	0/4
CA	Mud Slough, Unit 5	37°04′N	120°46′W	02/02/1998	1	4	2	2	1	2/4	2/2	3/4	2/2

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TABLE 1. Continued.

State	Wetland	Lat.	Long.	Date	Ser[a]	Total isolates		Isolates tested[c]		Male mort.[d]		Female mort.[d]	
						W[b]	S[b]	W	S	W	S	W	S
CA	Merced NWR, Mariposa	37°11′N	120°40′W	02/18/1999	1	2	1	2	1	7/8[h]	1/2	—	0/2
CA	San Luis NWR, Page Lake	37°40′N	121°12′W	01/15/1998	1	4	2	3	1	5/7	4/4	2/5	—
CA	San Luis NWR, Page Lake E.	37°40′N	121°12′W	01/15/1998	1	9	0	3	0	8/9	—	2/3	—
NE	Eckhardt	40°28′N	97°54′W	03/18/1996	1	1	0	1	0	3/3	—	1/1	—
NE	Eckhardt	40°28′N	97°54′W	03/06/1998	1	1	0	1	0	0/2	—	1/2	—
NE	Hansen	40°27′N	97°51′W	03/06/1998	3	1	0	1	0	0/4	—	—	—
NE	Hansen	40°27′N	97°51′W	03/06/1998	11	1	0	1	0	0/1	—	0/3	—
NE	Harvard	40°37′N	98°11′W	03/17/1997	3/4	0	1	0	1	—	0/2	—	0/2
NE	Harvard	40°37′N	98°11′W	03/17/1997	10	0	1	0	1	—	0/1	—	0/3
NE	Mallard Haven	40°27′N	97°45′W	03/17/1996	1	1	0	1	0	2/4[f]	—	—	—
NE	Mallard Haven	40°27′N	97°45′W	03/20/1997	1	0	1	0	1	—	1/2	—	2/2
NE	Massie	40°29′N	98°02′W	03/05/1998	1	1	0	1	0	3/4	—	—	—
NE	McMurtry	40°34′N	98°11′W	03/05/1998	1	1	0	1	0	1/1	—	3/3	—
NE	Sandpiper	40°38′N	97°58′W	03/17/1996	1	3	1	2	1	5/8[i]	4/4	—	—
NE	Sandpiper	40°38′N	97°58′W	03/17/1996	3	0	1	0	1	—	0/4[j]	—	—
NE	Sandpiper	40°38′N	97°58′W	03/20/1997	1	1	0	1	0	2/3	—	0/1	—
NE	Smartweed	40°20′N	98°01′W	03/17/1997	1	8	0	3	0	5/7	—	5/5	—
NE	Funk	40°31′N	99°13′W	03/19/1997	1	0	1	0	1	—	1/2	—	1/2
NE	Funk	40°31′N	99°13′W	04/02/1998	1	2	0	1	0	—	—	3/4	—
NE	Johnson	40°34′N	99°20′W	04/03/1998	1	2	0	1	0	3/3	—	1/1	—

[a] Ser = serotype.
[b] W/S = source of isolates, water or sediment.
[c] Tested = number of isolates that were challenged in ducks.
[d] Mort. = number of ducks that died/number challenged with *P. multocida* isolates recovered from wetland water and sediment samples.
[e] NWR = National Wildlife Refuge.
[f] Sex of the two challenged survivors unknown. Value represents total mortality. Sex of the two that died was male.
[g] WMA = Waterfowl Management Area.
[h] Value represents total mortality. Five males, one female, and one bird of unknown sex died of *P. multocida,* one female survived challenge.
[i] Value represents total mortality from the two water isolates. Four males and one female died of *P. multocida.* One female and two with sex undetermined survived challenged.
[j] Value represents total mortality. Sex of all four challenged survivors unknown.

TABLE 2. DNA characterization, differentiated by *Hha*I[a] and *Hpa*II restriction endonucleases, of *Pasteurella multocida* serotype 1 isolated from wetlands sampled in the Pacific and Central flyways from 1996–99.

Flyway	*Hpa*II	1996	1997	1998	1999	Total
Pacific	003	1	0	0	0	1
	004	0	7	35	23	65
Central	003	0	0	0	—[b]	0
	004	5	6	5	—	16
Total		6	13	40	23	82

[a] All isolates have *Hha*I 001 profiles which matches the somatic reference strain X-73.
[b] No samples were taken.

lates died due to *P. multocida* infection. All of these non-serotype 1 isolates were obtained from the Central flyway during winter 1997–98. In the 82 challenge trials with serotype 1 isolates, 0 ducks died in two trials, one duck died in 10 trials, two ducks died in 15 trials, three ducks died in 27 trials, and all four ducks died in 28 trials. All but one of the 232 ducks that died from *P. multocida* serotype 1 challenge produced isolates of serotype 1. One hundred five of the 115 euthanized ducks that survived challenge were tested for re-isolation of *P. multocida.* None of the 16 ducks challenged with non-serotype 1 produced *P. multocida* isolates, serotype 1 was re-isolated from 42 of the 89 ducks challenged with serotype 1, and six ducks challenged with serotype 1 and four with non-serotype 1 were not tested for re-isolation of *P. multocida.*

DNA fingerprinting using *Hha*I and *Hpa*II restriction endonucleases were performed on the same 87 isolates that were tested for virulence. The 82 serotype 1 isolates had *Hha*I 001 profiles that were identical to the reference somatic serotype 1, strain X-73 from the culture collection at the NVSL. One isolate from the Pacific flyway in 1996 had a *Hpa*II 003 profile and the remaining 81 isolates had a *Hpa*II 004 profile (Table 2). The three isolates identified as serotypes 3/4, 10, and 11 produced three *Hha*I DNA profiles identified as 1041, 1040, and 1065, respectively. The two isolates identified as serotype 3 produced 1039 and 1040 *Hha*I profiles.

In all, 224 (71.8%) of 312 Pekin ducks of known sex died from avian cholera when challenged with *P. multocida* serotype 1 isolated from wetlands. Virulence (odds of dying from *P. multocida* infection) was 1.51 (95% CI 1.02–2.24) times greater for challenged males than females. Virulence did not vary between isolates from the Pacific and Central flyways (odds ratio 0.92 [95% CI 0.73–1.16]), collected during El Niño events (odds ratio 0.91 [95% CI 0.76–1.09]), or whether the isolate was from an outbreak or control site (odds ratio 0.95 [95% CI 0.80–1.14]). The probability of death within 24 hr post-inoculation was 2.93 times greater for males than females (95% CI 1.42–6.06), but did not vary between Pacific and Central flyways (odds ratio 0.99 [95% CI 0.77–1.27]), for isolates collected during El Niño events (odds ratio 0.87 [95% CI 0.65–1.15]), or whether the isolate was from an outbreak or control site (odds ratio 0.99 [95% CI 0.81–1.21]). Virulence of isolates collected from water and sediment of the same wetlands ($n=14$) were not different (odds ratio 0.77 [95% CI 0.37–1.61]), after controlling for greater mortality that occurred in males then females (odds ratio 2.35 [95% CI 1.10–5.0]).

DISCUSSION

Avian cholera epizootics were more common and widely distributed in the Pacific and Central flyways during the El Niño winter (1997–98), the year we collected the most *P. multocida* serotype 1 isolates. Although two of the isolates found in the Pacific flyway during that year did not kill challenged Pekin ducks, there was no general indication that wetland isolates obtained during the El Niño event were less virulent than isolates obtained in other years. A large number of *P. multocida* isolates were also collected during the winter of 1998–99, when avian cholera occurred frequently in the Pacific flyway. We isolated other serotypes of *P. multocida* only

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from wetlands in the Rainwater Basin, Nebraska (Central flyway). Waterfowl are a likely source of the serotype 1 *P. multocida* that we isolated during avian cholera outbreaks. However, the origin of other serotypes recovered in the Rainwater Basin is unclear and may be associated with waterfowl (Samuel et al., 1999b) or from domestic livestock (Brogden and Packer, 1979). Further investigation on the source of these serotypes is needed to determine the origin of different *P. multocida* serotypes and whether these bacteria are distributed by waterfowl or by livestock.

DNA fingerprinting of *P. multocida* isolates collected from wild birds suggest there has been a shift in DNA profiles from *Hha*I 001/*Hpa*II 002 to *Hha*I 001/*Hpa*II 004 between 1978–1993 (Wilson et al., 1995). DNA fingerprinting for our wetland isolates collected from 1996–99 followed this trend with one *Hha*I 001/*Hpa*II 003 and 81 *Hha*I 001/*Hpa*II 004 DNA profiles (Table 2). We found no differences in DNA profiles between the Pacific and Central flyways, with the exception of the single *Hha*I 001/*Hpa*II 003 isolate from the Pacific flyway in 1996. These results support the conclusion that *P. multocida* with similar DNA *Hha*I/*Hpa*II profiles are widely distributed in the western United States. However, it should be considered that DNA fingerprinting has a limited ability to classify *P. multocida* isolates because of the considerable genetic variation within serotypes.

We found that virulence of serotype 1 isolates collected from wetlands varied from 0 to 100% in Pekin ducks. The only consistent pattern was higher mortality in males than in females, both in rapidity of death and total mortality. This finding is consistent with previous reports of higher mortality in male snow geese (McLandress, 1983) and in male Canada geese (*Branta canadensis*; Windingstad et al., 1998), but different than the pattern reported in American coots (*Fulica americana*; Mensik and Botzler, 1989). Consequently, further research should consider sex when testing virulence of *P. multocida* isolates. Overall, almost all of the serotype 1 isolates we tested killed Pekin ducks. In contrast, none of the non-serotype 1 isolates were virulent in Pekin ducks, however, we caution that none of these isolates have been tested in avian species which may be affected by these serotypes. Until improved methods are developed from characterizing virulence of *P. multocida* we recommend that isolates are serotyped and tested for virulence in live animals.

We were not able to find a consistent difference in serotypes or virulence between *P. multocida* isolates collected from water or sediments of wetlands. We recognize that samples we obtained from wetland sediments may have become contaminated with bacteria from the water during our sample collection. However, the potential for cross contamination seems limited because we seldom obtained *P. multocida* from both sediment and water at the same collection site. We suggest there is little evidence to support the hypothesis that *P. multocida* isolates from water or sediments of wetlands differ in their virulence. We suspect this concept is based on a hypothesized trade-off between survival of bacteria and level of virulence. However, based on other investigations we believe that *P. multocida* does not generally survive well in wetland environments and we have found no evidence that these bacteria can persist sufficiently long following outbreaks for wetlands to serve as a year-round reservoir for avian cholera (Samuel, unpubl.).

In addition, we were unable to demonstrate a difference in virulence of *P. multocida* isolated from outbreak or non-outbreak sites. However, this comparison may be limited because we isolated bacteria from only a limited number of control wetlands (where avian mortality was low). Interestingly, the two serotype 1 isolates that failed to kill challenged Pekin ducks were both from wetlands where avian cholera epizootics were not reported. In contrast, isolates collected from the same wetland

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in different winters or from proximate wetlands in the same year were highly virulent (up to 100% mortality) in Pekin ducks. Our present results do not support the concept that some wetlands have virulent isolates that result in mortality events and other wetlands have less virulent isolates that only produce minimal mortality. We suspect that mortality events are likely related to other factors that include particular avian species, bird densities, and potential stress factors that may initiate mortality events.

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