

PASTEURELLA HAEMOLYTICA CYTOTOXIN-DEPENDENT KILLING OF NEUTROPHILS FROM BIGHORN AND DOMESTIC SHEEP

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ABSTRACT: Peripheral blood neutrophils from Rocky Mountain bighorn sheep (*Ovis canadensis canadensis*) and domestic sheep were exposed to culture supernatants from *Pasteurella haemolytica* isolates recovered from these two sheep species. Six culture supernatants from bighorn sheep isolates and two from domestic sheep isolates were tested for cytotoxicity as determined by the release of lactate dehydrogenase. Two of the bacterial culture supernatants from bighorn sheep were not cytotoxic, while the other four bighorn sheep culture supernatants were effective cytotoxins on both bighorn (>95% cell death at 150 µg of cytotoxin) and domestic sheep neutrophils (55 to 95% cell death at 150 µg of cytotoxin). Two culture supernatants of *P. haemolytica* from domestic sheep were effective cytotoxins on both bighorn (>95% cell death at 150 µg of cytotoxin) and domestic sheep (70 to 75% cell death at 150 µg of cytotoxin) neutrophils. Potency of cytotoxins derived from *P. haemolytica* isolates from bighorn sheep was three to seven-fold higher when tested with bighorn sheep neutrophils as compared to domestic sheep neutrophils. Cytotoxins derived from *P. haemolytica* isolates from domestic sheep were five to six-fold more potent when tested with bighorn sheep neutrophils than when domestic sheep cells were used.

Key words: *Pasteurella haemolytica*, cytotoxin, bighorn sheep, *Ovis canadensis canadensis*, domestic sheep.

INTRODUCTION

Respiratory disease remains the most serious disease problem affecting survival of bighorn sheep (*Ovis canadensis canadensis*) populations in the Western United States and Canada. Domestic sheep mortalities caused by respiratory viruses and bacteria occur as well (Gilmour, 1980; Robinson, 1983; Lehmkuhl et al., 1989). Because bighorn and domestic sheep share public rangelands, disease transmission from one species to another is a major management concern.

The most common bacterial organism isolated from the lungs of bighorn sheep which have died of respiratory disease is *Pasteurella haemolytica* (Onderka and Wishart, 1984; Spraker et al., 1984; Coggins, 1988). Many strains of this bacterium are commonly isolated from domestic sheep (Frank, 1982). *Pasteurella haemolytica* has been isolated from pneumonic bighorn sheep following experimental co-pasturing with domestic sheep (Foreyt and Jessup, 1982; Foreyt, 1988; Onderka et al., 1988; Onderka and Wishart, 1988), as well as from pneumonic and normal bighorn sheep populations without any known con-

tact with domestic animals (Spraker et al., 1984; Onderka and Wishart, 1984; Clark et al., 1985; Miller et al., 1991).

An important question is whether there are differences in virulence between the *P. haemolytica* strains isolated from these two sheep species. *Pasteurella haemolytica* is particularly invasive due to a soluble toxin released into the area surrounding colonization (Baluyut et al., 1981; Berggren et al., 1981). This soluble toxin appears to be species specific (Shewen and Wilkie, 1982; Sutherland et al., 1983) and may contribute to strain virulence. Moreover, because the soluble toxin is considered an important bacterial virulence factor, its study is the focus of research attempts designed to block the pathogenesis of *P. haemolytica* infections (Sutherland et al., 1989; Henricks et al., 1990).

Recently, we found similar numbers of phagocytic cells in the alveolar spaces of both species, and observed no differences in phagocytic or bactericidal activities of alveolar macrophages between these two sheep species (Silflow et al., 1989). However, metabolism of arachidonic acid varied in alveolar macrophages obtained from

bighorn and domestic sheep; thus, bighorn sheep may be more susceptible to respiratory infections (Silflow et al., 1991).

Neutrophils represent a critical cellular constituent of host defense against infectious agents (Martin, 1986). Within the alveolar spaces of the lung, neutrophils and other alveolar cells damaged by cytotoxins derived from *P. haemolytica* may release cytotoxic and proteolytic molecules, thus leading to further damage to the lung tissue. As a result of these events, an optimal environment for bacterial colonization is created, a process which can result in fulminating infection and death of the animal.

Our objective was to test the hypothesis that *P. haemolytica* isolates produce cytotoxins with differing potencies, and furthermore, that neutrophils from bighorn sheep are more susceptible to cytotoxin-mediated destruction than neutrophils from domestic sheep.

MATERIALS AND METHODS

Five Rocky Mountain bighorn sheep (*Ovis canadensis canadensis*) and five domestic sheep were used as sources of neutrophils for this study. All animals were clinically healthy when samples were collected. The bighorn sheep (2 males, 3 females) ranged in age from 1 to 6 yr and were from a captive herd (Washington State University, Pullman, Washington, USA). The conventionally-reared domestic sheep (2 males, 3 females) used ranged in age from 9 mo to 3 yr.

Peripheral blood samples were collected by jugular venipuncture into citrate phosphate dextrose solution (Sigma Chemical Company, St. Louis, Missouri, USA). Following centrifugation at $850 \times g$ 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 $10\times$ phosphate buffered saline. Following centrifugation at $600 \times g$ for 10 min, the lysis and centrifugation steps were repeated, and the final cell pellets were 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 quantitated using a hemocytometer (American Optical Corporation, Buffalo, New York) and cell viability was determined by

trypan blue exclusion (Boyse et al., 1964). Typical yields were $>98\%$ neutrophils, and these cells exhibited $>90\%$ viability. For each experiment, cells were adjusted to a concentration of 5×10^6 cells/ml in HBSS and 1% FBS.

Isolates of *P. haemolytica* from six pneumonic bighorn sheep and two healthy domestic sheep were used. Cytotoxins were isolated from culture supernatants using the method of Shewen and Wilkie (1982). Individual *P. haemolytica* isolates, representing a variety of serotypes and ribotypes from bighorn and domestic sheep (Table 1), were streaked onto 5% sheep blood agar plates (Beckton Dickinson Microbiological Systems, Cockeysville, Maryland, USA) and incubated for 18 hr at 37 C. A negative control bacteria, *Enterobacter*, isolated from a healthy domestic sheep was handled identically. Several morphologically similar colonies were used to inoculate 100 ml of brain-heart infusion broth (Difco Laboratories, Detroit, Michigan, USA) which was incubated for 5 hr at 37 C. This period of time permitted the cultures to reach early logarithmic growth. Bacteria were centrifuged for 10 min at $6,000 \times g$ to a pellet, and resuspended in 30 ml of RPMI-1640 media (Gibco Laboratories, Grand Island, New York) containing 7% FBS. Following incubation for 1 hr at 37 C, the bacteria again was centrifuged at $6,000 \times g$ for 10 min, and the culture supernatants were removed and filter sterilized in a $0.22 \mu\text{m}$ filter (Sigma Chemical Company, St. Louis, Missouri). Culture supernatants were dialyzed to exhaustion against distilled water and lyophilized.

We characterized the relative potency of toxins produced by various *P. haemolytica* isolates by adding bacterial culture supernatants to neutrophils in vitro. Neutrophils from every animal were tested with every supernatant. 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, 50, 5, 0.5, 0.05 and $0.005 \mu\text{g}/50 \mu\text{l}$, respectively. All of the samples were resuspended in HBSS containing 1% FBS prior to the assay. Fifty μl of each supernatant preparation containing cytotoxin was added to the wells of 96-well plates, followed by the addition of 2.5×10^5 neutrophils in $50 \mu\text{l}$ of HBSS containing 1% FBS to each well. Following 1 hr incubation at 37 C, $100 \mu\text{l}$ 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

TABLE 1. Characteristics of bacterial culture supernatants tested for cytotoxicity and their 50% effective dose (ED₅₀).

Culture supernatant	Sheep species	Enterobacter or <i>Pasteurella haemolytica</i>		ED ₅₀ (μg) neutrophils	
		Biotype/serotype	Ribotype	Bighorn	Domestic sheep
<i>Enterobacter</i>					
1	Domestic	ND*	ND	1,000	4,000
<i>Pasteurella haemolytica</i>					
2	Bighorn	untypable	2	4,000	4,000
3	Bighorn	A2	3	15	62
4	Bighorn	T3	2	1,500	4,000
5	Bighorn	T4	ND	19	110
6	Bighorn	T10	4	19	140
7	Bighorn	T3, 4, 10	4	22	120
8	Domestic	A1	3	18	100
9	Domestic	A6	ND	18	90

* ND = not determined.

detergent saponin (Sigma Chemical Company, St. Louis, Missouri) (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 (ED₅₀). The ED₅₀ represents the intersection of supernatant concentration and 50% neutrophil death as determined from the graphic plot of these two factors.

Variables in this study were analyzed for conformity to a normal distribution using the Wilk-Shapiro statistic (Statistix 3.5, Analytical Software, St. Paul, Minnesota, USA). The unpaired Student's *t*-test was used to evaluate differences between experimental groups (Statistix 3.5, Analytical Software).

RESULTS

The negative control, *Enterobacter*, was essentially non-cytotoxic at all doses tested (Fig. 1). When *P. haemolytica* isolates from bighorn sheep were tested, two of six of the culture supernatants exhibited dose-response curves for bighorn and domestic sheep neutrophils which did not differ significantly ($P > 0.05$) from that exhibited by the control *Enterobacter* (Figs. 1, 2). Both isolates were ribotype 2. The other four supernatants from bighorn sheep isolates of *P. haemolytica* were cytotoxic for bighorn sheep neutrophils, at doses of 150 and 50 μg, in comparison to the control *Enterobacter* ($P < 0.001$). These latter four

supernatants from bighorn sheep isolates were effective cytotoxins for domestic sheep neutrophils at a dose of 150 μg ($P < 0.02$), but only one of these supernatants was significantly different from the control ($P < 0.03$) when tested at a dose of 50 μg (Fig. 2). These four isolates were confined to ribotypes 3 and 4.

Two supernatants from domestic sheep isolates of *P. haemolytica* were effective cytotoxins for bighorn and domestic sheep neutrophils at 150 μg, when compared to the negative control ($P < 0.001$) (Figs. 3, 4). Although the supernatants from these two isolates from domestic sheep were effective cytotoxins for bighorn sheep neutrophils at 50 μg ($P < 0.001$) (Fig. 3), they were not effective cytotoxins for domestic sheep neutrophils at the same dose ($P > 0.05$) (Fig. 4).

The ED₅₀'s for all isolates ranged from 15 to 4,000 μg when tested with bighorn sheep neutrophils, and from 62 to 4,000 μg when tested with domestic sheep neutrophils (Table 1). Cytotoxins from the most effective *P. haemolytica* ribotypes (3 and 4) had a mean ED₅₀ of 18.5 μg of cytotoxin on bighorn neutrophils and 105.5 μg on domestic sheep neutrophils. Both of these ribotypes (3 and 4) were 5.7 times more potent on bighorn sheep neutrophils. In general, the cytotoxins derived from *P.*

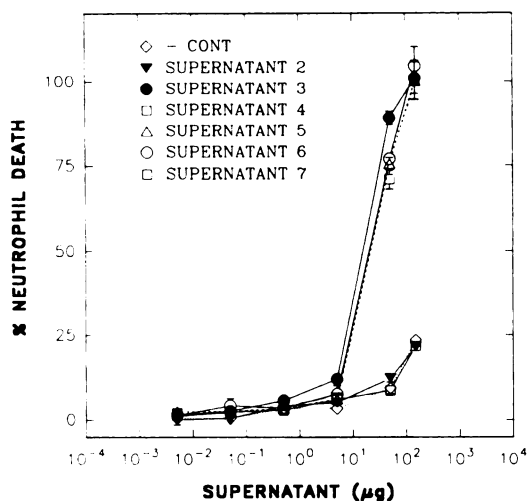


FIGURE 1. Dose response curves (mean \pm SE, $n = 6$) of supernatants from bighorn sheep isolates of *Pasteurella haemolytica* tested for cytotoxicity on bighorn sheep neutrophils. Comparison is made with the negative control (-Cont) *Enterobacter*. See Table 1 for characteristics of supernatants 2 to 7.

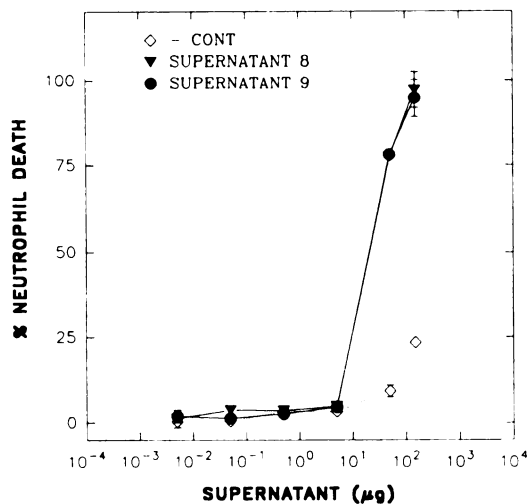


FIGURE 3. Dose response curves (mean \pm SE, $n = 6$) of supernatants from domestic sheep isolates of *Pasteurella haemolytica* tested for cytotoxicity on bighorn sheep neutrophils. Comparison is made with the negative control (-Cont) *Enterobacter*. See Table 1 for characteristics of supernatants 8 and 9.

haemolytica isolates from bighorn sheep were three to seven times more potent when tested with bighorn sheep neutrophils than when tested with domestic sheep neutrophils (Table 1). Further, the cyto-

toxins derived from *P. haemolytica* isolates from domestic sheep were four to five times more potent when tested with bighorn sheep neutrophils than when domestic sheep neutrophils were used.

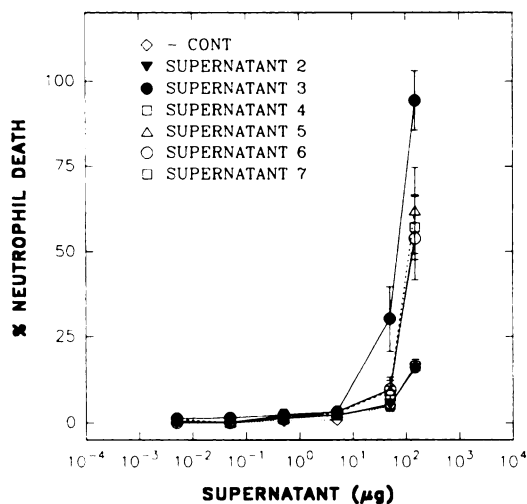


FIGURE 2. Dose response curves (mean \pm SE, $n = 6$) of supernatants from bighorn sheep isolates of *Pasteurella haemolytica* tested for cytotoxicity on domestic sheep neutrophils. Comparison is made with the negative control (-Cont) *Enterobacter*. See Table 1 for characteristics of supernatants 2 to 7.

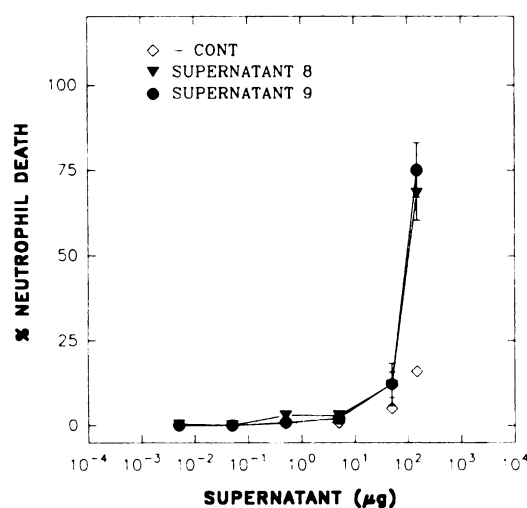


FIGURE 4. Dose response curves (mean \pm SE, $n = 6$) of supernatants from domestic sheep isolates of *Pasteurella haemolytica* tested for cytotoxicity on domestic sheep neutrophils. Comparison is made with the negative control (-Cont) *Enterobacter*. See Table 1 for characteristics of supernatants 8 and 9.

DISCUSSION

Our results support the hypothesis that different *P. haemolytica* isolates have differing cytotoxic potencies, a potency that may be linked to specific ribotypes. Further, neutrophils from bighorn sheep were more susceptible to cytotoxin-mediated lysis than neutrophils from domestic sheep. Therefore, it seems likely that the *P. haemolytica* cytotoxin is an important virulence factor, one capable of compromising the host neutrophil function in both of these sheep species. Although bighorn sheep neutrophils are more sensitive to the cytotoxins, the mechanisms for such an enhanced sensitivity remain unclear.

Different serotypes of *P. haemolytica* can be isolated routinely from the upper respiratory tract and tonsils of healthy sheep. Although there is some understanding of the factors involved in compromising host cellular defenses against *P. haemolytica* colonization of the lower respiratory tract, little is known regarding whether different ribotypes have different potential for causing disease. In this study, we show that *P. haemolytica* isolates from bighorn sheep and domestic sheep can be classified as more or less virulent based upon the potency of their cytotoxins *in vitro*. Moreover, though it appears that the potency of cytotoxin correlates with the specific ribotype of the isolate, additional testing is necessary to confirm this initial observation.

Our results may reflect either a reduction in quantity of cytotoxin synthesized by different ribotypes of *P. haemolytica*, or a difference in potency of the cytotoxins themselves. Quantitation of the cytotoxins in each isolate and determination of dose response curves would permit determination of which of these alternatives were true. Alternatively, there may be qualitative or quantitative differences in neutrophil membrane receptors for the cytotoxins involved. Isolation of cytotoxins from each *P. haemolytica* ribotype, determination of cell receptor number and affinity

for ligand, as well as competitive inhibition of binding would resolve the possibility of neutrophil receptor differences.

Our findings may assist in the explanation of why a greater susceptibility to respiratory disease is observed in bighorn sheep when compared to domestic sheep. As a result, future decisions regarding management of these two species will be facilitated. Finally, we emphasize that the ability to distinguish the presence or absence of virulent *P. haemolytica* isolates may be strengthened by a transition from the traditional serotype classification of bacteria, to that of genomic fingerprinting and classification by ribotype.

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