

EVALUATION OF THE CYTOTOXICITY OF VARIOUS ISOLATES OF *PASTEURELLA HAEMOLYTICA* FROM BIGHORN SHEEP AND OTHER UNGULATE POPULATIONS.

RONALD M. SILFLOW, Department of Veterinary Microbiology and Pathology, Washington State University, Pullman, WA 99164

WILLIAM J. FOREYT, Department of Veterinary Microbiology and Pathology, Washington State University, Pullman, WA 99164

JOHN E. LAGERQUIST, Department of Veterinary Microbiology and Pathology, Washington State University, Pullman, WA 99164

Abstract: An assay has been developed for the purpose of evaluating the cytotoxic potencies of various isolates of *Pasteurella haemolytica*. The assay allows measurement of the potency of a soluble toxin released by different *P. haemolytica* organisms isolated from a variety of ungulates including bighorn sheep (*Ovis canadensis*), domestic sheep, Dall sheep (*Ovis dalli dalli*), mouflon sheep (*Ovis musimon*), mountain goats (*Oreamnos americana*), domestic goats, and elk (*Cervus elaphus*). Peripheral blood neutrophils were used as target cells for cytotoxin-dependent killing. Cytotoxicity was quantitated by assessing the release of lactate dehydrogenase by neutrophils into the culture medium. Results are shown from a variety of *P. haemolytica* isolates collected from bighorn sheep representing geographically distinct populations, and from other ungulate species including: deer (*Odocoileus* spp.), elk, Dall sheep, mountain goats, mouflon sheep and domestic sheep. Neutrophil susceptibility to cytotoxin-mediated injury is compared among Dall sheep, mountain goats, bighorn sheep, domestic sheep, deer, and elk. The information gained can facilitate management decisions necessary for maintaining healthy bighorn sheep and other wildlife populations.

Respiratory disease caused by pasteurellosis results in mortality in both bighorn sheep and domestic sheep, yet it is reported less frequently in deer or elk (Thorne 1982, Franson and Smith 1988). To our knowledge, bacterial pneumonia has not been reported in Dall sheep or mountain goats. *P. haemolytica* can be isolated from many ruminant species whether respiratory disease symptoms exist or not (Frank 1982, Clark et al. 1985, Miller et al. 1991, Wild and Miller 1991). Bighorn sheep are more susceptible to respiratory infections than domestic sheep (Foreyt 1988, Onderka and Wishart 1988, Onderka et al. 1988, Callan et al. 1991) and this difference in susceptibility can be partially explained by the greater sensitivity of bighorn sheep neutrophils to cytotoxin-dependent killing by *P. haemolytica* organisms (Silflow et al. 1993).

Contact transmission of *P. haemolytica* from domestic sheep to bighorn sheep populations may have devastating consequences to bighorn sheep survival. The observation of mortality losses in free ranging bighorn sheep exposed to domestic sheep is confirmed by the results of experimental trials in

which transmission of *P. haemolytica* between these two sheep species were tested on captive bighorn sheep (Foreyt 1988, Onderka and Wishart 1988, Onderka et al. 1988, Callan et al. 1991). These experiments, conducted by three different investigative teams, all resulted in high mortality losses in bighorn sheep but not in domestic sheep. Important questions extending from these results include whether other wild species, such as Dall sheep, mountain goats, deer and elk, also are susceptible to mortality losses due to *P. haemolytica*, and whether Dall sheep, mountain goats, deer or elk serve as a reservoir of *P. haemolytica* which can be transmitted to bighorn sheep. Previous experiments involving deer and elk contact with bighorn sheep did not result in respiratory disease in any of the animals (Foreyt 1992).

The cytotoxin produced by some *P. haemolytica* isolates is an important virulence factor in the development of respiratory disease in many ruminant species. Colonization of the lower respiratory tract with *P. haemolytica* results in

exogenous release of a soluble toxin capable of exacerbating the acute inflammation which is characteristic of pasteurellosis (Baluyut et al. 1981, Berggren et al. 1981). Previous studies have focused primarily on the neutrophil as the target phagocytic cell susceptible to cytotoxin mediation (Baluyut et al. 1981, Confer et al. 1990, Czuprynski et al. 1991, Silflow et al. 1993). Studies with the cytotoxin isolated from cattle have shown that, at high concentrations, the cytotoxin causes lysis of the neutrophil resulting in the release of intracellular components which can cause damage to the integrity of the lung (Czuprynski et al. 1991). At low concentrations, the cytotoxin can activate neutrophils to release oxygen intermediates and granule constituents capable of causing host lung damage which may lead to morbidity or mortality losses (Czuprynski et al. 1991). In addition to the neutrophil, cytotoxin mediation occurs in other immune cells, including suppressed proliferation of bovine peripheral mononuclear cells (Czuprynski and Ortiz-Carranza 1992) and lymphocytes (Majury and Shewen 1991), and lethal and sublethal effects on alveolar macrophages from cattle (Markham and Wilkie 1980, Markham et al. 1982) and sheep (Sutherland et al. 1983).

Alveolar macrophages are the phagocytic cells responsible for initial defense of the lung against any infectious or non-infectious agent entering the lower airways (Liggitt 1985). Previous comparisons of bighorn and domestic sheep species revealed no differences in the numbers of phagocytic cells in the alveolar spaces, nor were there any differences in the phagocytic or bactericidal activities of alveolar macrophages (Silflow et al. 1989). During early exposure of the lower respiratory tract to *P. haemolytica*, the first phagocytic cells to encounter cytotoxin are alveolar macrophages. However, we have shown that alveolar macrophages are more resistant to killing by *P. haemolytica* supernatants than are neutrophils (Silflow and Foreyt 1994).

Our objectives were: 1) to measure the potency of cytotoxin released by different *P. haemolytica* organisms isolated from a variety of ungulates on neutrophils, 2) to measure the potency of cytotoxin from a variety of *P. haemolytica* isolates collected from bighorn sheep representing geographically distinct populations, and 3) to compare the sensitivity of neutrophils from different species to cytotoxin-dependent lysis.

METHODS

Animals

Rocky Mountain bighorn sheep (*Ovis canadensis canadensis*) from the captive herd at Washington State University were used as a source of neutrophils for the assays performed in this study. For each assay, neutrophils from 3 bighorn sheep (ewes and rams ranging in age from 1-4 years) were tested for cytotoxin-dependent lysis by *P. haemolytica* isolates. In addition, for the experiment to compare different species for neutrophil sensitivity to cytotoxin-dependent lysis, neutrophils were collected from mountain goats ($n = 2$), Dall sheep ($n = 3$), domestic sheep ($n = 3$), deer ($n = 4$) and elk ($n = 6$). All animals were clinically healthy when samples were collected.

Neutrophil Collection and Purification

Peripheral blood samples were collected into citrate phosphate dextrose solution (Sigma Chemical Company, St. Louis, Missouri, USA) by jugular venipuncture. Following centrifugation at 850 x g for 15 min, the plasma and buffy coats were discarded. Hypotonic lysis of red cells was accomplished by the addition of 45 ml distilled water for 45 sec followed by the addition of 5 ml of 10X phosphate buffered saline. Following centrifugation at 600 x g for 10 min, the lysis and centrifugation steps were repeated, and the final cell pellets were resuspended in Hanks Balanced Salt Solution (HBSS) + 1% fetal bovine serum (FBS). Cells were quantitated using a hemocytometer, and cell viability was determined by trypan blue exclusion. Typical yields were > 90% neutrophils, and these cells exhibited > 90% viability. For each experiment, cells were adjusted to a concentration of 5×10^6 cells/ml in HBSS + 1% FBS.

Cytotoxin Preparation

Cytotoxins were isolated from culture supernatants using the method of Shewen and Wilkie (Shewen and Wilkie 1982). The bacterial isolates were characterized according to biotype and serotype at the Washington State Disease Diagnosis Laboratory in Pullman, Washington. Individual *P. haemolytica* isolates were streaked onto 5% blood agar plates (Beckton Dickinson Microbiological Systems, Cockeysville, Maryland, USA) and incubated for 18 hr at 37 C. A negative control bacterium, *Enterobacter* (ATCC #35030), was handled identically. Several morphologically similar colonies were used to inoculate 100 ml of brain-heart infusion broth (Difco Laboratories,

Table 1. Summary of the cytotoxicity status of *Pasteurella haemolytica* isolates recovered from a variety of ungulate species.

Species	# isolates tested	# cytotoxic isolates	Biotype/ serotype of cytotoxic isolates
Bighorn sheep	112	29	A2, A5,11, A11, A(UT)*
Domestic sheep	23	13	A2, A(UT), T3
Dall sheep	19	6	A2
Mountain goat	4	2	A6
Domestic goat	13	0	NA ^b
Mouflon sheep	16	1	A(UT)
Elk	10	0	NA

* UT = serotype untypable due to autoagglutination.

^b NA = not applicable.

Detroit, Michigan, USA) which was incubated at 37 C until cultures reached early logarithmic growth. To quantitate the number of bacteria, the optical densities of the cultures were measured at a wavelength of 600 nm (OD_{600}) until $1 OD_{600}$ (8×10^8 bacteria/ml) was reached. Bacteria were centrifuged for 10 min at 6000 x g to a pellet, and resuspended in 30 ml of RPMI-1640 media (Gibco Laboratories, Grand Island, New York, USA) containing 7% FBS. Following incubation for 1 hr at 37 C, the bacteria again were centrifuged at 6000 x g for 10 min, and the culture supernatants were removed and filter sterilized in a 0.45 μ m filter (Sigma Chemical Company, St. Louis, Missouri, USA). Culture supernatants were dialyzed to exhaustion against distilled water and lyophilized.

Cytotoxicity Assay

We characterized the relative potency of toxins produced by various *P. haemolytica* isolates by adding bacterial culture supernatants to neutrophils *in vitro*. Cytotoxicity was quantitated by assessing the release of lactate dehydrogenase (LDH) into the culture medium (Korzeniewski and Callewaert 1983). Cytotoxicity was determined at final concentrations of supernatant of 150, 100, 50, 5, 0.5, and 0.05 μ g/50 μ l. All of the samples were resuspended in HBSS containing 1% FBS prior to the assay. Fifty μ l of each supernatant preparation containing cytotoxin was added to the wells of 96-well plates, followed by the addition of 2.5×10^6 neutrophils in 50 μ l of HBSS containing 1% FBS to

each well. Following 1 hr incubation at 37 C, 100 μ l of LDH substrate was added. Quantitation of the reduced LDH substrate was based on a Titertek 96-well plate reader (Flow Laboratories, McLean, Virginia, USA) coupled to an on-line IBM-XT computer (International Business Machines, Boca Raton, Florida, USA). All samples were compared to neutrophils treated with a 0.5% solution of the detergent Triton-X (Sigma Chemical Company, St. Louis, Missouri, USA) (maximal release) and untreated cells (background release) and the results recorded as a percentage of LDH released from untreated cells. The potency of the various cytotoxins was determined by comparing the 50% effective dose (ED_{50}). The ED_{50} represents the intersection of supernatant concentration and 50% cell death as determined from the graphic plot of these two factors.

RESULTS AND DISCUSSION

A total of 197 *P. haemolytica* isolates from 7 different ungulate species was tested for cytotoxicity on neutrophils from bighorn sheep (Table 1). For an isolate to be considered cytotoxic, according to our definition, it must have an ED_{50} of < 150 μ g/50 μ l of supernatant. Therefore, any isolates which had supernatants unable to kill at least 50% of the neutrophil population in 1 hr were classified as non-cytotoxic. At least one cytotoxic isolate was recovered from bighorn sheep.

Table 2. Cytotoxicity status of *Pasteurella haemolytica* isolates collected from geographically distinct bighorn sheep herds.

Herd location	Total # isolates	# cytotoxic	Biotype/ serotype of cytotoxic isolates
Sluice Creek, OR	4	1	A5, 11
Lostine, OR	7	0	NA
Central Oregon	8	3	A(UT)
Wenaha, OR	2	1	A11
Hall Mt., WA	3	0	NA
Curlew, WA	6	1	A(UT)
Umfanum, WA	5	0	NA
Swakane, WA	8	1	A(UT)
Wildhorse Is., MT	8	0	NA

* NA = not applicable.

* UT = serotype untypable due to autoagglutination.

domestic sheep, Dall sheep, mountain goats, and mouflon sheep. To date, no cytotoxic isolates have been identified in domestic goats ($n=13$) or elk ($n=10$). These results are from a small sample size; however, and should be interpreted carefully. Cytotoxin-producing isolates were identified most frequently following recovery from the lung and viscera of pneumonic animals, but occasionally they were also recovered from pharyngeal swabs of healthy animals. Biotype A isolates were more likely to be cytotoxic than biotype T isolates. We did recover biotype A isolates which were non-cytotoxic including A11, A1,8, A1,2,7,8,11,12, and A(untypable). In our experience, the *Pasteurella* organisms most frequently recovered from an animal which has died of pneumonia have been A2. To date, we have not recovered an A2 organism from a healthy bighorn sheep.

Geographically distinct herds of bighorn sheep were tested for the presence of cytotoxic *P. haemolytica* isolates. Of 9 different herds tested, cytotoxic isolates were recovered from 5 herds (Table 2). In 4 of these 5 herds testing positive for cytotoxin, all of the animals appeared to be healthy and these 4 herds consisted of California bighorn sheep (*Ovis canadensis californiana*). In 1 case, the cytotoxic isolate was recovered from a Rocky Mountain bighorn sheep which had died of pneumonia, while the remainder of the herd

appeared healthy. Whether neutrophils from California bighorn sheep are more resistant to cytotoxin damage than neutrophils from Rocky Mountain bighorn sheep is not known and will be tested in future research efforts.

Neutrophils were collected from 6 different ungulate species and tested for sensitivity to an A2 isolate from a domestic sheep (Table 3). Neutrophils from elk and deer were not sensitive to the cytotoxin at the doses of supernatant routinely used. However, neutrophils from bighorn sheep, Dall sheep, mountain goats and domestic sheep were sensitive to cytotoxin-dependent killing. The order of sensitivity to cytotoxin killing (from most sensitive to least) is Dall sheep, bighorn sheep, mountain goat, domestic sheep.

Testing geographically distinct herds of ungulates for neutrophil sensitivity to *P. haemolytica* cytotoxin requires transportation of blood samples to a location with adequate laboratory facilities. To determine how long after blood collection the neutrophil viability is adequate to use in the cytotoxicity assay, blood samples were collected into anti-coagulant and stored at either 4 C or 22 C for 0, 12, 24, and 48 hrs before neutrophils were isolated and viability was measured. No differences were observed for effects of temperature. Neutrophil viability steadily declined with time, though 90% were still alive by 12 hr post-collection

Table 3. Relative sensitivity of neutrophils from different ungulate species to cytotoxin lysis by a *Pasteurella haemolytica* isolate (A2) from a domestic sheep. The species are listed in order from most to least sensitive to lysis.

Species	ED ₅₀ ^a (µg/50µl)
Dall sheep	3
Bighorn sheep	12
Mountain goat	25
Domestic sheep	58
Elk	NC ^b
Deer	NC ^b

^a 50% effective dose representing the intersection of supernatant concentration and 50% neutrophil death.

^b Considered to be non-cytotoxic since the ED₅₀ exceeds the highest concentration of supernatant used in the assay.

(Figure 1). In situations where blood samples can be shipped to a laboratory within 24 hr of collection, adequate numbers of viable neutrophils will be available to use in a cytotoxin assay.

Cytotoxin production is a major virulence factor in the pathogenesis of pneumonia, yet the recovery of cytotoxic organisms from healthy animals indicates that it may not be the only factor involved. Others, such as the presence or absence of a capsule, and the role of lipopolysaccharide, should be investigated. In addition, the production of chemotactic signals by bacterial organisms, alveolar macrophages, or both, should be considered. Depletion of peripheral blood neutrophils prior to inoculation of *P. haemolytica* in calves blocked the development of respiratory disease (Slocombe et al. 1985). Therefore, if the chemotactic signals could be prevented or diminished, thus preventing the influx of neutrophils from peripheral blood into alveolar spaces, lung damage could be minimized.

MANAGEMENT CONSIDERATIONS

Since the cytotoxin assay can be performed routinely, wildlife managers and biologists should consider collecting pharyngeal swabs from captured animals to screen for the presence of *P. haemolytica* organisms. Furthermore, the status of cytotoxin production by these organisms should be tested. The information gained may influence

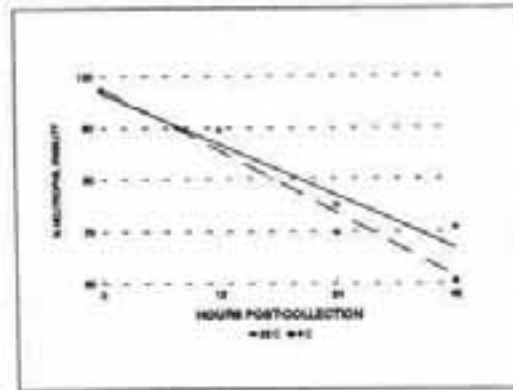


Figure 1. Peripheral blood samples were collected from bighorn sheep ($n = 3$) into citrate phosphate dextrose solution and stored at either 4 C or 22 C for 0, 12, 24, and 48 hours before neutrophils were isolated and tested for viability.

decisions related to transplantation of animals. Furthermore, the information of the status of a herd regarding the presence or absence of cytotoxic organisms may have epizootiologic value which may be used to predict or anticipate disease risk within a herd.

LITERATURE CITED

- BALUYUT, C. S., R. R. SIMONSON, W. J. BEMRICK AND S. K. MAHESWARAN. 1981. Interaction of *Pasteurella haemolytica* with bovine neutrophils: Identification and partial characterization of a cytotoxin. *Am. J. Vet. Res.* 42:1920-1926.
- BERGGREN, K. A., C. S. BALUYUT, R. R. SIMONSON, W. J. BEMRICK AND S. K. MAHESWARAN. 1981. Cytotoxic effects of *Pasteurella haemolytica* on bovine neutrophils. *Am. J. Vet. Res.* 42:1383-1388.
- CALLAN, R. J., T. D. BUNCH, G. W. WORKMAN AND R. E. MOCK. 1991. Development of pneumonia in desert bighorn sheep after exposure to a flock of exotic wild and domestic sheep. *J. Am. Vet. Med. Assoc.* 198:1052-1056.
- CLARK, R. K., D. A. JESSUP, M. D. KOCK AND R. A. WEAVER. 1985. Survey of desert bighorn sheep in California for exposure to selected infectious diseases. *J. Am. Vet. Med. Assoc.* 187:1175-1179.
- CONFER, A. W., K. R. SIMONS, M. T. BARRIE AND K. D. CLINKENBEARD. 1990. Effects of *Pasteurella haemolytica* leukotoxin on neutrophils from

- white-tailed deer and several exotic ruminant species. *Vet. Res. Commun.* 14:175-180.
- CZUPRYNSKI, C. J., E. J. NOEL, O. ORTIZ-CARRANZA AND S. SRIKUMARAN. 1991. Activation of bovine neutrophils by partially purified *Pasteurella haemolytica* leukotoxin. *Infect. Immun.* 59:3126-3133.
- _____, AND O. ORTIZ-CARRANZA. 1992. *Pasteurella haemolytica* leukotoxin inhibits mitogen-induced bovine peripheral blood mononuclear cell proliferation in vitro. *Microb. Pathog.* 12:459-463.
- FOREYT, W. J. 1989. Fatal *Pasteurella haemolytica* pneumonia in bighorn sheep following direct contact with normal domestic sheep. *Am. J. Vet. Res.* 50:341-344.
- _____. 1992. Experimental contact association between bighorn sheep, elk, and deer with known *Pasteurella haemolytica* infections. *Bienn. Symp. North. Wild Sheep Goat Council.* 8:213-218.
- FRANK, G. H. 1982. Serotypes of *Pasteurella haemolytica* in sheep in the midwestern United States. *Am. J. Vet. Res.* 43:2035-2037.
- FRANSON, J. C. AND B. L. SMITH. 1988. Septicemic pasteurellosis in elk (*Cervus elaphus*) on the United States National Elk Refuge, Wyoming. *J. Wildl. Dis.* 24:715-717.
- KORZENIEWSKI, C. AND D. M. CALLEWAERT. 1983. An enzyme-release assay for natural cytotoxicity. *J. Immunol. Methods.* 64:313-320.
- LIGGITT, H. D. 1985. Defense mechanisms in the bovine lung. *Vet. Clin. North Am. Food Anim. Pract.* 1:347-366.
- MAJURY, A. L. AND P. E. SHEWEN. 1991. Preliminary investigation of the mechanism of inhibition of bovine lymphocyte proliferation by *Pasteurella haemolytica* A1 leukotoxin. *Vet. Immunol. Immunopathol.* 29:57-68.
- MARKHAM, R. J. AND B. N. WILKE. 1980. Interaction between *Pasteurella haemolytica* and bovine alveolar macrophages: cytotoxic effect on macrophages and impaired phagocytosis. *Am. J. Vet. Res.* 41:18-22.
- _____, M. L. RAMNARAIN AND C. C. MUSCOPLAT. 1982. Cytotoxic effect of *Pasteurella haemolytica* on bovine polymorphonuclear leukocytes and impaired production of chemotactic factors by *Pasteurella haemolytica*-infected alveolar macrophages. *Am. J. Vet. Res.* 43:285-288.
- MILLER, M. W., N. T. HOBBS AND E. S. WILLIAMS. 1991. Spontaneous pasteurellosis in captive Rocky Mountain bighorn sheep (*Ovis canadensis canadensis*): clinical, laboratory, and epizootiological observations. *J. Wildl. Dis.* 27:534-542.
- ONDERKA, D. K. AND W. D. WISHART. 1988. Experimental contact transmission of *Pasteurella haemolytica* from clinically normal domestic sheep causing pneumonia in Rocky Mountain bighorn sheep. *J. Wildl. Dis.* 24:663-667.
- _____, S. A. RAWLUK AND W. D. WISHART. 1988. Susceptibility of Rocky Mountain bighorn sheep and domestic sheep to pneumonia induced by bighorn and domestic livestock strains of *Pasteurella haemolytica*. *Can. J. Vet. Res.* 52:439-444.
- SHEWEN, P. A. AND B. N. WILKE. 1982. Cytotoxin of *Pasteurella haemolytica* acting on bovine leukocytes. *Infect. Immun.* 35:91-94.
- SILFLOW, R. M. AND W. J. FOREYT. 1994. Susceptibility of phagocytes from elk, deer, bighorn sheep and domestic sheep to *Pasteurella haemolytica* cytotoxins. *J. Wildl. Dis.* (Submitted for Publication).
- _____, S. M. TAYLOR, W. LAEGREID, W. H. D. LIGGITT AND R. W. LEID. 1989. Comparison of pulmonary defense mechanisms in Rocky Mountain bighorn sheep (*Ovis canadensis canadensis*) and domestic sheep. *J. Wildl. Dis.* 25:514-520.
- _____, _____, AND R. W. LEID. 1993. *Pasteurella haemolytica*, cytotoxin-dependent killing of neutrophils from bighorn and domestic sheep. *J. Wildl. Dis.* 29:30-35.
- SLOCOMBE, R. F., J. MALARK, R. INGERSOLL, F. J. DERKSEN AND N. E. ROBINSON. 1985. Importance of neutrophils in the pathogenesis of acute pneumonic pasteurellosis in calves. *Am. J. Vet. Res.* 46:2253-2258.
- SUTHERLAND, A. D., E. GRAY AND P. W. WELLS. 1983. Cytotoxic effect of *Pasteurella haemolytica* on ovine bronchoalveolar macrophages in vitro. *Vet. Microbiol.* 8:3-15.
- THORNE, E. T. 1982. Bacteria. Pages 36-38 in E. T. Thorne, N. Kingston, W. R. Jolley and R. C. Bergstrom. (eds.), *Diseases of Wildlife in Wyoming*. Cheyenne, Wyoming. 353 pp.
- WILD, M. A. AND M. W. MILLER. 1991. Detecting nonhemolytic *Pasteurella haemolytica* infections in healthy Rocky Mountain bighorn sheep (*Ovis canadensis canadensis*): influences of sample site and handling. *J. Wildl. Dis.* 27:53-60.