SHORT COMMUNICATIONS

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Fusobacterium necrophorum in North American Bighorn Sheep (*Ovis canadensis*) Pneumonia

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ABSTRACT: *Fusobacterium necrophorum* has been detected in pneumonic bighorn sheep (BHS; *Ovis canadensis*) lungs, in addition to the aerobic respiratory pathogens *Mannheimia haemolytica, Bibersteinia trehalosi, Pasteurella multocida*, and *Mycoplasma ovipneumoniae*. Similar to *M. haemolytica*, *F. necrophorum* produces a leukotoxin. Leukotoxin-induced lysis and degranulation of polymorphonuclear leukocytes (PMNs) and macrophages are responsible for acute inflammation and lung tissue damage characteristic of *M. haemolytica*-caused pneumonia. As one approach in elucidating the role of *F. necrophorum* in BHS pneumonia, we determined the frequency of the presence of *F. necrophorum* in archived pneumonic BHS lung tissues, and susceptibility of BHS leukocytes to *F. necrophorum* leukotoxin. A species-specific PCR assay detected *F. necrophorum* in 37% of pneumonic BHS lung tissues (total tested n=70). Sequences of PCR amplicons were similar to the less virulent *F. necrophorum* subsp. *funduliforme. Fusobacterium necrophorum* leukotoxin exhibited cytotoxicity to BHS PMNs and peripheral blood mononuclear cells. As with the *M. haemolytica* leukotoxin, *F. necrophorum* leukotoxin was more toxic to BHS PMNs than domestic sheep PMNs. It is likely that *F. necrophorum* enters the lungs after *M. haemolytica* and other aerobic respiratory pathogens enter the lungs and initiate tissue damage, thereby creating a microenvironment that is conducive for anaerobic bacterial growth. In summary, *Fusobacterium* leukotoxin is highly toxic for BHS leukocytes; however, based on the PCR findings, it is unlikely to play a direct role in the development of BHS pneumonia.

Key words: Bighorn sheep, *Fusobacterium necrophorum*, leukotoxin, *Mannheimia haemolytica*, pneumonia.

Pneumonia is a major factor in the drastic decline of bighorn sheep (BHS; *Ovis canadensis*) populations in North America (Miller 2001). The respiratory pathogens commonly detected in pneumonic BHS lungs include *Mannheimia haemolytica*, *Bibersteinia trehalosi, Pasteurella multocida,* and *Mycoplasma ovipneumoniae* (Miller 2001; Besser et al. 2008). *Mycoplasma ovipneumoniae* can predispose BHS to *M. haemolytica* infection (Dassanayake et al. 2010). Lysis and degranulation of polymorphonuclear leukocytes (PMNs) and macrophages by *M. haemolytica* leukotoxin are responsible for the acute inflammation and lung injury characteristic of *M. haemolytica*-caused pneumonia (Slocombe et al. 1985). Several anaerobic bacteria have also been reported in pneumonic BHS lungs (Besser et al. 2008, 2012). The role of these anaerobic bacteria, including *Fusobacterium necrophorum* in BHS pneumonia is not clear. *Fusobacterium necrophorum* is a gram-negative, nonmotile, nonspore-forming, strictly anaerobic organism that causes a variety of suppurative and necrotic infections, generally called necrobacillosis (Nagaraja et al. 2005). It is one of the common anaerobic bacteria isolated from abscesses, respiratory tract infections, and other necrotizing infections in domestic livestock, wild mammals, and humans (Nagaraja et al. 2005). It is commonly found in foot rot in domestic sheep (*Ovis aries*; Roberts and Egerton 1969). *Fusobacterium necrophorum* produces several virulence factors which include leukotoxin, endotoxin, hemolysin, and hemagglutinin (Tan et al. 1996). Of these, leukotoxin is considered to be the major virulence determinant (Nagaraja et al. 2005). Leukotoxin is cytotoxic to leukocytes, hepatocytes, and ruminal epithelial cells of cattle and domestic sheep (Ishii et al. 1988; Tan et al. 1992). Herein we determine the presence of *F. necrophorum* in archived pneumonic BHS lung tissues and the susceptibility of BHS leukocytes to *F. necrophorum* leukotoxin.

For the study we obtained 70 pneumonic lung tissue samples from dead or euthanized BHS submitted to, and archived in, Washington Animal Disease Diagnostic Laboratory (Pullman, Washington, USA). Normal lung tissue samples from five BHS that did not show signs of pneumonia and lacked gross or microscopic lesions distinctive of pneumonia on necropsy were used as negative controls. Pharyngeal swabs from 42 apparently healthy BHS were also examined. Genomic DNA was extracted from pneumonic and normal BHS lung tissues and healthy BHS pharyngeal swabs using QIAamp DNA Mini Kit (Qiagen, Valencia, California, USA). To detect *F. necrophorum*, we performed a PCR assay specific for *F. necrophorum* RNA polymerase beta subunit gene (*rpoB*) using the forward primer (TP1F 5′-TCTACGTATGCCTCACG-GAT-3′) and the reverse primer (TP2R 5′-AGGAATATGAGGATGAGGAT-3′; Narongwanichgarn et al. 2003). Using PCR, we detected *F. necrophorum* in 37% of 70 pneumonic lung tissues, 40% of five non-pneumonic lung tissues, and 31% of 42 pharyngeal swabs.

There are two subspecies of *F. necrophorum*: subsp. *necrophorum* and subsp. *funduliforme* (Shinjo et al. 1991). Although both cause illnesses, subsp. *necrophorum* is considered more pathogenic than *funduliforme* (Lechtenberg et al. 1988). The difference in virulence has been attributed to differences in the amount of leukotoxin produced, and quantity and composition of lipopolysaccharide between the two subspecies (Scanlan et al. 1986). Subsp. *necrophorum* has been more frequently encountered in infections than subsp. *funduliforme* (Lechtenberg et al. 1988). To determine the subspecies of *F. necrophorum*, the 925 base-pair PCR amplicons from the lung tissue (n=26) and pharyngeal samples (n=13), were sequenced. The sequences of PCR amplicons were either identical to, or bearing a single base pair difference from, *F. necrophorum* subsp. *funduliforme* (GenBank accession AJSY01000008), but differing from subsp. *necrophorum* by 13 or 14 base pairs.

The presence of *F. necrophorum* in 37% of pneumonic BHS lungs raises the question as to whether it is a significant pathogen in BHS pneumonia. We previously tested these 70 samples for *Pasteurellaceae* species, particularly *M. haemolytica* (Shanthalingam et al. 2014). The PCR analysis in that study revealed that 77% of these samples carried *M. haemolytica*. Presence of *F. necrophorum* in only 37% of pneumonic lung tissues, 40% of healthy lung tissues, and 31% of pharyngeal swabs from healthy BHS suggests that this bacterium is not a major pathogen in BHS pneumonia. The detection of subsp. *funduliforme* in 100% of the *F. necrophorum*-positive samples tested is in agreement with previous observations that subsp. *funduliforme* occurs more frequently in mixed infections (Lechtenberg et al. 1988; Tan et al. 1996).

To detect the *F. necrophorum* leukotoxin gene in lung tissues and pharyngeal swab extracts, we performed a leukotoxin-specific PCR assay using the primers lktA-F 5′-AAATGGTGAAAGAATGACAA-3′ and lktA-R 5′-TGCATAATTTCCTACTCCTG-3′ (Tadepalli et al. 2008). All *F. necrophorum*-positive pharyngeal swab extracts (n=13) and normal lung tissues (n=2) had the leukotoxin gene as confirmed by sequencing of PCR amplicons. Sixty-nine percent (18/26) of *F. necrophorum*-containing pneumonic lung tissue samples were positive for the leukotoxin gene. Ludlam et al. (2009) reported that 92% of subsp. *funduliforme* isolates of bovine origin possessed the leukotoxin gene. Therefore, it is likely that most of the *F. necrophorum* isolates from BHS also have the leukotoxin gene.

Because *F. necrophorum* subsp. *funduliforme* was detected in BHS pneumonic lungs, it was of interest to us to determine the susceptibility of BHS leukocytes to the *F. necrophorum* leukotoxin. Leukotoxin was produced from an isolate of *F. necrophorum* subsp. *funduliforme* and *M. haemolytica* (Gentry and Srikumaran 1991; Narayanan et al. 2002). We isolated PMNs and peripheral blood mononuclear cells (PBMCs) from peripheral blood of BHS and domestic sheep by density gradient centrifugation and determined cytotoxicity of leukotoxin from *F. necrophorum* and *M. haemolytica* by the

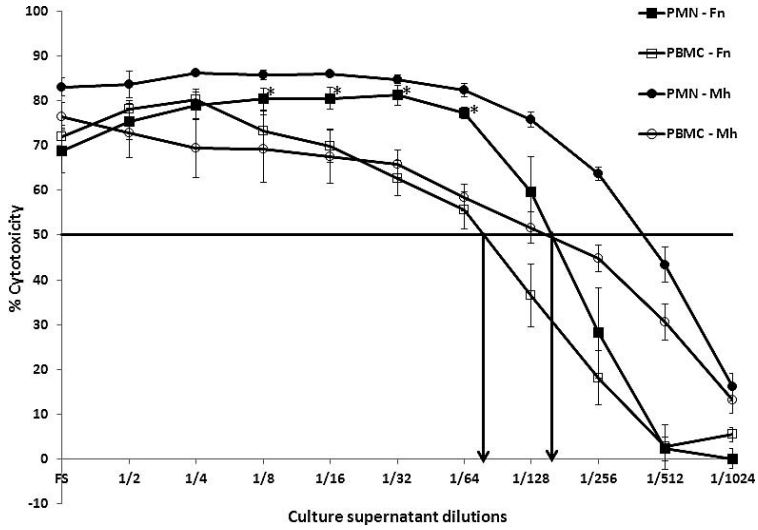

FIGURE 1. Susceptibility of bighorn sheep (BHS; *Ovis canadensis*) polymorphonuclear leukocytes (PMNs) and peripheral blood mononuclear cells (PBMCs) to leukotoxins produced by *Fusobacterium necrophorum* (Fn) and *Mannheimia haemolytica* (Mh). MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] dye-reduction cytotoxicity assay was performed to determine the susceptibility of PMNs and PBMCs of bighorn sheep to leukotoxins produced by Fn and Mh. An asterisk (*) indicates Fn leukotoxin-induced cytotoxicity of BHS PMNs is significantly different ($P<0.05$) from that of BHS PBMCs. Results shown are the means of three independent experiments. The error bars indicate standard deviation of the means.

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] dye-reduction cytotoxicity assay (Gentry and Srikumaran 1991). For analyzing cytotoxicity of *F. necrophorum* and *M. haemolytica* leukotoxin to BHS and domestic sheep PMNs and PBMCs, we used a repeated-measures analysis of variance. Results were considered statistically significant at $P<0.05$.

Bighorn sheep PMNs and PBMCs were susceptible to *F. necrophorum* leukotoxin-induced cytotoxicity in a dose-dependent manner. Percent cytotoxicity of BHS PMNs caused by *F. necrophorum* leukotoxin was significantly higher than that of BHS PBMCs at leukotoxin dilutions 1:8 to 1:64. As with *M. haemolytica* leukotoxin, *F. necrophorum* leukotoxin was twofold more cytotoxic to BHS PMNs than to PBMCs, as judged by leukotoxin concentrations causing 50% cytotoxicity to PMNs and PBMCs (Fig. 1). The plausible explanation for enhanced cytotoxicity of *M. haemolytica* leukotoxin to BHS PMNs is that CD18, the receptor for *M. haemolytica* leukotoxin (Deshpande et al. 2002), is expressed to a higher level on PMNs than on PBMCs. Differential susceptibility of BHS PMNs and PBMCs to *F. necrophorum* leukotoxin cannot be deciphered until the receptor for this leukotoxin on BHS leukocytes is identified. Similar to *M. haemolytica* leukotoxin (Silflow and Foreyt 1994), *F. necrophorum* leukotoxin was fourfold more cytotoxic to BHS PMNs than to domestic sheep PMNs, as judged by leukotoxin concentrations causing 50% cytotoxicity to the PMNs (Fig. 2). Identification of the leukocyte receptor for *F. necrophorum* leukotoxin will help elucidate the molecular basis for this differential susceptibility.

Various pulmonary lesions have been associated with *F. necrophorum* in domestic animals. Pulmonary lesions have also been reported in white-tailed deer (*Odocoileus virginianus*) and pronghorn (*Antilocapra americana*; Wobeser et al. 1975; Edwards et al. 2001). Lung is a richly oxygenated and a highly defended organ. Therefore, as an anaerobe, *F. necrophorum* must overcome high oxygen concentrations in the lungs, in

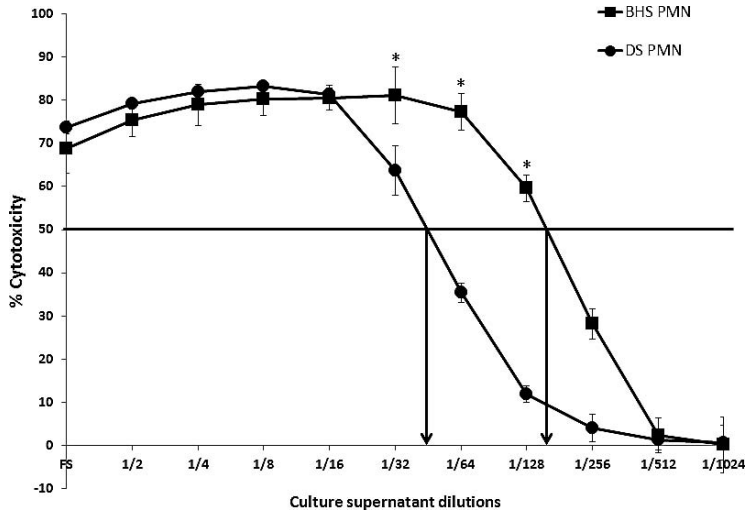

FIGURE 2 Comparison of *Fusobacterium necrophorum* leukotoxin-induced cytotoxicity of bighorn sheep (BHS; *Ovis canadensis*) and domestic sheep (DS; *Ovis aries*) polymorphonuclear leukocytes (PMNs). MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] dye-reduction cytotoxicity assay was performed to determine the susceptibility of PMNs of BHS and DS to leukotoxin produced by *F. necrophorum*. An asterisk (*) indicates cytotoxicity of BS PMNS was significantly different ($P<0.05$) from that of DS PMNs. Results shown are the mean of three independent experiments. The error bars indicate standard deviation of the means.

addition to the phagocytic mechanisms to survive, proliferate, and produce its virulence factors. It is conceivable that the lysis of alveolar macrophages and PMNs by leukotoxin produced by *M. haemolytica* abrogates or mitigates the host defense mechanisms. More importantly, killing of PMNs could lead to parenchymal cell damage, abscess formation, and sequestration leading to an anaerobic environment in lungs. *Fusobacterium necrophorum* is likely to enter the lungs after *M. haemolytica* enters the lungs and initiates tissue damage, creating a microenvironment more conducive for growth of anaerobic bacteria. The leukotoxin of *F. necrophorum* would also help protect it from phagocytosis. In conclusion, although *Fusobacterium* leukotoxin can lyse BHS leukocytes, it is unlikely that this bacterium plays a direct role in the development of pneumonia. Further studies are necessary to definitively ascertain the role of *F. necrophorum* in BHS pneumonia.

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