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A Cryopreservation Method for *Pasteurella multocida* from Wetland Samples

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ABSTRACT: A cryopreservation method and improved isolation techniques for detection of *Pasteurella multocida* from wetland samples were developed. Wetland water samples were collected in the field, diluted in dimethyl sulfoxide (DMSO, final concentration 10%), and frozen at −180 C in a liquid nitrogen vapor shipper. Frozen samples were transported to the laboratory where they were subsequently thawed and processed in *Pasteurella multocida* selective broth (PMSB) to isolate *P. multocida*. This method allowed for consistent isolation of 2 to 18 organisms/ml from water seeded with known concentrations of *P. multocida*. The method compared favorably with the standard mouse inoculation method and allowed for preservation of the samples until they could be processed in the laboratory.

Key words: Avian cholera, cryopreservation method, mouse inoculations, *Pasteurella multocida*, wetland samples.

Avian cholera, caused by the bacterium *Pasteurella multocida*, is among the most common diseases affecting wild waterfowl. However, basic epizootiological information about this infectious disease, including bacterial survival in the environment, disease reservoirs, and transmission, have not been systematically investigated (Botzler, 1991; Wobeser, 1992). Enzootic wetland sites have been suggested as one of the important reservoirs of this disease in waterfowl, and field investigations have usually attempted to isolate *P. multocida* from wetland samples (water and sediment) using mouse inoculation procedures. Backstrand and Botzler (1986) used mouse inoculations to isolate *P. multocida* from pond water at 3 and 10 days following an avian cholera epizootic in California (USA), although it was not isolated from any soil samples collected before, during, or after the epizootic. Price and Brand (1984) used mouse inoculations to isolate *P. multocida* from several wetlands during an avian cholera epizootic in Nebraska (USA). Rosen (1969) isolated *P. multocida*, by mouse inoculation, from a small pond 3 wk after 100 dead geese had been removed.

The mouse inoculation method has been used because of the sensitivity of mice to *P. multocida*, and the difficulty of isolating the organism from environmental samples using standard bacteriological media (Moore et al., 1994). However, the use of mice to isolate *P. multocida* typically requires transport and maintenance of mice in the field or sampling in close proximity to a laboratory. Because many avian cholera mortality sites are remote, and survival of the organism from wetland samples in standard transport media may be limited (National Wildlife Health Center, unpublished data), the difficulty in isolating the bacteria is increased. Therefore, we developed a simple method that allowed transport and long-term storage of wetland samples under relatively inert conditions prior to processing in a laboratory. This method included cryopreservation of samples in 10% dimethyl sulfoxide (DMSO) at −180 C in a liquid nitrogen vapor shipper (model SC 4/2V, Minnesota Valley Engineering, Inc., Bloomington, Minnesota, USA) prior to shipment to the laboratory and later processing using *Pasteurella multocida* selective broth (PMSB), an improved isolation medium (Moore et al., 1994).

To determine the sensitivity of this new cryopreservation method for isolating *P. multocida* from wetland samples, and for comparison of this method with the standard mouse inoculation method, we first prepared a stock culture of *P. multocida*. A single colony of the organism was in-

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cubated for 18 hr in brain heart infusion (BHI) broth (Difco, Detroit, Michigan, USA) with 5% CO_2 at 37 C. One ml aliquots of this stock culture were placed in cryovials and frozen at −180 C in liquid nitrogen. To determine the bacterial concentration in these stock cultures, 10 vials of the stock culture were removed, thawed at 18 to 21 C and mixed by inverting the vials. Ten-fold serial dilutions (1×10^{-2} to 1×10^{-8}) were prepared and samples were inoculated into five BHI broth tubes for each dilution and incubated for 48 hr at 37 C with 5% CO_2. The Most Probable Number (MPN) for all stock cultures was estimated at 1.76×10^6 cells/ml (standard error = 6.31×10^5 cells/ml) using the statistical formulation provided by Cochran (1950).

In a preliminary test, using this stock culture, we seeded and attempted to recover *P. multocida* from water collected from 7 wetlands that were part of a study on the relationship between avian cholera epizootics and wetland characteristics. For each of these wetlands, a cryovial of the frozen stock of *P. multocida* was thawed at 18 to 21 C, serially diluted (1×10^{-3} to 1×10^{-7}) in nonsterile marsh water, followed by storage and processing using the cryopreservation method. An aliquot of 4 ml from each of the dilutions and from a nonsterile marsh water control (unseeded), was added to separate 1 ml quantities of 50% DMSO (diluted in distilled water) in cryovials (Corning, 5 ml, #430663) to obtain a final concentration of 10% DMSO. The vials were mixed by inverting 10 times, and frozen at −180 C in a liquid nitrogen vapor shipper. The vials were later removed, thawed at 18 to 21 C, and mixed by inverting. A 2 ml aliquot from each vial was placed in 5 ml of BHI broth and incubated with orbital shaking for 2.5 hr at 37 C. After incubation, 2 ml of each culture was added to 5 ml of PMSB and incubated at 37 C for 12 to 16 hr with 5% CO_2. A sterile cotton swab was then inserted into the PMSB tube, allowed to moisten completely, swabbed and streaked onto blood agar (BA) (Remel, Lenexa, Kansas, USA), which was then incubated at 37 C with 5% CO_2 for 16 to 24 hr. Suspect colonies were identified by the methods described by Samuel et al. (1997). We consistently (7 of 7 samples) isolated *P. multocida* from these seeded samples at estimated densities of 17.6 cells/ml and less frequently (two of 7 samples) at 1.76 cells/ml, while no *P. multocida* was isolated from the nonsterile control samples.

To further test the sensitivity of the cryopreservation method and to compare it to the mouse inoculation method, we seeded water from an additional 12 wetlands (Table 1). Nonsterile wetland water samples were seeded with the frozen stock of *P. multocida* (average MPN of 1.76×10^6 cells/ml) in 10-fold dilutions from 1×10^{-3} to 1×10^{-7}, and processed concurrently using both the cryopreservation and mouse inoculation methods. Unseeded nonsterile wetland water was used as a control in both methods. Two mice were injected intraperitoneally with 0.2 ml from each dilution, including controls. Mice were observed daily for mortality for ≥72 hr and those that did not die were euthanized. Livers of all mice were removed and cultured on BA for *P. multocida*. Suspect colonies were identified using the methods of Samuel et al. (1997) and isolates from the highest dilutions of the cryopreservation (or mouse inoculation) method where growth (or death of mice) occurred were identified by the API 20E identification system (bioMerieux Vitek, Inc., Hazelwood, Missouri, USA).

Using the cryopreservation method, we were able to recover *P. multocida* from 12 of 12 samples at densities of 17.6 cells/ml, from two of 12 samples at 1.76 cells/ml, and from three of 12 samples at 0.176 cells/ml. Although *P. multocida* was isolated at low concentrations of the seeded sample, most of the plates were covered with abundant growth of contaminants, with only a few *P. multocida* colonies. When the mouse inoculation method and the cryopreservation method were com-

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TABLE 1. Recovery of *Pasteurella multocida* isolates from seeded wetland water samples using the cryopreservation method (first row results) and standard mouse inoculation (second row results).

Wetland/Location	Date collected	Concentration (cells/ml)				
		1.76×10^3	1.76×10^2	1.76×10	1.76	0.176
Funk	12 September 1996	+[a]	+	+	−[b]	+
Phelps County, Nebraska		+(2)[c]	+(1)	+(2)	−	−
Harvard	13 September 1996	+	+	+	−	−
Clay County, Nebraska		+(2)	+(1)	+(1)	−	−
Big Water	16 September 1996	+	+	+	−	−
Churchill County, Nevada		+(2)	−	+(1)	−	−
MARC reservoir	16 September 1996	+	−	+	−	−
Clay County, Nebraska		+(2)	+(2)	+(2)	+(1)	−
Tule Lake	18 September 1996	+	+	+	+	−
Siskiyou County, California		+(2)	+(2)	+(1)	−	−
Sutter NWR T-2	20 September 1996	+	+	+	−	−
Sutter County, California		+(2)	+(2)	−	+(1)	−
Sacramento NWR	16 October 1996	+	+	+	−	−
Glenn County, California		+(2)	+(2)	+(1)	−	−
Butte Sink	17 October 1996	+	+	+	+	−
Sutter County, California		+(2)	+(2)	+(1)	−	−
Page Lake	23 October 1996	+	+	+	−	+
Stanislaus County, California		+(2)	+(1)	+(1)	−	−
North Dehaven	15 December 1996	+	+	+	−	−
Humboldt County, California		+(2)	+(2)	−	−	−
Triangle Marsh	15 December 1996	+	+	+	−	+
Humboldt County, California		+(2)	+(2)	+(2)	−	−
West Marsh	15 December 1996	+	+	+	−	−
Humboldt County, California		+(2)	+(2)	+(2)	−	−

[a] +, *P. multocida* recovered.
[b] −, *P. multocida* not recovered.
[c] Number in parentheses indicates how many mice, of two tested, died from *P. multocida*.

pared, both methods were able to detect *P. multocida* at the same concentrations in four (33%) of the 12 wetlands tested. In six (50%) of the wetlands, we detected *P. multocida* at lower concentrations using the cryopreservation method than with mouse inoculations. Two (17%) of the wetlands tested yielded better results with the mouse inoculation method. Based on the highest serial dilution (i.e., 1×10^{-3} through 1×10^{-7}) with positive identification of *P. multocida,* we used a Wilcoxon matched-pairs signed-rank test (Daniel, 1978) to compare the cryopreservation and mouse inoculation methods; the cryopreservation method was marginally better ($T = 1.86$, $n = 12$, $P = 0.089$) than the mouse inoculation method. *Pasteurella multocida* was isolated from livers of all mice that died, but not from any that were euthanized. All controls were negative for both the cryopreservation and mouse inoculation methods.

We found the cryopreservation method to be consistent, with isolation routinely possible down to a concentration of 2 to 18 organisms/ml. However, at lower concentrations of *P. multocida,* considerable experience is needed to identify *P. multocida* in its varied forms and to isolate the organism from the accompanying contaminants. The major advantages of the cryopreservation method are that little processing is required in the field, samples can be conveniently stored before laboratory processing, and the use of live animals is avoided. We have successfully used this method to isolate *P. multocida* from wet-

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land samples (both sediment and water) where avian cholera epizootics have occurred (National Wildlife Health Center, unpublished data).

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