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Authors: Foreyt, William J., and Silflow, Ronald M.

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ATTEMPTED PROTECTION OF BIGHORN SHEEP (*OVIS CANADENSIS*) FROM PNEUMONIA USING A NONLETHAL CYTOTOXIC STRAIN OF *PASTEURELLA HAEMOLYTICA*, BIOTYPE A, SEROTYPE[11]

William J. Foreyt and Ronald M. Silflow
Department of Veterinary Microbiology and Pathology, Washington State University, Pullman, Washington 99164-7040, USA

ABSTRACT: Between February and April, 1994, we tested the hypothesis that bighorn sheep (*Ovis canadensis canadensis*) inoculated with a cytotoxic isolate of *Pasteurella haemolytica* biotype A, serotype 11 (A11) could withstand challenge inoculation with a cytotoxic strain of *P. haemolytica* A2 of domestic sheep origin known to cause lethal pneumonia in bighorn sheep. On experimental day 0, two bighorn sheep were inoculated intratracheally with 6×10^9 colony forming units (cfu) of a cytotoxic strain of *P. haemolytica* A11 (group 1); two bighorn sheep were inoculated intratracheally with 6×10^9 cfu of a noncytotoxic *P. haemolytica* A11 (group 2), and two control bighorn sheep were inoculated intratracheally with a similar volume of brain heart infusion (BHI) broth (group 3). After inoculation, all bighorn sheep remained healthy. On experimental day 16, group 1 bighorn sheep each were given the same intratracheal inoculation as on day 0, and groups 2 and 3 bighorn sheep each were inoculated with BHI broth at the same volume as group 1. All bighorn sheep remained healthy following inoculations. On experimental day 42, bighorn sheep in groups 1 and 3 each were challenged with an intratracheal inoculation of 6×10^9 cfu of *P. haemolytica* A2 of domestic sheep origin known to be lethal in bighorn sheep. Group 2 sheep each were inoculated intratracheally with BHI broth at the same volume as groups 1 and 3. The four bighorn sheep in groups 1 and 3 that received the challenge inoculation died from acute bronchopneumonia within 72 hours after challenge inoculation, and cytotoxic *P. haemolytica* A2 was isolated from the four dead bighorn sheep. Both cytotoxic or noncytotoxic strains of *P. haemolytica* A11 were not lethal and did not cause pneumonia in the experimentally inoculated bighorn sheep. However, previous inoculation with cytotoxic *P. haemolytica* A11 did not protect the bighorn sheep against later experimental challenge inoculation with a known lethal strain of cytotoxic *P. haemolytica* A2 under the conditions defined in these experiments.

Key words: Bighorn sheep, *Ovis canadensis*, pneumonia, cytotoxicity, *Pasteurella haemolytica*, experimental infection.

INTRODUCTION

Pneumonia, primarily caused by *Pasteurella haemolytica*, is the major cause of mortality of free ranging bighorn sheep (*Ovis canadensis*) in North America (Foreyt et al., 1994). *Pasteurella haemolytica* has two distinct biotypes, A and T, and at least 15 known serotypes (Tsai et al., 1988) within the biotypes. In addition, numerous isolates of *P. haemolytica* crossreact in antisera to two or more serotypes, or do not react in antisera to any of the known serotypes (Foreyt et al., 1994). Different ribotypes also have been described within the same serotype (Foreyt et al., 1994), indicating the complexity of *P. haemolytica*. On evaluating potency of cytotoxins (leukotoxins) produced by specific serotypes of *P. haemolytica* from bighorn sheep and domestic sheep against blood neutrophils, Sweeney et al. (1994) found that some serotypes produce potent toxins that lyse neutrophils, and other serotypes produce toxins that result in low toxicity to neutrophils. From previous experiments, we determined that cytotoxic serotypes of *P. haemolytica*, especially A2, are likely to kill healthy bighorn sheep, whereas noncytotoxic serotypes of *P. haemolytica*, usually in the T biotype, are not likely to cause respiratory disease in healthy bighorn sheep, and often occur as normal flora in healthy bighorn sheep (Foreyt et al., 1994). Cytotoxic *P. haemolytica* A2 from healthy domestic sheep is lethal to healthy bighorn sheep in less than 48 hr following intratracheal inoculation (Foreyt et al.,

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TABLE 1. Biotypes and serotypes of *Pasteurella haemolytica* isolates recovered from bighorn sheep before and after inoculations, April 1994.

	Bighorn number	Experimental Day 0[a]	Experimental Day 16[b]	Experimental Day 42[c]	At necropsy	Pneumonia
Group 1	1	T3, 4	T3, 4 T3, 4, 10	A7, 11 T3, 4	A2 (lung, tonsil) T3 (tonsil) T3, 4 (tonsil)	+
	2	T3, 4	None	T4	A2 (lung)	+
Group 2	3	T3, 4	T3, 4, 10	T3, 4, 10	NA[d]	−
	4	T3, 4	T3, 4	A11 T3, 4	NA	−
Group 3	5	T3, 4	None	A6, 11	A2 (lung) T3, 4, 15 (lung)	+
	6	T3, 4	T(unt)[e]	A11	A2 (lung) A1 (spleen) T3, 4 (lung)	+

[a] Group 1 inoculated with 6×10^9 colony forming units (cfu) of cytotoxic *P. haemolytica* A11 of bighorn sheep origin; Group 2 inoculated with 6×10^9 cfu of noncytotoxic *P. haemolytica* A11 of bighorn sheep origin; Group: 3, controls.
[b] Group 1 inoculated with 6×10^9 cfu of cytotoxic *P. haemolytica* A11 of bighorn sheep origin; Group 2 and Group 3 controls.
[c] Group 1 and Group 3 inoculated with 6×10^9 cfu of cytotoxic *P. haemolytica* A2 of domestic sheep origin; Group 2 controls.
[d] NA, Not applicable.
[e] Unt, Untypeable.

1994); based on these experimental data, *P. haemolytica* A2 is the most serious primary pathogen in the respiratory disease complex in bighorn sheep. Some success has been achieved in protecting domestic sheep against experimental *P. haemolytica* infection using a crude cytotoxin vaccine (Sutherland et al., 1989), but protection of bighorn sheep against *P. haemolytica* infections has not been accomplished. We recently isolated cytotoxic and noncytotoxic strains of *P. haemolytica* A11 from dead free-ranging bighorn sheep. Our objectives were to determine if these organisms were lethal in healthy bighorn sheep, and if the cytotoxic A11 strain would protect bighorn sheep against a challenge inoculation of cytotoxic *P. haemolytica* A2 from domestic sheep known to be lethal in bighorn sheep.

MATERIALS AND METHODS

Six bighorn sheep (*O. canadensis canadensis*) were used in these experiments between February and April 1994, and were divided into three groups of two animals each. Group I consisted of a yearling male (bighorn 1) and a male lamb (bighorn 2), group 2 consisted of a yearling male (bighorn 3) and a 2 ½ yr-old male (bighorn 4), and group 3 consisted of two male lambs (bighorns 5 and 6) (Table 1). All bighorn sheep had been in captivity for over 6 mo, and were maintained together in a 0.4-ha pen with shelter at Washington State University, Pullman, Washington (USA). All bighorn sheep were clinically healthy at the beginning of the experiment, and contact with other ruminants was not allowed. Feed consisted of free-choice alfalfa hay and alfalfa pellets, supplemented with pasture grasses within the pen. Mineralized salt and water were available at all times.

Microbiology and cytotoxicity techniques

Three isolates of *P. haemolytica* were used for inoculation in this experiment. A noncytotoxic isolate of *P. haemolytica* A11 was obtained from the lung of a 2-yr-old male bighorn killed by Oregon Department of Fish and Wildlife personnel in Hells Canyon, Oregon (USA) (47°27′N, 116°36′W) after it was observed commingling with domestic sheep. A cytotoxic isolate of *P. haemolytica* A11 was obtained from the lung of a mature male bighorn found dead near Troy, Oregon (45°56′N, 117°29′W). A cytotoxic isolate of *P. haemolytica* A2 was obtained from a pharyngeal swab sample from a healthy domestic sheep maintained at the University of Idaho Sheep Center, Moscow, Idaho (USA) (Foreyt et al., 1994). Isolates were originally frozen in phosphate buffered glycerol (PBG) (Foreyt and Lagerquist, 1994) at −70 C until they were regrown on 5% sheep blood

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agar (Becton Dickinson Microbiology Systems, Cockeysville, Maryland, USA). Cytotoxic isolates of *P. haemolytica* were defined (Silflow et al., 1994) as causing >50% neutrophil death in 1 hr at a toxin concentration of ≤150 μg/50μl of Hanks balanced salt solution (HBSS) (Gibco Laboratories, Grand Island, New York, USA) containing 1% fetal bovine serum (FBS) (Hyclone Laboratories, Logan, Utah, USA).

Peripheral blood neutrophils from four captive Rocky Mountain bighorn sheep (*O. canadensis canadensis*) were tested for sensitivity to cytotoxins produced by the various strains of *P. haemolytica*. The four bighorn sheep blood donors consisted of two yearling females, a yearling male, and an adult male which were maintained in captivity at Washington State University, Pullman, Washington. Peripheral blood samples (approximately 40 ml) were obtained from each bighorn sheep at each bleeding by jugular venipuncture into acid citrate anticoagulant solution. Samples were centrifuged at 850 × *G*, plasma and buffy coats discarded, and red blood cells hypotonically lysed by adding distilled water followed by phosphate buffered saline. Lysis and centrifugation steps were repeated, and final cell pellets resuspended in HBSS containing 1% FBS. Cells were counted using a hemocytometer (American Optical Corporation, Buffalo, New York), and cell viability determined by trypan blue exclusion (Boyse et al., 1964). Yields were generally >90% neutrophils with >90% viability. Neutrophils from each animal were adjusted to a final concentration of 5×10^6 cells/ml (Silflow et al., 1993).

Cytotoxins were partially purified from bacterial culture supernatants using the method of Shewen and Wilkie (1982). For the cytotoxin evaluation, individual *P. haemolytica* isolates were streaked onto 5% sheep blood agar plates and incubated for 18 to 24 hr at 37 C. A negative control bacterium, *Enterobacter cloacae* (American Type Culture Collection No. 35030), was cultured identically. Morphologically similar bacteria colonies of *P. haemolytica* were inoculated into 100 ml of brain-heart infusion (BHI) broth (Difco Laboratories, Detroit, Michigan, USA), which was incubated for 3 to 5 hr at 37 C to attain early logarithmic growth until a concentration of 8×10^8 bacterial cells/ml was reached. Bacterial concentration was evaluated using a spectrophotometer (Beckman Inc., Palo Alto, California, USA) at a wavelength of 600 nm. Bacteria were centrifuged at 6,000 × *G* for 10 min, the supernatant discarded, and the bacterial pellet resuspended in 30 ml of Roswell Park Memorial Institute (RPMI)-1640 medium (Gibco Laboratories) containing 7% FBS. After incubation for 1 hr at 37 C, bacteria again were centrifuged, and the culture supernatant removed and filter-sterilized by passage through a 0.45 μm filter (Sigma Chemical Company, St. Louis, Missouri, USA).

Cytotoxic potencies of *P. haemolytica* isolates were assessed by adding bacterial culture supernatants to bighorn sheep neutrophils in vitro. Neutrophils from each of the four bighorn sheep were tested individually with each supernatant. Cytotoxicity was quantitated by measuring the release of lactate dehydrogenase (LDH) into the culture medium (Korzeniewski and Callewaert, 1983). Bacteria culture supernatants were diluted in HBSS containing 1% FBS to concentrations of 150, 100, 50, 5, and 0.5 μg/50 ul for determination of toxicity. Higher concentrations of cytotoxin were not tested because of potential interference of proteins from the FBS. Fifty μl of each supernatant preparation was added to the wells of 96-well plates, followed by addition to each well of 2.5×10^5 neutrophils in 50 μl of HBSS containing 1% FBS. Each concentration of cytotoxin was tested three times against neutrophils from each of the four bighorn sheep. After 1 hr incubation, 100 μl of LDH substrate was added. Release of LDH enzyme (equated to neutrophil death) was quantitated with a 96-well plate reader coupled to an on-line computer. Samples were compared to neutrophils treated with a 0.5% solution of saponin detergent (Sigma Chemical Company) (maximal LDH release) and untreated cells (background LDH release). Samples were also compared to a negative control (*Enterobacter cloacae*) and positive control (a domestic sheep isolate of *P. haemolytica* A2 known to be cytotoxic). Results were recorded as a percentage of LDH released from lysed cells.

Cytotoxic potency of various *P. haemolytica* isolates were determined by the 50% lethal concentration (LC_{50}), represented by the graphic intersection of supernatant concentration and 50% neutrophil death (Silflow et al., 1993), with a curve fitted to the graph. Differences between groups were compared graphically.

Nasal swab samples (Marion Scientific Viral Culturette, Marion Scientific, Kansas City, Kansas, USA) were collected for virus evaluation on the day the inoculation experiment began. Specimens were inoculated onto ovine embryonic tracheal cells (American Type Culture Collection No. CCL 44) and bovine turbinate cells (American Type Culture Collection No. CRL 1390) for two passages at 10-day intervals and were examined daily for cytopathic effect (Castro, 1992). Additional specimens were tested for respiratory syncytial virus by use of solid phase-enzyme immunoassay (Ab-

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bott RSV EIA, Abbott Laboratories, South Pasadena, California). Isolation of *Chlamydia* spp. was not attempted. Fecal samples from all six bighorn sheep were evaluated for lungworm larvae by a modified Baermann technique (Beane and Hobbs, 1983).

Bighorn sheep that died were submitted to the Washington Animal Disease Diagnostic Laboratory (WADDL), Pullman, Washington. Standard necropsy, microbiological and pathological techniques were used by WADDL personnel.

Samples of lung, heart, cervical and tracheobronchial lymph nodes, brain, spleen, kidney, jejunum, and adrenal gland were fixed in 10% buffered formalin. They then were embedded in paraffin, sectioned at 5 μm and stained with hematoxylin and eosin.

Experimental design

Bacterial cultures of the *P. haemolytica* A2 and the two *P. haemolytica* A11 isolates for bighorn sheep inoculation were grown in BHI broth at 37 C for approximately 16 hr before inoculation. Estimated concentration of bacteria in the BHI broth culture just prior to inoculation was determined by the McFarland Method (Balows et al., 1991), and final concentration of *P. haemolytica* in the inoculum was measured in colony forming units (cfu) per ml by culturing ten-fold serial dilutions retrospectively after inoculations had been given. Bighorn sheep were captured in a drive net, and while animals were restrained physically, the inoculations were injected intratracheally with a 3 cc syringe and 20 gauge needle.

On experimental day 0, group 1 bighorn sheep each were inoculated intratracheally with 6×10^9 cfu of the cytotoxic strain of *P. haemolytica* A11; group 2 bighorn sheep each were inoculated intratracheally with 6×10^9 cfu of the noncytotoxic *P. haemolytica* A11, and group 3 control bighorn sheep each were inoculated intratracheally with BHI broth. The volume of inoculum given to each group was 0.5 ml. At the time of inoculation (day 0), group 3 bighorn sheep were moved to an adjacent 0.4 ha pen which was separated from the first pen by 4 m to prevent nose to nose contact. Husbandry was essentially identical. After inoculation, all bighorn sheep remained healthy. On experimental day 16, group 1 bighorn sheep each were given the same intratracheal inoculation as on day 0, and groups 2 and 3 bighorn sheep each were inoculated with BHI broth at the same 0.5 ml volume as group 1. All bighorn sheep remained healthy following inoculations. On experimental day 42, bighorn sheep in groups 1 and 3 each were challenged with an intratracheal inoculation of 6×10^9 cfu of *P. haemolytica* A2 of domestic sheep origin known to be lethal in bighorn sheep. Group 2 sheep each were inoculated intratracheally with BHI broth at the same 0.5 ml volume as groups 1 and 3. At the time of inoculation, group 2 bighorn sheep were moved to pen 2, and group 3 bighorn sheep were moved back to pen 1 with group 1 bighorn sheep to separate the two noninoculated controls from the four challenge inoculated bighorn sheep.

On experimental days 0, 16, and 42, pharyngeal swab samples were collected from all bighorn sheep with sterile cotton-tipped swabs (Foreyt et al., 1994), and transported in phosphate buffered glycerol (PBG) to WADDL within 30 min after collection. All pharyngeal swab samples were streaked on sheep blood agar plates within 2 hr of collection to maximize isolation of *P. haemolytica* (Wild and Miller, 1991). Isolates of *P. haemolytica* were identified according to the methods of Foreyt et al. (1994). Biotyping (Biberstein, 1978) and serotyping (Frank and Wessman, 1978) of *P. haemolytica* isolates also were done. When isolates of *P. haemolytica* reacted in antisera to several serotypes, all cross-reacting serotypes were listed. Isolates of *P. haemolytica* recovered from bighorn sheep were frozen in PBG at −70 C until they were regrown on 5% sheep blood agar and tested for cytotoxic activity.

RESULTS

All bighorn sheep remained healthy after the first two inoculations of cytotoxic and noncytotoxic *P. haemolytica* A11, or BHI broth. Before inoculation, only serotype T3,4 was isolated from each bighorn sheep (Table 1). On experimental day 16, serotype T3,4 was isolated from two sheep, T3,4,10 from two sheep, an untypeable T from one sheep, and no *P. haemolytica* was isolated from two sheep. On day 42, a variety of *P. haemolytica* serotypes were isolated including T3,4, T4, T3,4,10, A11, A6,11, A7,11 (Table 1). Viruses or *Protostrongylus* sp. larvae were not isolated from any of the sheep.

After challenge with *P. haemolytica* A2, all four challenge-inoculated bighorn sheep died from pneumonia within 72 hr, and the two BHI inoculated control bighorn sheep remained healthy. At necropsy, all sheep were in moderate to good body condition. Lesions were similar in all

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sheep and were an acute fibrinohemorrhagic bronchopneumonia and pleuritis. Approximately 0.5 to 1.0 l of serosanguinous fluid was in the thoracic cavity. Cranial portions of ventral lung lobes were most severely affected. Up to 80% of lung volume was dark red and firm. Cervical and tracheobronchial lymph nodes were consistently enlarged. Histologically, lesions of acute bronchopneumonia were in all sheep, and included necrosis of bronchiolar epithelium and fibrinopurulent exudate in distal airways. Basophilic bacterial colonies were mixed with fibrinocellular exudate in alveoli of all bighorn sheep and in the liver of one sheep. Cytotoxic *P. haemolytica* A2 was isolated from the lungs of all four challenge inoculated bighorn sheep (Table 1). Other noncytotoxic isolates recovered from one or more sheep at necropsy included T3, T3,4, and T3,4,15 (Table 1). The three A biotype isolates could not be regrown on blood agar for toxicity testing.

DISCUSSION

Based on our results the cytotoxic and noncytotoxic strains of *P. haemolytica* A11 used in these experiments were not lethal in bighorn sheep when administered intratracheally, and premunization of bighorn sheep with this novel cytotoxic strain of *P. haemolytica* A11 was unsuccessful in protecting bighorn sheep from death caused by cytotoxic *P. haemolytica* A2 of domestic sheep origin. We conducted this experiment with the knowledge that bighorn sheep and domestic sheep contact likely results in fatal pneumonia in the bighorn sheep due to *P. haemolytica* A2 (Foreyt et al., 1994). Furthermore, the A11 serotype was chosen because the cytotoxins produced by biotype T isolates are not recognized by monoclonal antibodies developed against biotype A isolates (Gerbig et al., 1992). Because *P. haemolytica* A2 commonly is carried by domestic sheep (Frank, 1982) and is rare in healthy bighorn sheep (Dunbar et al., 1990; Silflow et al., 1993), protection of bighorn sheep from *P. haemolytica* A2 would be very beneficial for management and survival of bighorn sheep in areas where domestic sheep contact is possible. Based on our experience, cytotoxic serotypes of *P. haemolytica* are uncommon in healthy free-ranging bighorn sheep populations, and the survival of our captive bighorn sheep inoculated with the novel cytotoxic serotype A11 provided justification to test this organism as a potential protective premunizing agent against cytotoxic *P. haemolytica* A2 in bighorn sheep. Although the numbers of experimental animals was limited due to humane concerns, the experiment obviously failed, and may corroborate data that most or all serotypes found in bighorn sheep are not protective against the virulent A2 serotype found commonly in domestic sheep. We are unaware of virulent *P. haemolytica* A2 existing in healthy free-ranging bighorn sheep. Current management techniques to prevent pneumonia caused by *P. haemolytica* A2 in bighorn sheep populations mandate the separation of domestic and bighorn sheep to prevent nose to nose transmission of *P. haemolytica,* primarily A2, between species (Foreyt, 1989).

The cytotoxicity assay used in this study has been used by our laboratory for comparing cytotoxin dependent killing potential of different serotypes of *P. haemolytica,* and for comparing neutrophil susceptibility of different animal species to cytotoxin mediated injury (Silflow and Foreyt, 1994; Silflow et al., 1994). Based on the *P. haemolytica* cytotoxin killing of neutrophils in one study (Silflow et al., 1994), bighorn sheep and Dall sheep (*Ovis dalli dalli*) were more sensitive to pneumonia caused by cytotoxic *P. haemolytica* than mountain goats (*Oreamnos americana*) and domestic sheep. Elk (*Cervus elaphus*) and white-tailed deer (*Odocoileus virginianus*) were not sensitive to cytotoxin-dependent killing and were considered to be resistant species. We envision the assay to be useful in the potential identification of bighorn sheep herds with resis-

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tance to pneumonia caused by *P. haemolytica,* and as a method to evaluate serotypes of *P. haemolytica* in terms of virulence. Cytotoxicity is a major virulence factor of pasteurellosis in cattle (Whiteley et al., 1992) and likely also in bighorn sheep. Because the cytotoxic A11 used in two of our bighorn sheep did not result in mortality under the conditions described in our experiment, the cytotoxin may not be the only virulence factor involved in pasteurelloses in bighorn sheep. Other factors that also may be important in the development of pasteurellosis in bighorn sheep are the role of lipopolysaccharide, presence or absence of a capsule (Wilson et al., 1992), and production of specific chemotactic signals by bacteria and inflammatory cells (Silflow et al., 1989; Czuprynski et al., 1991; Henricks et al., 1992).

Documentation of bacterial profiles present within herds of bighorn sheep, as well as sensitivity of neutrophils to cytotoxin-dependent lysis may be helpful in assessing the overall health risk of bighorn sheep populations. Based on the importance of respiratory disease in bighorn sheep populations, additional research to determine methods of preventing disease transmission between domestic and bighorn sheep is necessary, especially in areas where contact between the two species is possible.

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