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COMPARATIVE LEUKOTOXICITIES OF *PASTEURELLA HAEMOLYTICA* ISOLATES FROM DOMESTIC SHEEP AND FREE-RANGING BIGHORN SHEEP (*OVIS CANADENSIS*)

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ABSTRACT: Twenty-eight isolates of *Pasteurella haemolytica* from domestic sheep ($n = 14$ isolates) and bighorn sheep ($n = 14$ isolates) were evaluated for leucotoxicity against peripheral blood neutrophils of bighorn sheep by adding bacterial culture supernatants to bighorn sheep neutrophils in vitro. Leukotoxic isolates of *P. haemolytica*, defined as causing >50% neutrophil death as measured by release of lactate dehydrogenase into culture supernatants, were identified from eight of 14 domestic sheep isolates and from 0 of 14 bighorn sheep isolates. The in vitro assay of isolates of *P. haemolytica* may provide a valid predictive measure of strain virulence of *P. haemolytica*, and of potential pneumonic episodes in bighorn sheep populations.

Key words: *Pasteurella haemolytica*, bighorn sheep, *Ovis canadensis*, leukotoxicity, cytotoxin, in vitro test.

INTRODUCTION

Respiratory disease is a serious problem affecting survival of bighorn sheep (*Ovis canadensis*) populations throughout western North America. Lungworm infection, augmented by susceptibility to pulmonary viral and bacterial pathogens, and environmental stressors, promotes respiratory tract disease and mortality from bacterial pneumonia, principally caused by *Pasteurella haemolytica* (Spraker et al., 1984; Onderka and Wishart, 1984). Domestic sheep often are asymptomatic carriers of leukotoxic strains of *P. haemolytica* (Silflow et al., 1993), and there is both circumstantial and experimental evidence that direct contact between domestic and bighorn sheep has resulted in fatal epizootics of pneumonia in bighorn populations (Foreyt and Jessup, 1982; Foreyt, 1989). In areas where these two species share habitat, potential pathogen transmission exists from domestic to bighorn sheep.

Pasteurella haemolytica has been isolated from pneumonic bighorn sheep after pasturing with domestic sheep (Foreyt, 1989, 1992), and from pneumonic and normal bighorn sheep without any known domestic sheep contact (Spraker et al., 1984; Miller et al., 1991). Based on the lack of clinical signs in healthy bighorn sheep from which *P. haemolytica* has been isolated (Foreyt, unpubl.), animals may acquire immunity from previous exposure, have increased genetic resistance, or strains of *P. haemolytica* with reduced virulence may be involved. A major virulence factor contributing to the pathogenesis of pneumonic pasteurellosis is a soluble leukotoxin (cytotoxin) produced by *P. haemolytica* (Lo, 1990). We have shown previously that bighorn sheep are more susceptible than domestic sheep to neutrophil lysis by leukotoxin (Silflow et al., 1993).

Our objective was to compare the relative leukotoxicities of *P. haemolytica* isolates from eight clinically healthy domestic sheep, and seven clinically healthy free-ranging bighorn sheep from a population in northeastern Washington (USA). We estimated the prevalence of potentially virulent strains of *P. haemolytica* within these two groups of animals and quantified differences in leukotoxicity in bighorn sheep. Our research is intended to enhance understanding of the transmission of *P. haemolytica* between domestic and bighorn sheep and to improve success in managing populations through selective reintroductions.

MATERIALS AND METHODS

Peripheral blood neutrophils from four captive Rocky Mountain bighorn sheep (*O. canadensis canadensis*) were tested for sensitivity to

leukotoxins produced by various strains of *P. haemolytica* isolated from the respiratory tracts of domestic and bighorn sheep. The bighorn sheep consisted of two yearling ewes, a yearling ram, and an adult ram which were maintained in captivity at Washington State University, Pullman, Washington.

Peripheral blood samples (approximately 40 ml) were obtained from each bighorn sheep by jugular venipuncture into acid citrate 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 Hanks Balanced Salt Solution (HBSS) (Gibco Laboratories, Grand Island, New York, USA) containing 1% fetal bovine serum (FBS) (Hyclone Laboratories, Logan, Utah, USA). 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).

Twenty-eight isolates of *P. haemolytica*, including 14 from domestic sheep and 14 from bighorn sheep, were compared for leukotoxic activity. Domestic sheep isolates were obtained in July 1993 from eight adult Suffolk ewes in a research herd at University of Idaho, Moscow, Idaho (USA). Bighorn sheep isolates were obtained in December 1992 from seven adult ewes in a free-ranging herd at Hall Mountain (48°50′N, 117°15′W) in northeastern Washington. All domestic and bighorn sheep sampled appeared clinically healthy. Isolates were obtained from the pharyngeal area of the sheep with sterile cotton-tipped swabs, and transported in phosphate buffered glycerol (PBG) to the Washington Animal Disease Diagnostic Laboratory, Pullman, Washington. Biotyping (Biberstein, 1978) and serotyping (Frank and Wessman, 1978) of *P. haemolytica* isolates were done, and the isolates were frozen in PBG at −70 C until they were regrown and tested for leukotoxic activity. When an isolate reacted in antisera to several serotypes, all cross-reacting serotypes were listed.

Leukotoxins were partially purified from bacterial culture supernatants using the method of Shewen and Wilkie (1982). Individual *P. haemolytica* isolates, representing a variety of biotypes and serotypes from domestic and bighorn sheep, were streaked onto 5% sheep blood agar plates and incubated for 18 to 24 hr at 37 C. A negative control bacterium, *Enterobacter cloacae* (ATCC #35030), was cultured identically. Morphologically similar bacterial colonies of *P. haemolytica* were inoculated into 100 ml of brain-heart infusion broth (Difco Laboratories, Detroit, Michigan, USA), and incubated for 3 to 5 hr at 37 C to attain early logarithmic growth. 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 RPMI-1640 medium (Gibco Laboratories, Grand Island, New York) 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). Culture supernatants were dialyzed (6,000 to 8,000 molecular weight cutoff) (Spectrum Medical Industries, Los Angeles, California) to exhaustion against distilled water at 4 C, and then lyophilized.

Leukotoxic potencies of *P. haemolytica* isolates from domestic and bighorn sheep 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. Leukotoxicity was quantitated by measuring the release of lactate dehydrogenase (LDH) into the culture medium (Korzeniewski and Callewaert, 1983). Supernatants were diluted to concentrations of 100, 50, 10, 5, and 1 μg/50 μl by resuspending lyophilized samples in HBSS containing 1% FBS. 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 leukotoxin 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, St. Louis, Missouri) (maximal LDH release) and untreated cells (background LDH release) as well as to negative (*Enterobacter cloacae*) and positive (a domestic sheep isolate of *P. haemolytica* A2 known to be leukotoxic) controls. Results were recorded as a percentage of LDH released from saponin lysed cells.

Leukotoxic potency of various *P. haemolytica* isolates from domestic and bighorn sheep were determined from the 50% lethal concentration

TABLE 1. Leukotoxicity of domestic sheep *Pasteurella haemolytica* isolates to bighorn sheep neutrophils. Eight of the 14 isolates caused >50% release of lactate dehydrogenase (LDH) from exposed neutrophils.

Isolate	Biotype/serotype	% LDH release at leukotoxin concentration (μg/50 μl)					Lethal concentration$_{50}$ (μg/50 μl)
		100	50	10	5	1	
Positive control	A2	91	74	54	33	1	9
Negative control	*Enterobacter* sp.	8	4	0	1	0	—
4	A2	75	66	38	10	2	27
6	NT[a]	79	66	4	0	1	40
20	A2	76	58	24	6	0	41
7	NT	80	59	18	3	1	42
9	NT	60	48	3	0	0	60
5	T3	61	34	0	0	0	80
17	NT	56	35	2	1	0	86
2	NT	52	26	1	0	0	96
8	NT	15	5	2	2	0	—
15	NT	14	2	1	1	0	—
21	NT	14	5	0	0	0	—
16	NT	11	7	4	1	0	—
3	T3,4,10	8	3	0	0	0	—
24	A11	7	2	0	0	0	—

[a] NT = not typeable.

(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.

RESULTS

Culture supernatants of eight of 14 *P. haemolytica* isolates from the domestic sheep had leukotoxic activity (defined as ≥50% LDH release) when tested with bighorn sheep neutrophils (Table 1). Leukotoxic culture supernatants of *P. haemolytica* isolates were detected from five of eight domestic sheep, and from none of the seven bighorn sheep. Culture supernatants from six of 14 domestic sheep isolates were nonleukotoxic (defined as <50% LDH release) (Table 1), and all 14 culture supernatants from the bighorn sheep were nonleukotoxic (Table 2). Range of the LC_{50} for leukotoxic *P. haemolytica* isolates was 27 to 96 μg/50 μl (Table 1); whereas, the LC_{50} for the nonleukotoxic isolates was greater than 100 μg/50 μl and could not be determined in the study because 100 μg/50 μl was the highest concentration used (Fig. 1).

Most *P. haemolytica* isolates from domestic sheep could not be serotyped due to autoagglutination (Table 1). Two of eight isolates with leukotoxic activity were identified as biotype/serotype A2 and one was identified as biotype/serotype T3. Nonleukotoxic isolates from domestic sheep included one T3,4,10 and one A11; the rest were untypeable. Of the 14 nonleukotoxic isolates from bighorn sheep, eight were T3,4,10, two were T3,4, two were T4, and two were untypeable (Table 2).

DISCUSSION

There was a higher prevalence of leukotoxic activity for isolates of *P. haemolytica* from domestic sheep than for isolates from bighorn sheep (57% vs. 0%) when tested in vitro with bighorn sheep neutrophils. Absence of leukotoxicity (defined as low toxicity under the specific conditions of these experiments) in bighorn sheep isolates of *P. haemolytica* was consistent with previous observations that healthy free-ranging bighorn sheep populations usually do not carry detectable leukotoxic strains of *P. haemolytica* (Foreyt, unpubl.); whereas, healthy domestic sheep populations often carry leukotoxic strains (Silflow

TABLE 2. Leukotoxicity of bighorn sheep *Pasteurella haemolytica* isolates to bighorn sheep neutrophils. None of the 14 isolates caused >20% release of lactate dehydrogenase (LDH) from exposed neutrophils.

Isolate	Biotype/serotype	% LDH release at leukotoxin concentration (μg/50 μl)					Lethal concentration$_{50}$ (μg/50 μl)
		100	50	10	5	1	
Positive control	A2	100	89	45	21	2	15
Negative control	*Enterobacter* sp.	9	5	2	0	0	—
12	NT*	17	6	0	0	0	—
5	T3,4,10	12	5	0	0	0	—
11	T3,4	11	7	1	0	0	—
1	T4	10	5	0	0	2	—
25	T3,4	10	3	0	0	0	—
26	T3,4,10	10	5	0	0	0	—
2	T3,4,10	9	4	0	0	0	—
4	T4	9	5	0	0	0	—
6	T3,4,10	8	3	0	1	0	—
9	T3,4,10	7	4	1	0	0	—
7	T3,4,10	5	2	2	0	0	—
24	T3,4,10	5	2	0	0	0	—
46	NT	5	2	0	0	0	—
45	T3,4,10	4	4	0	0	0	—

* NT = not typeable.

et al., 1993). Although the amount of toxin may vary over time (Gentry et al., 1988), we only examined for toxin after 1 hr of incubation to be consistent with previous studies (Shewen and Wilkie, 1982). Based on experimental and field data, we believe that healthy domestic sheep often carry leukotoxic strains of *P. haemolytica*, and when those strains are transferred to bighorn sheep through close contact or experimental inoculation (Foreyt et al., 1994); the bighorn sheep die from pneumonia because of their increased susceptibility to respiratory disease (Silflow et al., 1991, 1993). The Hall Mountain herd of free-ranging bighorn sheep from which the samples were collected never has had a known respiratory disease problem, and has been used for numerous transplants.

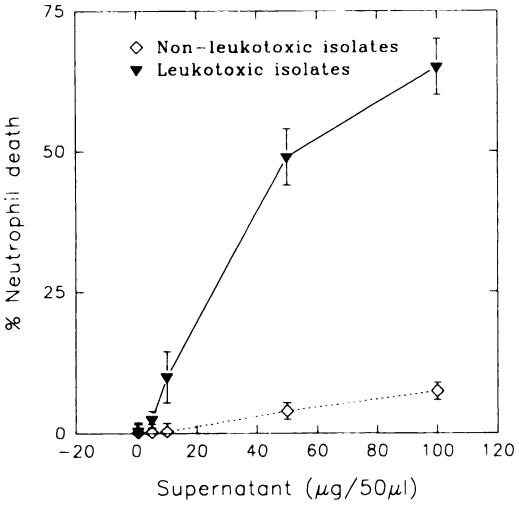

FIGURE 1. Dose response curves of supernatants from *Pasteurella haemolytica* isolates obtained from domestic sheep and bighorn sheep, and tested for leukotoxicity on bighorn sheep neutrophils. The curves represent the mean ± SE for all leukotoxic isolates (n = 8) and all nonleukotoxic isolates (n = 20).

Past experiments involving domestic sheep and bighorn sheep contact have resulted in death in a high proportion of the bighorn sheep (Foreyt, 1989, 1992). In our experiment, eight of 14 *P. haemolytica* isolates from domestic sheep were leukotoxic, but six isolates were nonleukotoxic. This finding is consistent with our belief that some domestic sheep carry less virulent or avirulent strains of *P. haemolytica* that are not pathogenic to bighorn sheep. However, based on our results, the probability of recovering a leukotoxic, and therefore potentially lethal *P. haemolytica*

isolate from domestic sheep is high. In contrast, our ability to detect a leukotoxic *P. haemolytica* isolate from a healthy bighorn sheep population was unsuccessful in this experiment. Whether this pattern is represented in other bighorn sheep populations with different serotypes and ribotypes (Snipes et al., 1992) warrants further study.

In this study we used isolates of *P. haemolytica* from only seven bighorn sheep and eight domestic sheep from distinct populations at one point in time. We suggest additional testing of isolates from sympatric and isolated populations of bighorn and domestic sheep to assess prevalence of leukotoxic and nonleukotoxic strains of *P. haemolytica* and to determine whether prevalence of leukotoxic strains changes with herd disease status. In vitro testing for leukotoxicity of *P. haemolytica* isolates from pneumonic and healthy bighorn sheep, combined with challenge experiments in live animals, would indicate whether in vitro assays of leukotoxicity provide a valid predictive measure of strain virulence in *P. haemolytica*. The ability to distinguish virulent and avirulent strains of *P. haemolytica* and to compare the *P. haemolytica* profiles of potential donor and recipient herds will assist wildlife managers in selecting bighorn sheep for reintroduction or herd supplementation, and will provide data to assist in land use decisions regarding copasturing bighorn sheep with animals that carry *P. haemolytica*. Until additional data are evaluated, it would be wise to minimize contact between domestic and bighorn sheep because of the potential for transmission of pathogenic strains of *P. haemolytica* to bighorn sheep, resulting in fatal pneumonia. Likewise, bighorn sheep that are identified as carriers of leukotoxic *P. haemolytica* strains should be kept isolated from other healthy bighorn sheep populations.

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