Growth of *Mannheimia haemolytica*: Inhibitory agents and putative mechanism of inhibition

By

ABIRAMI KUGADAS

A dissertation submitted in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

WASHINGTON STATE UNIVERSITY
College of Veterinary Medicine

DECEMBER 2014
To the Faculty of Washington State University

The members of the committee appointed to examine the dissertation of ABIRAMI KUGADAS find it satisfactory and recommend that it be accepted.

________________________________________
Subramaniam Srikumaran, BVSc, Ph.D., Chair

________________________________________
Kelly A. Brayton, Ph.D.

________________________________________
Douglas R. Call, Ph.D.

________________________________________
Donald P. Knowles, DVM, Ph.D.
ACKNOWLEDGMENT

I would like to express my deepest appreciation to my mentor Dr. Subramaniam Srikumaran for his continuous support and guidance during my graduate student period. I admired the way he simplifies the scientific questions, coherently presenting the results in an appealing style to the scientific community, correcting manuscripts with lots of patience and providing inputs on alternative ways of presenting the results. I would like to thank him for providing the necessary freedom to think and execute experiments on my own and making me an independent researcher.

I would like to extend my sincere gratitude to my committee members Drs. Douglas Call, Kelly Brayton and Don Knowles for their valuable time and guidance during the preparation for the preliminary examination. I thank Dr. Brayton for the time spent for the bioinformatics analysis, support and encouragement to keep me focused in the research even in the difficult times. I greatly appreciate Dr. Call for helping me to find different approaches to answer the research question and his willingness to help me whenever there is a need for a technical or intellectual assistance.

Next, I would like to thank all the past and present members of the Sri lab for their technical assistance and support. I appreciate Dr. Humann and Dr. Highlander for helping me with some bioinformatics work. I thank the DVM students for helping me with animal handling. I also offer my utmost gratitude to the faculty and staff of VMP for helping me in many ways and for their friendship. I thank Dr. Shah and Dr. Lau for letting me use the instruments of their lab.
I like to extend my special thanks to all my friends outside VMP for their moral support and the kind friendship during my graduate training period. I am very thankful for parents and my sister for their endless love, support and dedication that enabled me to complete my PhD program successfully.
Growth of *Mannheimia haemolytica*: Inhibitory agents and putative mechanism of inhibition

Abstract

by Abirami Kugadas, Ph.D.
Washington State University
December 2014

Chair: Subramaniam Srikumaran

Pneumonia is the most important disease of bighorn sheep (BHS). Pathogens detected from pneumonic BHS lungs include *Mannheimia haemolytica*, *Bibersteinia trehalosi*, *Pasteurella multocida* and *Mycoplasma ovipneumoniae*. Although leukotoxin-producing *M. haemolytica* consistently causes fatal pneumonia in BHS under experimental conditions, *B. trehalosi* and *P. multocida* are isolated more frequently than *M. haemolytica* from pneumonic BHS lungs by culture methods. In this study, I extended the previous findings from our laboratory that *B. trehalosi* and *P. multocida* inhibit the growth of *M. haemolytica*. I hypothesized that the inhibitory phenotype of *B. trehalosi* is conserved across *B. trehalosi* strains. Fifty-five *B. trehalosi* isolates were tested by bacterial competition assays. All of them inhibited *M. haemolytica* is consistent with a conserved phenotype. No plasmids were isolated from any of the 30 *B. trehalosi* isolates tested, suggesting that the effectors are chromosomally-encoded.

The observation that *M. haemolytica* was not isolated frequently even from pneumonic BHS lungs that did not carry *B. trehalosi* or *P. multocida* led to my second hypothesis that bacteria other than *B. trehalosi* and *P. multocida* inhibit the growth of *M. haemolytica*. Bacterial competition assays showed that *Escherichia coli* also inhibits the growth of *M. haemolytica* via a
proximity-dependent mechanism. *Streptococcus* spp. and *Staphylococcus* spp. did not have any inhibitory effect.

Fatal pneumonia in BHS often results from acquisition of leukotoxin-positive *M. haemolytica* from domestic sheep (DS). I hypothesized that intranasal inoculation of *B. trehalosi* will eliminate or reduce shedding of *M. haemolytica* by DS. Domestic sheep were oro-nasally inoculated with *B. trehalosi* following antibiotic treatment and these DS shed reduced numbers of *M. haemolytica*, post-treatment. However, healthy BHS commingled with *B. trehalosi*-treated DS acquired *M. haemolytica* around day 14 post-commingling which resulted in the development of leukotoxin-neutralizing antibodies. They did not develop pneumonia for 211 days. However, they died of pneumonia when commingled with DS that carried leukotoxin-positive *M. haemolytica*, *B. trehalosi* and *M. ovipneumoniae*. These findings suggest that *B. trehalosi* can be used to reduce the shedding of *M. haemolytica* by DS. However, infection with leukotoxin-positive *M. haemolytica*, *B. trehalosi* and *M. ovipneumoniae* synergistically causes fatal pneumonia in BHS.
# TABLE OF CONTENTS

ACKNOWLEDGEMENTS ................................................................................................. iii

ABSTRACT ......................................................................................................................... v

LIST OF TABLES ................................................................................................................ ix

LIST OF FIGURES ............................................................................................................ x

GENERAL INTRODUCTION .............................................................................................. 1

REFERENCES ...................................................................................................................... 4

CHAPTER ONE

1. ABSTRACT ..................................................................................................................... 8

2. INTRODUCTION .......................................................................................................... 9

3. MATERIALS AND METHODS .................................................................................... 11

4. RESULTS .................................................................................................................... 14

5. DISCUSSION ............................................................................................................. 18

6. CONCLUSION ........................................................................................................... 22

7. REFERENCES ............................................................................................................ 23

8. TABLES ..................................................................................................................... 29

9. FIGURES ................................................................................................................... 31

CHAPTER TWO

1. ABSTRACT ..................................................................................................................... 41

2. INTRODUCTION .......................................................................................................... 42

3. MATERIALS AND METHODS .................................................................................... 44
4. RESULTS AND DISCUSSION ................................................................. 50

5. REFERENCES ..................................................................................... 56

6. TABLES ............................................................................................. 62

7. FIGURES ........................................................................................... 65

CONCLUSION .......................................................................................... 69
LIST OF TABLES

CHAPTER ONE
1. Inhibition of growth of *M. haemolytica* by *E. coli* .........................................................29
2. *Staphylococcus aureus* does not inhibit the growth of *M. haemolytica* ...............................30
3. Inhibition of growth of *M. haemolytica* by 55 *B. trehalosi* isolates ........................................36

CHAPTER TWO
1. Commingling of DS and BHS: Experimental design ...............................................................62
2. Lkt-neutralizing antibody titers of the commingled BHS ........................................................63
3. Nasopharyngeal microbial profile of the commingled DS and BHS from day 105 to the end of the study ..................................................................................................................64
LIST OF FIGURES

CHAPTER ONE
1. $\text{EC186}^{\text{Nal}}$ inhibits the growth of $\text{M. haemolytica} \ 10/2/3$ in co-culture..............................31
2. Killed $\text{B. trehalosi}$ does not inhibit the growth of $\text{M. haemolytica}$.................................32
3. $\text{E. coli}$ inhibits the growth of $\text{M. haemolytica}$ via a proximity-dependent mechanism .....34
4. $\text{S. agalactiae}$ does not inhibit the growth of $\text{M. haemolytica}$ ........................................35
5. Analysis of plasmid preps ($<$10 Kb) by gel electrophoresis................................................38
6. Analysis of plasmid preps ($>$10 Kb) by gel electrophoresis................................................39

CHAPTER TWO
1. Intranasal inoculation of $\text{B. trehalosi}$ reduces shedding of $\text{M. haemolytica}$ by DS........65
2. In spite of the reduced shedding by DS, BHS acquire leukotoxin-positive $\text{M. haemolytica}$
   from DS in 14 days of commingling...........................................................................................66
3. Histopathology of lungs of the rams that died on day 47 and 84 confirms the absence of
   pneumonia in these animals...........................................................................................................67
4. Histopathology of lungs of the rams that died on day 14 and 47 of phase 3 confirms bacterial
   pneumonia in these animals...........................................................................................................68
GENERAL INTRODUCTION

Pneumonia is considered the most important disease responsible for the decline of bighorn sheep (*Ovis canadensis*, BHS) populations (Buechener 1960; Foreyt and Jessup 1982; Coggins 1988; Valdez and Krausman 1999; Miller 2001 and Smith et al. 2014). *Mannheimia haemolytica* is an important pathogen that causes bronchopneumonia in BHS, domestic sheep (*Ovis aries*, DS) and other ruminants (Foreyt. et al., 1994; Ackermann and Brogden 2000; Dassanayake et al., 2009 and Subramaniam et al., 2011). Compared to DS, BHS are much more susceptible to pneumonia caused by *M. haemolytica* (Dassanayake et al. 2009). Leukotoxin (Lkt) produced by *M. haemolytica* is the most important virulence factor of this bacterium. This notion is based on the observation that Lkt-deletion mutants of *M. haemolytica* cause mild lesions, but no mortality in BHS (Dassanayake et al. 2009). Although BHS and DS carry *M. haemolytica* as a commensal bacterium in their nasopharynx, BHS carry this bacterium less frequently than DS. Furthermore, BHS carry mostly Lkt-negative strains whereas DS carry mostly Lkt-positive strains (Miller et al. 2011; Shanthalingam et al. 2014). This differential distribution of Lkt-positive *M. haemolytica* strains in DS and BHS is likely to be a factor responsible for the high serum titers of Lkt-neutralizing antibodies observed in DS and negligibly low titers in BHS. Consequently, when BHS acquire Lkt-positive strains of *M. haemolytica* from DS or elsewhere, they almost always develop fatal pneumonia.

Although, Lkt-producing *M. haemolytica* consistently causes fatal pneumonia in BHS under experimental conditions, it is not frequently detected in the field pneumonic lung samples by culture-based methods. *B. trehalosi* and *P. multocida* are more frequently isolated along with a few others including *Actinobacillus sp.*, *Staphylococcus sp.*, *Streptococcus sp.*, *Moraxella sp.*,
and *Escherichia coli* (Besser et al. 2008 and Besser et al. 2012). Studies conducted in our laboratory demonstrated that *B. trehalosi* and *P. mutocida* inhibit the growth of *M. haemolytica* via a proximity-dependent mechanism (Dassanayake et al. 2010; Bavananthasivam et al., 2013). It is not clear, however, if the inhibitory phenotype of *B. trehalosi* is conserved across various strains of *B. trehalosi*. The inhibitory phenotype is often due to antimicrobial peptides such as bacteriocins and microcins that are encoded on plasmids or chromosomes of the strains that inhibit the growth of other bacteria (Duquesne et al., 2007 and Gillor et al. 2004). I tested the hypothesis that the inhibitory phenotype of *B. trehalosi* is conserved in the genome of different strains of *B. trehalosi*. Although *B. trehalosi* and *P. mutocida* inhibit the growth of *M. haemolytica*, there are reports of failure to detect *M. haemolytica* even in pneumonic BHS lungs that did not carry *B. trehalosi* or *P. multocida* (Besser et al. 2008 and Besser et al. 2012). This observation led to my second hypothesis: Bacteria other than *B. trehalosi* and *P. multocida* also inhibit the growth of *M. haemolytica*. I tested the effect of co-culture of *M. haemolytica* with *Staphylococcus* spp., *Streptococcus* spp., and *Escherichia coli*. The manuscript describing the results of this study forms the first chapter of this dissertation.

Several anecdotal reports suggested that BHS often died following contact with domestic sheep (Foreyt. W. J. and Jessup. D. A., 1982; Foreyt. et al., 1994; George et al., 2008). A study from our laboratory clearly demonstrated the transmission of *M. haemolytica* from DS to BHS by employing green fluorescent protein-tagged organisms (Lawrence et al., 2010). Previous studies from our laboratory and my study described in chapter 1 revealed that *B. trehalosi*, *P. multocida* and *Escherichia coli* inhibit the growth of *M. haemolytica* by a proximity-dependent mechanism. This observation led to my third hypothesis: Oro-nasal administration of Lkt-
negative *B. trehalosi* will result in the elimination or reduction of Lkt-positive *M. haemolytica* from the nasopharynx of DS. In the first phase of this study, I tested the inhibitory effect of *B. trehalosi* on *M. haemolytica* in the nasopharynx of the DS. In the second phase, these DS were commingled with BHS to determine the transmission of Lkt-positive *M. haemolytica* from DS to BHS. Although *M. haemolytica* alone can cause pneumonia in BHS, *M. ovipneumoniae* has been shown to be a predisposing factor (Dassanayake et al. 2010). However, studies that address the natural transmission of *M. haemolytica* and *M. ovipneumoniae* from DS and the role of these organisms in the pathogenesis of BHS pneumonia are limited. Therefore, in the third phase of this study, the BHS that were commingled with *B. trehalosi*-treated DS were subsequently commingled with DS that carry *M. haemolytica, B. trehalosi* and *M. ovipneumoniae*. The results of this study are described in the manuscript that forms the second chapter of this dissertation.
REFERENCES


Growth of *Mannheimia haemolytica*: Inhibitory agents and putative mechanism of inhibition

Abirami Kugadas, Jessica Poindexter, Mee-La Lee, Jegarubee Bavananthasivam, Douglas R. Call, Kelly A. Brayton, and Subramaniam Srikumaran *

Department of Veterinary Microbiology and Pathology, Washington State University, Pullman, Washington 99164-7040.

Running title: Growth inhibition of *Mannheimia haemolytica*.

Note: This manuscript was accepted for publication in Veterinary Microbiology, 2014

* Corresponding author:

Mailing address:

Dr. S. Srikumaran,

Department of Veterinary Microbiology and Pathology,

College of Veterinary Medicine

Washington State University

Pullman, WA 99164-7040, USA

Phone: 509-335-4572; Fax: 509-335-8529

E-mail: ssrikumaran@vetmed.wsu.edu
ABSTRACT

Leukotoxin-producing *Mannheimia haemolytica* consistently causes fatal pneumonia in bighorn sheep (BHS) under experimental conditions. Surprisingly, by culture methods, it has been isolated from pneumonic BHS lungs less frequently than other bacteria. However, in one study PCR assays detected *M. haemolytica* from over 70% of the pneumonic lung samples that were negative for this organism by culture, suggesting that the growth of *M. haemolytica* is inhibited by other bacteria. Previously, we have shown that *Bibersteinia trehalosi* inhibits the growth of *M. haemolytica*. Herein we report that 100% of a diverse panel of *B. trehalosi* isolates (n=55) tested in a bacterial competition assay inhibited the growth of *M. haemolytica*, suggesting that the inhibitory phenotype is conserved. Further, no plasmids were isolated from any of the 30 *B. trehalosi* isolates tested, suggesting that the effectors are chromosomally-encoded. An earlier study by us showed that *Pasteurella multocida* also inhibits the growth of *M. haemolytica*. However, *M. haemolytica* has not been isolated even from pneumonic BHS lungs that did not carry *B. trehalosi* or *P. multocida*. Consequently, we tested *Staphylococcus* spp., *Streptococcus* spp., and *Escherichia coli*, the bacteria that have been detected frequently in pneumonic BHS lungs, for possible inhibition of *M. haemolytica*. Neither the *Staphylococcus* spp. nor the *Streptococcus* sp. strains inhibited the growth of *M. haemolytica*. *E. coli* inhibited the growth of *M. haemolytica* by a proximity-dependent mechanism. Growth inhibition of *M. haemolytica* by several bacterial species is likely to contribute to the infrequent detection of this bacterium from pneumonic BHS lungs by culture.
Pneumonia is an important cause for the drastic decline of bighorn sheep (BHS) from an estimated two million animals last century to less than 70,000 animals at the present time (Buechner 1960, Foreyt and Jessup 1982, Coggins 1988, Valdez and Krausman 1999 and Miller 2001). *Mannheimia haemolytica, Bibersteinia trehalosi, Pasteurella multocida,* and *Mycoplasma ovipneumoniae* have been detected in the pneumonic lungs of BHS (Besser et al., 2008, Besser et al., 2012, Bavananthasivam et al., 2013, and Shanthalingam et al., 2014). Leukotoxin-producing *M. haemolytica* consistently causes fatal pneumonia in BHS under experimental conditions (Dassanayake et al., 2009, Lawrence et al., 2010 and Subramaniam et al., 2011). Surprisingly, by culture methods, *M. haemolytica* has been isolated from pneumonic BHS lungs less frequently than *B. trehalosi* and *P. multocida* (Dassanayake et al., 2010b and Shanthalingam et al., 2014). However, in one study PCR assays detected *M. haemolytica* from over 70% of the pneumonic lung samples that were negative for this organism by culture (Shanthalingam et al., 2014). These findings suggest that the growth of *M. haemolytica* is inhibited by other bacterial species during advanced lung infections.

A previous study from our laboratory showed that *B. trehalosi* inhibits the growth of *M. haemolytica* (Dassanayake et al., 2010b). It is not clear, however, whether the inhibition is a strain-specific or a conserved phenomenon. Therefore the first objective of this study was to determine whether the inhibitory phenotype is conserved across the different strains of *B. trehalosi*. Antimicrobial peptides such as bacteriocins and microcins are encoded on plasmids or chromosomes of the strains that inhibit the growth of other bacteria (Duquesne et al., 2007 and Gillor et al. 2004). Therefore the second objective was to determine whether the *B. trehalosi*
effectors of inhibition are encoded on a plasmid or on the chromosome. Another study by us has shown that *P. multocida* also inhibits the growth of *M. haemolytica* (Bavananthasivam et al., 2013). However, there are reports of failure to detect *M. haemolytica* even in pneumonic BHS lungs that did not carry *B. trehalosi* or *P. multocida* (Besser et al. 2008 and Besser et al. 2012). We hypothesized that bacteria other than *B. trehalosi* and *P. multocida* inhibit the growth of *M. haemolytica*. Therefore, the third objective of this study was to determine whether bacteria other than *B. trehalosi* and *P. multocida* that are frequently detected in pneumonic BHS lungs also have an inhibitory effect on the growth of *M. haemolytica*. In this study, we focused on *Staphylococcus* spp., *Streptococcus* spp., and *Escherichia coli*, because these organisms have been detected in several pneumonic BHS lungs (Besser TE, et al. 2008 and Besser TE, et al. 2012).
2. MATERIALS AND METHODS

2.1. Bacterial Strains and culture conditions. Fifty-five field isolates of *B. trehalosi*, one *Streptococcus agalactiae*, one *Streptococcus* sp. and one *Staphylococcus aureus* isolate archived in the Washington Animal Disease Diagnostic Laboratory (WADDL) depository were used in this study. The *M. haemolytica* isolates included an ampicillin-resistant (25 µg/ml) lktA mutant serotype 1 (Murphy et al., 1995), a rifampicin-resistant (10 µg/ml), leukotoxin-producing serotype 2 and two ampicillin-resistant (25 µg/ml) leukotoxin-producing isolates (10/2/3 and 7/3/1) of domestic sheep origin and tagged with green fluorescent protein and beta -lactamase gene (Lawrence et al., 2010). The *E. coli* isolates included an isolate of bighorn sheep origin (E6536) and two isolates of cattle origin (*E. coli*-186 and *E. coli*-25) described elsewhere (Eberhart et al., 2012 and Khachatryan et al., 2008). All isolates were revived from the freezer stock on brain heart infusion (BHI) agar plates. Single colonies were sub-cultured. All the *B. trehalosi* isolates and *M. haemolytica* isolates were further confirmed by species-specific PCR described previously (Dassanayake et al., 2010b and Shanthalingam et al., 2014). *E. coli*-186 and *E. coli*-25 were resistant to nalidixic acid (30µg/ml) and tetracycline (50µg/ml) respectively.

2.2 Quantification of bacteria. Bacterial quantification was completed by serial dilution and plating on BHI agar plates containing respective antibiotics. To precisely quantify the Colony Forming Units/ml (CFU/ml) of *M. haemolytica* in the co-cultures and eliminate false positive counts, the colonies harvested from the ampicillin (25µg/ml) plates were reconfirmed by *M. haemolytica*-specific PCR (Dassanayake et al., 2010b and Shanthalingam et al., 2014).
2.3 Bacterial competition assay. Competition assays were performed according to the previously described methods (Bavananthasivam et al., 2013 and Dassanayake et al., 2010b). Briefly, overnight cultures of the target and inhibitor strains were inoculated into 10 ml of BHI broth in a 1:10 ratio and incubated at 37°C on a shaker at 225 rpm over a period of 24 h. Aliquots taken at different time points were serially diluted and plated on appropriate antibiotic containing BHI agar plates to enumerate the bacterial counts. *B. trehalosi* and *E. coli* (1x10^6 or 1X10^8) were treated with 70% ethanol for 1 h at room temperature. Killed bacteria were resuspended in sterile BHI broth and used as an inoculum in competition assays designed to determine the effect of killed inhibitor strains.

2.4 Proximity-dependent inhibition assay. This assay was performed as described by us previously (Bavananthasivam et al., 2013 and Dassanayake et al., 2010b). Briefly, filters with two different pore sizes (0.4 and 8 µm) (BD Falcon; BD Biosciences, Franklin Lakes, NJ) were used to create upper and lower chambers in the wells of a six-well cell culture plate. When *M. haemolytica* (target) and a potential inhibitor strain were separated by 0.4 µm filter, the bacteria were confined to the respective chambers, whereas the soluble compounds and phage (if present) moved freely between the two chambers. When bacteria were separated by 8 µm filter, both the target and the inhibitor species moved freely between the chambers.

2.5 Bactericidal assay. Culture supernatants from mono- and co-culture *M. haemolytica* and *E.coli* were filter-sterilized using a 0.2 µm filter. Sterile culture supernatant (300 µl or 500 µl) was mixed with 1x10^6 mid- log phase *M. haemolytica* and incubated at 37°C for 20 min to facilitate adsorption of any bacteriophage present in the culture supernatant. Then 3 ml of 0.7%
soft molten agar (45°C) were added into each tube. The bacteria-agar suspension was mixed gently and was poured onto BHI agar plates that were preheated to 37°C with a swirling movement. As soon as the soft agar solidified, the plates were incubated either at room temperature (25°C) or at 37°C over a period of 24 h. Plates were examined for plaque formation. *M. haemolytica* with sterile BHI was used as a negative control.

2.6 Statistical analysis. Bacterial counts were enumerated and expressed as mean Log_{10} CFU/ml of culture media. Standard Error of Mean (SEM) of the counts was also determined at different time points. Bacterial counts at different time points were statistically analyzed by student *t*-test using GraphPad online software (GraphPad, La Jolla, CA).
3. RESULTS

3.1 Growth inhibition of *M. haemolytica* is conserved across *B. trehalosi* strains. Fifty-five *B. trehalosi* isolates from BHS that were healthy and/or died due to pneumonia were co-cultured with *M. haemolytica* to determine the growth inhibitory activity on *M. haemolytica*. Thirty out of fifty-five isolates (54.5%) resulted in consistent inhibition of *M. haemolytica* as evident by a 4 log reduction in the growth compared with mono-culture, while the rest of the isolates (45.5%) caused 2-4 log reduction as compared with the mono-culture (Supplementary Table 1). Inhibition by 100% of the isolates (*P*<0.0001), suggest that the inhibitory phenotype is conserved in *B. trehalosi*.

3.2 The *B. trehalosi* effector/s responsible for the growth inhibition are encoded on the chromosome. Inhibition of *M. haemolytica* by 100% of the *B. trehalosi* isolates tested prompted us to determine whether the effector/s causing the inhibition is/are encoded on the plasmids. Plasmid preps were made from 30 isolates that inhibited *M. haemolytica*. Two different methods were employed to extract plasmids: one method targeted plasmids that were less than 10 kb (Qiagen Plasmid mini Kit) and the other targeted plasmids greater than 10 kb in size (Kado and Liu, 1981). However both methods failed to yield any plasmids suggesting that the effector/s is/are encoded on the chromosome (Supplementary Fig. 1). This notion is supported by the genome sequence data from an isolate that inhibited *M. haemolytica*.

3.3 *E. coli* also inhibits the growth of *M. haemolytica*. The growth of *M. haemolytica* was inhibited by the *E. coli* isolate from BHS (E6536). A 2 log difference between mono- and co-cultured *M. haemolytica* was observed with this *E.coli* isolate (Table 1). Since antibiotic-
resistant *E. coli* isolates of cattle origin were available to us, we used them in subsequent experiments because of the ease of detection of antibiotic-resistant strains.

When *E.coli* (*E.coli*-186) and *M. haemolytica* were grown individually as mono-cultures, *E.coli*-186 grew slightly faster than *M. haemolytica* and yielded nearly 1 log CFU/ml more than *M. haemolytica* at the stationary phase (Fig. 1). When both species were co-cultured, *M. haemolytica* displayed relatively slow growth between 3-6 h. Thereafter, the growth of *M. haemolytica* was inhibited. At 24 h, there was a 4 log (CFU/ml) difference between mono- and co-cultured *M. haemolytica* (Fig. 1). Furthermore, co-culture of *E. coli*-186 with two other strains of *M. haemolytica* yielded similar results (Table 1). Another multidrug resistant *E. coli* strain, *E. coli*-25, was co-cultured with *M. haemolytica*. In mono-culture, *E. coli*-25 yielded 1 log more than *M. haemolytica*. At 24 h of co-culture, there was an average of 5 log (CFU/ml) difference between mono- and co-cultured *M. haemolytica* (Table 1).

### 3.4 Killed *E. coli* and *B. trehalosi* do not inhibit the growth of *M. haemolytica*

To determine the effect of surface-exposed cell wall components and the requirement of the presence of live inhibitor strains, killed *E. coli* or *B. trehalosi* was co-cultured with *M. haemolytica*. We employed ethanol treatment, instead of heat treatment, since it is relatively mild and does not severely alter the conformation of the surface-exposed proteins. There was no difference in growth between mono- and co-cultured *M. haemolytica* (Fig. 2). This observation indicated that live, but not dead, *E. coli* and *B. trehalosi* mediate the growth inhibition of *M. haemolytica*.  


3.5 Bacteriophage is not involved in the growth inhibition of *M. haemolytica* by *E. coli*.

Many *E. coli* strains are known to harbor lytic phages that kill related bacteria (Kropinski et al., 2013). To identify the presence of lytic phages, the culture supernatant of *E. coli*-186 was tested using a soft-agar assay with incubation at 25°C and 37°C. Absence of plaque-formation suggested that the growth inhibition of *M. haemolytica* by *E. coli* is not mediated by lytic phages (data not shown).

3.6 *E. coli* inhibits the growth of *M. haemolytica* by a proximity-dependent mechanism. To determine whether, physical proximity between *M. haemolytica* and *E. coli* is necessary for the inhibition, *E. coli* (*E. coli*-186) and *M. haemolytica* were cultured in cell culture chambers separated by filters with different pore-sizes. *E. coli* displayed similar growth irrespective of the size of the filter that separated it from *M. haemolytica* (Fig. 3). *M. haemolytica* that was separated from *E. coli* by 0.4 µm filter displayed similar growth as when cultured in mono-culture. In contrast, *M. haemolytica* that was separated from *E. coli* by 8 µm filter displayed a 2 log reduction (CFU/ml) compared to that of mono-cultured *M. haemolytica*. These observations suggest that physical proximity between these two strains is important for the growth inhibition of *M. haemolytica* (Fig. 3).

3.7 *Streptococcus* does not inhibit the growth of *M. haemolytica*.

Mono-cultures of *Streptococcus agalactiae*, *Streptococcus* sp. and *M. haemolytica* displayed similar growth patterns. However, we observed no significant difference in growth between *M. haemolytica* that was mono- and co-cultured with *Streptococcus agalactiae* or *Streptococcus* sp (Fig 4).
3.8 *Staphylococcus aureas* isolate does not inhibit the growth of *M. haemolytica*. The growth curves of mono-cultured *Staphylococcus aureus* and *M. haemolytica* did not differ from each other. When co-cultured with *Staphylococcus aureus*, no significant difference was observed between the mono- and co-cultured *M. haemolytica* (Table 2).
4. DISCUSSION

Identification of the bona fide etiological agent or agents of a disease is critical for the development of prevention and control strategies against that disease. Although leukotoxin producing *M. haemolytica* consistently causes fatal pneumonia in BHS under natural and experimental conditions (Dassanayake et al., 2009, Lawrence et al., 2010, Subramaniam et al., 2010), viable *M. haemolytica* is isolated much less frequently than other bacteria (Dassanayake et al., 2010 and Shanthalingam et al., 2014). An earlier study by us demonstrated that *B. trehalosi* inhibits the growth of *M. haemolytica* in vitro. Our subsequent demonstration of the presence of *M. haemolytica* by PCR assays in 70% of the pneumonic lung samples that were negative for *M. haemolytica* by culture (Shanthalingam et al., 2014), suggested that the growth inhibition of *M. haemolytica* observed in vitro, occurs in vivo as well. The present study extends our previous finding of *M. haemolytica* growth inhibition by *B. trehalosi*.

Typically, *M. haemolytica* serotype 1 and 2 are isolated from the pneumonic cattle and sheep respectively. Few *M. haemolytica* isolates from sheep are untyposable with the currently available antisera. Therefore, serotypes 1, 2 and the untyposable strains of *M. haemolytica* were used in the bacterial competition assays. All fifty-five *B. trehalosi* isolates tested were from BHS herds from different geographical regions in the USA and the majority of the isolates were from pneumonic BHS. Inhibition of growth of *M. haemolytica* by 100% of these *B. trehalosi* isolates indicates that the inhibitory phenotype is conserved across the *B. trehalosi* strains, which could explain at least partially, the failure to isolate *M. haemolytica* from pneumonic BHS lungs by culture. The finding that the inhibitory phenotype is not plasmid-encoded, but chromosomally encoded, is likely to be responsible for the conservation of the inhibitory phenotype. A previous study by us...
found that *P. multocida* also inhibits the growth of *M. haemolytica* (Bavananthasivam et al., 2013). However, *M. haemolytica* has not been isolated by culture, even from pneumonic BHS lungs that did not carry *B. trehalosi* or *P. multocida*, indicating the possibility that other agents could be inhibiting the growth of *M. haemolytica* thereby reducing the likelihood of recovering this pathogen from the late stage infections (Besser TE, et al. 2008 and Besser TE, et al. 2012).

Our finding that *E. coli* inhibits the growth of *M. haemolytica* is consistent with our hypothesis that bacteria other than *B. trehalosi* and *P. multocida* inhibit the growth of *M. haemolytica*. As with *B. trehalosi* and *P. multocida*, *E. coli* also inhibits the growth of *M. haemolytica* by a proximity-dependent mechanism. The growth inhibition of *M. haemolytica* was observed in broth cultures and not on solid agar plates (data not shown). Broth cultures simulate the growth conditions in pneumonic lungs more closely than solid agar plates. Thus it appears that a pneumonic lung is a conducive environment for the growth inhibition of *M. haemolytica* by *B. trehalosi*, *P. multocida* and *E. coli*.

Competition within and/or between bacterial species drives the composition of bacterial communities (Cotter et al., 2013) and presumably this is true in polymicrobial lung infection. Bacterial secretion systems, bacteriocins/microcins, toxin-antitoxins, and siderophore-mediated mechanisms have been reported to be responsible for inhibition of growth (Cotter et al., 2013, Zschüttig et al., 2012, Bellomio et al., 2007, 2, 4, 24, 27). Of these, bacteriocins are often used by bacteria to inhibit the growth of the closely related bacteria (Duquesne et al., 2007 and Cotter et al., 2013). In this study, supernatant from inhibitor strains had no effect on the growth of *M. haemolytica* suggesting that if the growth inhibition is driven by a soluble factor it is present in
very low concentration or it has a very short half-life. Either scenario is consistent with a requirement for close physical proximity of the target for the inhibition to occur. These notions are also supported by the observation that the inhibition is most prominent in liquid medium.

Phages are typically small and are able to pass through a 0.4 µm filter (Sawant et al., 2012). The growth of *M. haemolytica* was not inhibited when cultured with an inhibitor strain while being separated by a 0.4 µm filter. This observation suggests that growth inhibition of *M. haemolytica* by *E. coli* is not mediated by phages. Similar observations have been made for *B. trehalosi* (Dassanayake et al., 2010) and *P. multocida* (Bavananthasivam et al., 2013). Our observation that the growth of *M. haemolytica* was not inhibited by killed *B. trehalosi* or *E. coli* suggests that the inhibitor is produced by live bacteria only, and the inhibition is not mediated by the surface-exposed cell wall components.

Several *E. coli* strains inhibit the growth of genetically different *E. coli* via a contact- or proximity-dependent mechanism (Aoki et al. 2008, Eberhart et al., 2012, and Sawant et al., 2012). Aoki *et al* showed a specific receptor-ligand interaction between the inhibitor and the target strain (Aoki et al. 2008). CdiAB of the inhibitor strain of *E. coli* recognizes the receptor BamA on the target cells. Further, microcins such as E492 produced by *E. coli* and other organisms utilize FepA or CirA to get into the target cells (Aoki et al. 2008 and Cotter et al., 2013). BLAST analysis revealed the presence of homologs of BamA and FepA in *M. haemolytica*. Thus, it is plausible that a similar mechanism is utilized by *E. coli, B. trehalosi*, and *P. multocida* to inhibit the growth of *M. haemolytica*. 
Gram-positive bacteria can inhibit the growth of Gram-negative bacteria (Cálix-Lara et al., 2014). *Staphylococcus* species and *Streptococcus* species are commonly observed as commensal bacteria in the upper respiratory tract of domestic sheep and BHS (Safaee et al., 2006 and Weiser et al., 2009). Moreover, these organisms have been isolated from BHS pneumonic lungs (Safaee et al., 2006, Weiser et al., 2009 and Besser TE, et al. 2008). The lack of inhibition of growth of *M. haemolytica* in co-culture experiments with *Staphylococcus* spp. and *Streptococcus* spp suggests that these bacteria are not responsible for the failure to isolate *M. haemolytica* by culture, from pneumonic BHS lungs.
5. CONCLUSION

In summary, inhibition of *M. haemolytica* is conserved across the *B. trehalosi* strains. The inhibitory phenotype is chromosomally-encoded. *E. coli* also inhibits the growth of *M. haemolytica* in a manner that is consistent with proximity-dependent inhibition (Sawant et al., 2012, Bavananthasivam et al., 2013, Dassanayake et al., 2010) *Staphylococcus* and *Streptococcus*, also frequently isolated from pneumatic BHS lungs, do not inhibit the growth of *M. haemolytica*.

ACKNOWLEDGMENTS

This research was supported by funds from the Wild Sheep Foundation and its State Chapters, and US Forest service. Abirami Kugadas was supported by a Graduate Training Fellowship from Morris Animal Foundation. We thank Lisa Orfe, Rohana Dassanayake, Lauren Eberhart and Murugan Subbiah for technical help.
REFERENCES


Haldorson G.J., Foreyt W.J., Evermann J.F., Herrmann-Hoesing L.M., Knowles D.P.,
Srikumaran S. 2013. Role of Bibersteinia trehalosi, respiratory syncytial virus, and


Characterization of a novel microcin that kills enterohemorrhagic Escherichia coli O157:H7


of healthy bighorn sheep with Pasteurella haemolytica from healthy domestic sheep. Journal

19. Foreyt W.J., 1994. Effects of controlled contact exposure between healthy bighorn sheep and
llamas, domestic goats, mountain goats, cattle, domestic sheep, or mouflon sheep.
Proceedings of the Biennial Symposium of the Northern Wild Sheep and Goat Council 9:7–
14.


Inactivation of Pasteurella (Mannheimia) haemolytica leukotoxin causes partial attenuation


**Table 1:** Inhibition of growth of *M. haemolytica* by *E. coli*. Three different isolates of *M. haemolytica* were co-cultured with three different isolates of *E. coli*. Results of the bacterial counts are reported as log$_{10}$ of mean CFU/ml of two independent experiments at 24 h. Bacterial counts of mono- and co-culture *M. haemolytica* at 24 h post-culture are statistically significantly different for all comparisons (*P* < 0.05).

<table>
<thead>
<tr>
<th>Bacterial strain combination (E. coli, M. haemolytica)</th>
<th>M. haemolytica</th>
<th>E. coli</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mono-culture</td>
<td>Co-culture</td>
</tr>
<tr>
<td><em>E. coli</em> E6536, Mh1023</td>
<td>9.09</td>
<td>7.16</td>
</tr>
<tr>
<td><em>E. coli</em> 186, Mh731</td>
<td>7.14</td>
<td>5.08</td>
</tr>
<tr>
<td><em>E. coli</em> 186, Mh A1 mutant</td>
<td>7.24</td>
<td>3.80</td>
</tr>
<tr>
<td><em>E. coli</em> 25, Mh1023</td>
<td>10.12</td>
<td>4.24</td>
</tr>
<tr>
<td><em>E. coli</em> 25, Mh731</td>
<td>9.69</td>
<td>4.65</td>
</tr>
</tbody>
</table>
Table 2: *Staphylococcus aureus* does not inhibit the growth of *M. haemolytica*. Three different isolates of *M. haemolytica* were co-cultured with *Staphylococcus aureus*. Results of the bacterial counts are reported as $\log_{10}$ of mean CFU/ml of three independent experiments at 24 h. Bacterial counts of mono- and co-culture *M. haemolytica* at 24 h post-culture is not statistically significant ($P<0.05$).

<table>
<thead>
<tr>
<th>Bacterial strain combination (S. aureus, M. haemolytica)</th>
<th><em>M. haemolytica</em></th>
<th><em>S. aureus</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mono-culture</td>
<td>Co-culture</td>
</tr>
<tr>
<td>SA23N, Mh1023</td>
<td>8.25</td>
<td>7.47</td>
</tr>
<tr>
<td>SA23N, Mh A1 mutant</td>
<td>6.38</td>
<td>7.07</td>
</tr>
<tr>
<td>SA23N, Mh731</td>
<td>7.52</td>
<td>7.59</td>
</tr>
</tbody>
</table>
Figure 1. EC186\textsuperscript{Nal} inhibits the growth of \textit{M. haemolytica} 10/2/3 in co-culture. EC186 (■/□) and \textit{M. haemolytica}\textsuperscript{Amp} 10/2/3 (●/○) were cultured either as monoculture (■/●) or as co-culture (□/○) in BHI broth. Aliquots were serially diluted and plated on BHI agar plates to enumerate the bacterial colonies. Results are the $\log_{10}$ of mean CFU/ml of three independent experiments (± SEM). (p< 0.05)
Figure 2.A. Killed *B. trehalosi* does not inhibit the growth of *M. haemolytica*. \(1 \times 10^6\) CFU of *B. trehalosi* were killed by ethanol treatment and subsequently co-cultured with *M. haemolytica* serotypes A1 or A2. At 24 hours post-culture, no difference was observed between the *M. haemolytica* in mono-culture and the *M. haemolytica* in co-culture with killed *B. trehalosi*. Results are the Log\(_{10}\) of mean CFU/ml of three independent experiments (± SEM).
Figure 2.B. Killed *E. coli* does not inhibit the growth of *M. haemolytica*. 1x10⁶ CFU of *E. coli* were killed by ethanol treatment and subsequently co-cultured with *M. haemolytica* strain (10/2/3). At 24 hours post-culture, no difference was observed between the *M. haemolytica* in mono-culture and the *M. haemolytica* in co-culture with killed *E. coli*. Results are the Log₁₀ of mean CFU/ml of three independent experiments (± SEM).
Figure 3. *E. coli* inhibits the growth of *M. haemolytica* via a proximity-dependent mechanism.

*E. coli*<sup>Nal</sup> (■) and *M. haemolytica Amp* (10/2/3) (□) were cultured in a six-well culture plate as mono-culture or co-culture. The co-culture wells were separated into two chambers using cell culture filters with either 0.4 µm or 8 µm pore size. In chambers that were separated by 0.4 µm filter, the two bacterial species were confined to the respective chambers whereas in those separated by 8 µm filter, there was mixing of the two bacterial species. At 24 hours, no significant difference was observed between the *M. haemolytica Amp* in mono-culture wells and in co-culture wells separated by 0.4 µm filter. However, there was a significant difference (p<0.05) between *M. haemolytica Amp* in mono-culture wells and co-culture wells separated by 8 µm filter. Results are the Log<sub>10</sub> of mean CFU/ml of two independent experiments (± SEM).
Figure 4. A. *S. agalactiae* does not inhibit the growth of *M. haemolytica*. *S. agalactiae* (■/□) and *M. haemolytica* (●/○) were cultured either as mono- (●/■) or as co-culture (○/□) in BHI broth. B. *Streptococcus* sp. does not inhibit the growth of *M. haemolytica*. *Streptococcus* sp. (■/○) and *M. haemolytica* (●/□) were cultured either as mono-culture (■/●) or as co-culture (○/□) in BHI broth, and the CFU/ml were enumerated as described in the legend for Figure 1. Results are the Log$_{10}$ of mean CFU/ml of three independent experiments (± SEM).
**Supplementary Table 1:** Inhibition of growth of *M. haemolytica* by 55 *B. trehalosi* isolates.

*B. trehalosi* isolates were individually co-cultured with *M. haemolytica* and CFU counts of *M. haemolytica* in mono- and co-cultures were determined by serial dilution and plating. The CFU/ml counts of *M. haemolytica* in mono- and co-cultures is significantly different (p<0.0001).

**Inhibition of growth of *M. haemolytica* by 55 *B. trehalosi* isolates**

<table>
<thead>
<tr>
<th>Animal #</th>
<th>BT isolate #</th>
<th>Source</th>
<th>Difference between mono- and co-culture <em>M. haemolytica</em> $\log_{10}$CFU/ml at 24 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>272</td>
<td>4944</td>
<td>pharynx</td>
<td>3.03</td>
</tr>
<tr>
<td>101</td>
<td>4970</td>
<td>pharynx</td>
<td>2.07</td>
</tr>
<tr>
<td>105</td>
<td>4974</td>
<td>pharynx</td>
<td>4.48</td>
</tr>
<tr>
<td>185901</td>
<td>5039</td>
<td>pharynx</td>
<td>2.69</td>
</tr>
<tr>
<td>185903</td>
<td>5038</td>
<td>pharynx</td>
<td>4.82</td>
</tr>
<tr>
<td>185902</td>
<td>5045</td>
<td>pharynx</td>
<td>4.16</td>
</tr>
<tr>
<td>09TU88</td>
<td>5060</td>
<td>N/A</td>
<td>5.01</td>
</tr>
<tr>
<td>54</td>
<td>5061</td>
<td>N/A</td>
<td>4.09</td>
</tr>
<tr>
<td>185895</td>
<td>5064</td>
<td>pharynx</td>
<td>2.01</td>
</tr>
<tr>
<td>185891</td>
<td>5067</td>
<td>pharynx</td>
<td>2.79</td>
</tr>
<tr>
<td>185889</td>
<td>5076</td>
<td>lung</td>
<td>4.10</td>
</tr>
<tr>
<td>185889</td>
<td>5078</td>
<td>lung</td>
<td>2.82</td>
</tr>
<tr>
<td>185887</td>
<td>5184</td>
<td>lung</td>
<td>4.01</td>
</tr>
<tr>
<td>185899</td>
<td>5192</td>
<td>lung</td>
<td>4.62</td>
</tr>
<tr>
<td>185899</td>
<td>5194</td>
<td>lymphnode</td>
<td>2.62</td>
</tr>
<tr>
<td>A</td>
<td>5210</td>
<td>lung</td>
<td>4.54</td>
</tr>
<tr>
<td>A</td>
<td>5212</td>
<td>lymphnode</td>
<td>2.18</td>
</tr>
<tr>
<td>A</td>
<td>5214</td>
<td>tonsil</td>
<td>2.00</td>
</tr>
<tr>
<td>183</td>
<td>5226</td>
<td>N/A</td>
<td>4.01</td>
</tr>
<tr>
<td>B</td>
<td>5324</td>
<td>tonsil</td>
<td>2.85</td>
</tr>
<tr>
<td>2622</td>
<td>5345</td>
<td>nasal</td>
<td>4.10</td>
</tr>
<tr>
<td>2622</td>
<td>5348</td>
<td>lung</td>
<td>4.00</td>
</tr>
<tr>
<td>UM1</td>
<td>5351</td>
<td>lung</td>
<td>2.10</td>
</tr>
<tr>
<td>UM1</td>
<td>5354</td>
<td>tonsil</td>
<td>4.31</td>
</tr>
<tr>
<td>SB1</td>
<td>5356</td>
<td>lung</td>
<td>4.92</td>
</tr>
<tr>
<td>Code</td>
<td>ID</td>
<td>Type</td>
<td>Value</td>
</tr>
<tr>
<td>----------</td>
<td>--------</td>
<td>--------------</td>
<td>-------</td>
</tr>
<tr>
<td>SB1</td>
<td>5357</td>
<td>lymphnode</td>
<td>3.09</td>
</tr>
<tr>
<td>SB1</td>
<td>5358</td>
<td>tonsil</td>
<td>4.31</td>
</tr>
<tr>
<td>SB2</td>
<td>5359</td>
<td>lung</td>
<td>3.37</td>
</tr>
<tr>
<td>SB2</td>
<td>5360</td>
<td>lung</td>
<td>4.01</td>
</tr>
<tr>
<td>SB2</td>
<td>5377</td>
<td>tonsil</td>
<td>2.01</td>
</tr>
<tr>
<td>27</td>
<td>5459</td>
<td>pharynx</td>
<td>2.27</td>
</tr>
<tr>
<td>126</td>
<td>5476</td>
<td>oropharynx</td>
<td>4.22</td>
</tr>
<tr>
<td>127</td>
<td>5478</td>
<td>oropharynx</td>
<td>3.47</td>
</tr>
<tr>
<td>1</td>
<td>5484</td>
<td>lung</td>
<td>4.49</td>
</tr>
<tr>
<td>186063</td>
<td>5550</td>
<td>pharynx</td>
<td>4.62</td>
</tr>
<tr>
<td>106</td>
<td>5530</td>
<td>N/A</td>
<td>2.11</td>
</tr>
<tr>
<td>40190</td>
<td>5531</td>
<td>N/A</td>
<td>2.85</td>
</tr>
<tr>
<td>186061</td>
<td>5591</td>
<td>lung</td>
<td>4.01</td>
</tr>
<tr>
<td>186097</td>
<td>5601</td>
<td>lung</td>
<td>2.96</td>
</tr>
<tr>
<td>186055</td>
<td>5605</td>
<td>lung</td>
<td>4.48</td>
</tr>
<tr>
<td>186055</td>
<td>5606</td>
<td>lymphnode</td>
<td>2.91</td>
</tr>
<tr>
<td>186055</td>
<td>5607</td>
<td>tonsil</td>
<td>4.21</td>
</tr>
<tr>
<td>186139</td>
<td>5614</td>
<td>lymphnode</td>
<td>3.45</td>
</tr>
<tr>
<td>186151</td>
<td>5628</td>
<td>lymphnode</td>
<td>2.81</td>
</tr>
<tr>
<td>186150</td>
<td>5629</td>
<td>lung</td>
<td>2.71</td>
</tr>
<tr>
<td>186185</td>
<td>5695</td>
<td>pharynx</td>
<td>4.10</td>
</tr>
<tr>
<td>186173</td>
<td>5697</td>
<td>pharynx</td>
<td>2.20</td>
</tr>
<tr>
<td>3177</td>
<td>5759</td>
<td>lung</td>
<td>4.72</td>
</tr>
<tr>
<td>3177</td>
<td>5762</td>
<td>pharynx</td>
<td>4.21</td>
</tr>
<tr>
<td>3179</td>
<td>5765</td>
<td>lung</td>
<td>4.12</td>
</tr>
<tr>
<td>3179</td>
<td>5766</td>
<td>lung</td>
<td>4.93</td>
</tr>
<tr>
<td>ST-URS-B</td>
<td>5825</td>
<td>lung</td>
<td>4.73</td>
</tr>
<tr>
<td>EH-MORT-0218-4</td>
<td>5885</td>
<td>pharynx</td>
<td>3.02</td>
</tr>
<tr>
<td>DSURS-E</td>
<td>5940</td>
<td>lung</td>
<td>2.75</td>
</tr>
<tr>
<td>1862241</td>
<td>5962</td>
<td>lung</td>
<td>2.63</td>
</tr>
</tbody>
</table>

*B. trehalosi* isolates were individually co-cultured with *M. haemolytica* and CFU counts of *M. haemolytica* in mono- and co-cultures were determined by serial dilution and plating. The CFU/ml counts of *M. haemolytica* in mono- and co-cultures is significantly different (p<0.0001)

N/A-Not Available
Supplementary Figure 1. Analysis of plasmid preps by gel electrophoresis. A) Plasmids isolated by Qiagen plasmid extraction kit. Lane M: 10kb DNA ladder; Lanes 2-10: plasmid preps from *B. trehalosi* isolates; Lanes 11 and 12: positive control plasmid (pAM2425, 6.2kb).
Supplementary Figure 1. Analysis of plasmid preps by gel electrophoresis. B) Plasmids isolated by using the phenol-chloroform method described by Kado and Liu, 1981. Lanes M: Bac Tracker supercoiled DNA ladder; Lanes 2, 3, 4, 5, and 10: plasmid preps made from B. trehalosi isolates; Lanes 8 and 9: positive control plasmid preps (peH4H, ~140kb).
Effects of intranasal administration of *Bibersteinia trehalosi* on shedding of *Mannheimia haemolytica* by domestic sheep and survival of commingled bighorn sheep

Kugadas A. ¹, Bavananthasivam J. ¹, Raghavan B. ¹, Batra S. A. ¹, Von Hoey J. ¹, Haldorson G. ¹,
Call D. R. ¹, ², Shanthalingam S. ¹, Srikumaran S¹*. ¹

¹Department of Veterinary Microbiology and Pathology, ²Paul G. Allen School for Global Animal Health, Washington State University, Pullman, Washington 99164-7040.

Note: This manuscript is currently in preparation for submission to Veterinary Microbiology

*Corresponding author:
Mailing address:
Dr. S. Srikumaran,
Department of Veterinary Microbiology and Pathology,
College of Veterinary Medicine
Washington State University
Pullman, WA 99164-7040, USA
Phone: 509-335-4572; Fax: 509-335-8529
E-mail: ssrikumaran@vetmed.wsu.edu
ABSTRACT

Fatal pneumonia in bighorn sheep (BHS) often results from acquisition of leukotoxin (Lkt) - positive Mannheimia haemolytica from domestic sheep (DS). Previously, we demonstrated inhibition of growth of M. haemolytica by Bibersteinia trehalosi in vitro. We hypothesized that oro-nasal inoculation of B. trehalosi will eliminate or reduce shedding of M. haemolytica by DS. Domestic sheep carrying Lkt-positive M. haemolytica were oro-nasally inoculated with Lkt-negative B. trehalosi following antibiotic treatment. These DS shed reduced numbers of M. haemolytica, post-treatment. However, healthy BHS commingled with B. trehalosi-treated DS acquired M. haemolytica around day 14 post-commingling, but did not develop pneumonia during this phase of the study (phase 1, 106 days). On subsequent commingling with DS that carried the native, as well as an inoculated virulent strain of M. haemolytica, these BHS did not develop pneumonia during this phase of the study also (phase 2, 105 days). However, they died of pneumonia in phase 3 of this study when commingled with DS that carried Lkt-positive M. haemolytica and Mycoplasma ovipneumoniae. It is likely that the BHS acquired small doses of Lkt-positive M. haemolytica from B. trehalosi-treated DS in phase 1 that was adequate to induce an immune response but not fatal pneumonia. Lkt-neutralizing antibodies developed in phase 1 helped the BHS to survive commingling with DS inoculated with a virulent strain of M. haemolytica in phase 2. However, in phase 3, when they acquired M. ovipneumoniae which abrogates the muco-ciliary defense mechanism, an overwhelming dose of M. haemolytica entered the lungs and caused fatal pneumonia.
1. INTRODUCTION

Bacterial pneumonia is a fatal disease responsible for the substantial decline of bighorn sheep (Ovis canadensis; BHS) population and its subsequent slow recovery in North America (Buechner 1960; Foreyt and Jessup 1982; Coggins 1988; Valdez and Krausman 1999; Miller 2001 and Smith et al. 2014). Several anecdotal reports suggested that BHS often died following contact with domestic sheep (Ovis aries; DS; Foreyt. W. J. and Jessup. D. A., 1982; Foreyt. et al., 1994; George et al., 2008). Experimental commingling studies have confirmed the transmission of respiratory pathogens from DS to BHS (Foreyt and Jessup 1982; Foreyt, W. J. 1994; Lawrence et al. 2010). Mannheimia haemolytica is an important pathogen of BHS pneumonia (Foreyt. et al., 1994; Ackermann and Brogden 2000; Dassanayake et al., 2009 and Subramaniam et al., 2011). Our laboratory has clearly demonstrated the transmission of M. haemolytica from DS to BHS by employing green fluorescent protein-tagged organisms (Lawrence et al., 2010).

Bacteria detected in the lungs of pneumonic BHS from the field include M. haemolytica, Bibersteinia trehalosi, Pasteurella multocida and Mycoplasma ovipneumoniae (Rudolph et al., 2007; Besser et al., 2008; Besser et al., 2012; and Shanthalingam et al., 2014). Although M. haemolytica causes fatal bronchopneumonia in BHS under natural and experimental conditions B. trehalosi and P. multocida have been isolated from the pneumonic BHS lung tissues by cultural methods more frequently than M. haemolytica, (Shanthalingam et al., 2014). Nevertheless, in one study PCR assay detected M. haemolytica in 70% of BHS lung tissues that were negative for this organism by culture, suggesting that B. trehalosi, P. multocida, and possibly others, inhibit the growth of M. haemolytica (Shanthalingam et al., 2014). Subsequently,
we have demonstrated that \textit{B. trehalosi}, \textit{P. multocida} and \textit{Escherichia coli} inhibit the growth of \textit{M. haemolytica} by a proximity-dependent mechanism (Dassanayake et al., 2010; Bavananthasivam et al., 2013; Kugadas et al., 2014). We reasoned that this phenomenon of growth inhibition could be utilized to develop a strategy to prevent transmission of \textit{M. haemolytica} from DS to BHS. We hypothesized that intranasal inoculation of \textit{B. trehalosi} will eliminate or reduce shedding of \textit{M. haemolytica} by DS and hence eliminate or reduce transmission of this organism by DS to BHS.

Leukotoxin (Lkt) has been widely accepted as the most important virulence factor of \textit{M. haemolytica} based on the fact that Lkt-deletion mutants cause only mild pneumonic lesions, but do not cause death of BHS (Dassanayake et al., 2012). Therefore, the first objective of this study was to determine the effects of intra-nasal administration of Lkt-negative \textit{B. trehalosi} on the shedding of Lkt-positive \textit{M. haemolytica} by DS. Since DS have been shown to transmit respiratory pathogens to BHS, it was of interest for us to determine the effect of intra-nasal administration of Lkt-negative \textit{B. trehalosi} to DS on the transmission of Lkt-positive \textit{M. haemolytica} to BHS. Therefore the second objective was to determine whether BHS commingled with \textit{B.trehalosi}-inoculated DS acquire Lkt-positive \textit{M. haemolytica}. As a control experiment, the third objective was designed to determine the effects of commingling BHS with DS that were not inoculated with \textit{B. trehalosi}. Furthermore, we have shown previously that \textit{M. ovipneumoniae} can predispose BHS to \textit{M. haemolytica}-caused pneumonia (Dassanayake et al., 2010). The current study provided the opportunity to further examine the effect of commingling the BHS from Objective 3 with DS that were positive for \textit{M. ovipneumoniae} and \textit{M. haemolytica}. 
2. MATERIALS AND METHODS

2.1 Experimental Animals

Experiments were carried out in three phases (Table 1). Altogether, 4 BHS and 8 DS were used in this study. Experimental protocols involving animals were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at Washington State University. Eight clinically normal, female DS from the same flock that were positive for Lkt-positive *M. haemolytica* and *B. trehalosi*, but negative for *M. ovipneumoniae*, were selected for this study. These DS were randomly divided into two groups comprising four sheep in each.

2.2. Experimental design. **Phase 1:** One group of four DS was injected twice with ceftiofur (3 mg/lb., subcutaneously) at 72 hr interval. All sheep were intra-nasally inoculated with Lkt-negative *B. trehalosi* 4 days after the last ceftiofur treatment. These DS were commingled in a 60 ft x 125 ft pen with four clinically healthy BHS that were negative for *M. haemolytica* and *M. ovipneumoniae*. The untreated group of four DS was kept in a distant pen for use in phase 2. Nasal and pharyngeal swabs, and blood samples were collected from DS before the treatment with antibiotic, on the day of inoculation with *B. trehalosi*, 10 days post-*B. trehalosi* inoculation and on days 0, 55 and 106 of commingling (Table 1). **Phase 2:** Two clinically normal DS from the untreated group of four DS (negative for *M. ovipneumoniae*, but positive for Lkt-positive *M. haemolytica* and Lkt-positive *B. trehalosi*) were intra-nasally inoculated with *M. haemolytica* serotype 2 strain WSU-1. These two DS were then commingled with the BHS that survived in phase 1 (106 days). Nasal and pharyngeal swabs, and blood samples were collected from both BHS and DS on day 0, 48 and 105 post-commingling. **Phase 3:** Two clinically normal DS that were positive for *M. ovipneumoniae*, Lkt-positive *M. haemolytica* and Lkt-positive *B. trehalosi*
were commingled with the BHS that survived in phase 2. Nasal and pharyngeal swabs, and blood samples were collected from both BHS and DS on day 0 and 48 post-commingling, and at necropsy.

One set of the nasal swabs was submitted to Washington Animal Disease Diagnostic Laboratory (WADDL) at Washington State University for detection of *M. ovipneumoniae* by PCR assay. Serology was carried out to determine the antibody titers for *M. haemolytica* leukotoxin (LktA) and *M. ovipneumoniae*.

### 2.3. Isolation and quantification of *M. haemolytica*, *B. trehalosi* and *M. ovipneumoniae*

To compare the colony forming units (CFU) of *M. haemolytica* and *B. trehalosi* between sheep, CFU were normalized based on the mass of epithelial lining fluid collected in each sample. Mass of the epithelial lining fluid was estimated by weighing the swabs before and after swabbing. Bacteria from each swab were recovered by the addition of one ml of PBS in a tube and vortexing. The extract from each swab was serially diluted and plated on BHI agar plates and incubated overnight at 37°C. Fifty single colonies per swab were picked based on the colony morphology for sub-culturing overnight on BHI agar plate. Selected colonies were subjected to a colony PCR assay designed to identify *M. haemolytica*, *B. trehalosi* and/or lktA of *M. haemolytica*. Colonies positive for *B. trehalosi* were screened by another PCR assay to determine the presence of lktA. Leukotoxin was produced from randomly selected Lkt-positive colonies to determine their cytotoxicity.
2.4. Detection of *M. haemolytica*, *B. trehalosi* and *lktA* by polymerase chain reaction (PCR)

Species-specific primers were used to detect Lkt-positive *M. haemolytica* and *B. trehalosi* via a multiplex PCR assay (Dassanayake et al., 2010 and Shanthalingam et al., 2014). This PCR assay was performed in a 20 ul final reaction with GoTaq® PCR SuperMix (Promega Inc., Madison, Wisconsin, USA) with 0.2 µM of each primers and one and bacterial colony. The primers used were: Mh F: TGG GCA ATA CGA ACT ACT CGG G; Mh R: CTT TAA TCG TAT TCG CAG; Bt F: GCC TGC GGA CAA ACG TGT TG; Bt R: TTT CAA CAG AAC CAA AAT CAC GAA TG; LktA497 F: CTT ACA TTT TAG CCC AAC GTG; LktA497 R: TAA ATT CGC AAG ATA ACG GG. The following cycling conditions were used: denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 52°C for 40 s, extension at 72°C for 30 s, and a final elongation at 72°C for 10 min. Presence of *lktA* in *B. trehalosi* was determined by the previously described *lktA* gene-specific PCR (Dassanayake et al., 2010).

2.5. Lkt-negative *B. trehalosi* and *M. haemolytica* serotype 2 inoculum and inoculation

Rifampicin resistant Lkt-negative *B. trehalosi* strain Y31 was cultured overnight on BHI agar plate supplemented with 5% sheep blood (Remel, Lenexa, KS). Bacteria obtained from the plate were suspended in colorless RPMI 1640 medium to obtain an OD$_{600}$ of 0.5. The suspension was incubated at 37°C and 250 rpm for 2-2.5 hr until the OD$_{600}$ reached 1.5. Bacteria were pelleted by centrifugation at 2500xg for 20 min and re-suspended in colorless RPMI 1640 medium to approximately 1x10$^9$ CFU/ml. Five ml of the inoculum was administered intra-nasally and orally to each DS using an atomizer.
*M. haemolytica* serotype 2 strain WSU-1 inoculum was prepared according to the method described above. The final inoculum was adjusted to a concentration of ~$1 \times 10^8$ CFU/ml. Five ml of the inoculum was administered intra-nasally and orally to each DS as described above.

2.6. Production of leukotoxin

Presence of *lktA* on the genome of a *M. haemolytica* or *B. trehalosi* does not necessarily mean that the isolate produces a functional Lkt protein (unpublished observation). Therefore, Lkt was produced from Lkt+ *M. haemolytica* isolates that were isolated from the BHS to determine their cytotoxicity. Leukotoxin was produced according to a previously described protocol (Gentry, M. J., and S. Srikumaran. 1991). Briefly, bacteria grown to mid-logarithmic phase in BHI broth were pelleted and re-suspended in RPMI 1640 medium supplemented with 1.05 g/l bovine serum albumin. Bacteria in RPMI 1640 medium was incubated at 37°C for an additional 1-1.5 hr. Leukotoxin was harvested by removing the bacteria by centrifugation (2500xg) followed by sterilization of the leukotoxin-containing supernatant using a 0.22um syringe filter.

2.7. Determination of cytotoxicity of Lkt

Cytotoxicity of the toxin produced by *M. haemolytica* and *B. trehalosi* isolates was determined by a previously described 3-(4,5-dimethylthiazoyl-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) dye-reduction cytotoxicity assay (Gentry, M. J.; and S. Srikumaran. 1991). Briefly, 50 µl of serially diluted Lkt was incubated with bovine lymphoma cells (BL-3 cells; 50 µl of $5 \times 10^6$ cells/ml) at 37°C for 1 h. The plate was centrifuged at 600 xg for 5 min and supernatant fluid was decanted. The cells were re-suspended in 100 µl of colorless RPMI 1640 medium (Invitrogen, Carlsbad, CA) and 20 µl of MTT dye (Sigma Chemical Co., St. Louis, MO, 5 mg/ml) and incubated at 37°C for 1 hr. The
plate was centrifuged again at 600 xg for 5 min, the fluid was decanted, and 100 µl of acid isopropanol was added to dissolve the precipitate. The optical density at 540 nm (OD$_{540}$) of the final product was measured using an ELISA plate reader (Becton Dickinson). Percent cytotoxicity was calculated using the following formula: [1 - (OD of Lkt + BL-3/OD of BL-3 cells only) x 100].

2.8. Detection of antibodies against *M. haemolytica* Lkt and *M. ovipneumoniae*

The Lkt-neutralizing antibody titers were determined by a cytotoxicity inhibition assay, as previously described (Gentry, M. J.; and S. Srikumaran. 1991). Briefly, 50 µl of each serially diluted serum sample was incubated with 50 µl of log-phase culture supernatant fluid containing Lkt at a dilution that caused 50% cytotoxicity to BL-3 cells and incubated at 4°C for 1 hr. BL-3 cells (50 µl of 5 x 10$^6$ cells/ml) were then added to the serum-Lkt mixture and incubated at 37°C for another 1 hr. The plate was centrifuged at 600 xg for 5 min, and supernatant fluid was decanted. The cells were re-suspended in 100 µl of colorless RPMI 1640 medium (Invitrogen, Carlsbad, CA) and 20 µl of MTT dye (5 mg/ml) and incubated at 37°C for 1 h. The plate was centrifuged again at 600 xg for 5 min, the fluid was decanted, and 100 µl of acid isopropanol was added to dissolve the precipitate. The OD$_{540}$ of the final product was measured using an ELISA plate reader (Becton Dickinson). Percent inhibition was calculated as described previously (Gentry, M. J.; and S. Srikumaran. 1991) according to the following formula: (cytotoxicity$_{Lkt + \text{medium}}$ - cytotoxicity$_{Lkt + \text{serum}}$) / (cytotoxicity$_{Lkt + \text{medium}}$) x 100. The reciprocal of the highest dilution of the serum that yielded a minimum of 50% inhibition of Lkt-induced cytotoxicity was considered as the Lkt-neutralizing titer.
Antibody titers for *M. ovipneumoniae* were determined by WADDL via a competitive inhibition ELISA (cELISA) assay that used a *M. ovipneumoniae*-specific monoclonal antibody. Sera that caused >50% inhibition (I) were considered positive based upon 3 standard deviations from the mean percent inhibition of negative sera. Results are reported as antibody not detected (% I <40%); antibody detected at levels consistent with previous exposure or current infection with *M. ovipneumoniae* (% I >= 50%); antibody detection indeterminate to establishment of correlation with *M. ovipneumoniae* infection (% I 40% to 50%).
3. RESULTS AND DISCUSSION

3.1. Microbial profile of the nasopharynx of the naïve DS and BHS.

The baseline microbial profile of the nasopharynx of the experimental animals was obtained from at least three nasal and pharyngeal swabs collected during 8 weeks prior to the onset of phase 1 of this study. Isolation of bacteria followed by species-specific PCR assays revealed that all eight DS were positive for Lkt-positive *M. haemolytica* and negative for *M. ovipneumoniae*, whereas the four BHS were negative for both Lkt-positive *M. haemolytica* and *M. ovipneumoniae* but positive for Lkt-negative *B. trehalosi*.

3.2. Oro-nasal administration of *B. trehalosi* following antibiotic treatment reduces *M. haemolytica* in the nasopharynx of DS.

To enhance the colonization by our *B. trehalosi* strain, the DS were first treated with ceftiofur, a short-acting antibiotic that is bactericidal for Gram-negative bacteria. Following inoculation, the DS had high numbers of *B. trehalosi* (~5.5 log$_{10}$ CFU/g), which gradually decreased by day 105 (~4.2 log$_{10}$ CFU/g; Fig 1A). Only one out of the four DS shed *M. haemolytica* on day 14 post-*B. trehalosi* administration suggesting that *B. trehalosi* inhibits the growth of *M. haemolytica, in vivo* (Fig 1B). Although, there was a reduction in nasopharyngeal *M. haemolytica*, the DS gradually regained the pre-inoculation profile of *M. haemolytica* by day 28 post-*B. trehalosi* inoculation (Fig 1A). Spatial organization of bacterial community plays a significant role in the colonization of different bacterial species on the mucosa (Weyrich et al 2014). Different bacteria occupy different niches due to the effect of inhibitor substances produced by different species of bacteria and to avoid competition for resources (Zijng et al 2010; Gonzalez et al. 2011; Weyrich et al 2014). *B. trehalosi* typically colonizes the deeper region of the pharyngeal crypts.
Furthermore, our previous *in vitro* experiments showed that the proximity of *B. trehalosi* and *M. haemolytica* is necessary for the inhibition of the latter. Therefore, it is likely that the spatial separation of these organisms in the nasopharynx contributed to the reappearance of *M. haemolytica* to the pre-*B. trehalosi* inoculation levels.

### 3.3. Phase I: BHS commingled with *B. trehalosi*-inoculated DS do not develop pneumonia.

In almost all previous studies, BHS commingled with DS developed pneumonia and died within 100 days (Foreyt and Jessup 1982; Onderka and Wishart 1988; Besser 2008; Lawrence et al. 2010). In this study, two out of the four BHS commingled with *B. trehalosi*-inoculated DS did not develop any signs of pneumonia until the end of phase 1 of the study (day 106). Unfortunately the other two BHS died due to unrelated causes on day 47 and 82 post-commingling. One BHS (#4) died of severe enteritis and the other (#43) died of trauma to the spinal cord. Gross- and histo-pathological examination of the lungs of these two animals clearly indicated the absence of pneumonic lesions (Fig 3). *M. haemolytica* was isolated from the nasal and/or pharyngeal swabs collected from the commingled BHS on day 14 suggesting that they acquired this organism on day 14 or thereabout (Fig. 2). What enabled the two BHS (# 17 and 50) to survive in spite of infection by Lkt-positive *M. haemolytica*? It is very likely that they acquired Lkt-positive *M. haemolytica* in small doses from the *B. trehalosi*-treated DS which enabled them to develop immunity against them, particularly Lkt-neutralizing antibodies. This notion is supported by the Lkt-neutralizing antibody titers of these two animals. BHS #17 and 50 had titers of 1/640 and 1/800, respectively, on day 105 (Table. 2). This notion is further supported by our previous studies which have shown that Lkt-neutralizing antibodies and
antibodies to the surface antigens protect BHS against *M. haemolytica* challenge (Subramaniam et al 2011).

### 3.4. Phase II: BHS commingled with *B. trehalosi*-inoculated DS survive commingling with DS not inoculated with *B. trehalosi.*

As a control for the experiment in phase 1, we commingled the two surviving BHS with two DS that were not previously inoculated with *B. trehalosi*, but inoculated with a virulent strain of *M. haemolytica* serotype 2 (strain WSU-1) which is known to cause severe pneumonia in sheep (Dassanayake et al., 2010; Subramaniam et al., 2011). Although the two BHS were exposed to virulent Lkt-positive *M. haemolytica* and Lkt-positive *B. trehalosi* strains, neither one of them developed any respiratory signs until the end of this phase (day 105). It is likely that the immunity developed during the first phase of the study in which they were commingled with *B. trehalosi*-inoculated DS was protective against the virulent Lkt-positive *M. haemolytica* and *B. trehalosi* strains. Both BHS continued to be colonized with Lkt-positive *M. haemolytica* and *B. trehalosi* throughout phase II (Table 3). Moreover, the isolation of Lkt-positive *B. trehalosi* from BHS (#17) is indicative of the transmission of this organism from DS to BHS. It is noteworthy that the Lkt-neutralizing antibody titers of these two BHS increased further after 50 days post-commingling with the DS carrying *M. haemolytica* serotype 2. It is very likely that further exposure to Lkt-positive *M. haemolytica* and *B. trehalosi* in phase 2 resulted in this anamnestic response.
3.5. Phase III: Commingling with DS positive for Lkt-positive *M. haemolytica*, *B. trehalosi* and *M. ovipneumoniae* results in fatal pneumonia in BHS.

Previously, we have shown that *M. ovipneumoniae* can predispose BHS to *M. haemolytica*-caused pneumonia (Dassanayake et al, 2010). To further confirm our earlier finding, we commingled the BHS that were previously commingled with *B. trehalosi*-inoculated DS in phase 1 and subsequently commingled with DS not inoculated with *B. trehalosi* in phase 2 of this study, with two DS that carried *M. ovipneumoniae* in addition to Lkt-positive *M. haemolytica* and Lkt-positive *B. trehalosi*. Both BHS developed severe bronchopneumonia and died on day 14 and 47 of the third phase of commingling, respectively (Table 3). The BHS #50 that died on day 47 had profuse mucoid nasal discharge, coughing, and became weak after 14 days of commingling, while the BHS #17 that died on day 14 developed pneumonic symptoms abruptly. The gross- and histo-pathology of the lungs were consistent with bacterial pneumonia (Fig 4). Lkt-positive *M. haemolytica*, and *B. trehalosi* and *M. ovipneumoniae* were isolated from the lungs of both of BHS (Table 3). Both BHS had high titers of Lkt-neutralizing antibodies (Table 2). BHS #50 had detectable antibodies against *M. ovipneumoniae* whereas the BHS #17 did not seroconvert. It is plausible that BHS #17 did not survive long enough to produce detectable antibody titers against this organism. DS that carried Lkt-positive *M. haemolytica*, Lkt-positive *B. trehalosi* and *M. ovipneumoniae* at the beginning of the phase III continued to shed these three organisms but remained healthy until the end of the study (Table 3).

Lkt-positive *M. haemolytica*, by itself, can cause fatal pneumonia in BHS (Subramaniam et al. 2011). However, co-infection with Lkt-positive *M. haemolytica* and *M. ovipneumoniae* could enhance the spread and severity of the disease. Mucociliary clearance is a critical host defense
mechanism and the first line of defense against respiratory pathogens. This mechanism prevents/controls the movement of exogenous bacteria and the nasopharyngeal commensal bacteria from migrating to the lungs. *M. ovipneumoniae* has been detected in pneumonic and clinically healthy BHS (Besser et al., 2008 and 2012; Shanthalingam et al., 2014). It impairs the function of cilia by adhering to the cilia via its polysaccharide capsule and induces production of autoantibody to ciliary antigens (Niang et al. 1998a; 1998b). Furthermore, *M. ovipneumoniae* is known to alter or suppress the activity of macrophages and lymphocytes (Niang et al. 1997; Shahzad et al. 2010), which leads to an overall impairment of the immune response of the respiratory tract and predisposes the animals to infection by *M. haemolytica*, *B. trehalosi* and possibly other bacteria. In spite of the high serum neutralizing antibody titers against Lkt, the two BHS in this study developed pneumonia after exposure to *M. ovipneumoniae*. This observation suggests differential pathogenesis in BHS that have high Lkt-neutralizing antibody titers when exposed to Lkt-producing members of Pasteurellaceae alone or exposure to members of Pasteurellaceae in addition to *M. ovipneumoniae* at the same time. The number of animals in this study is admittedly small. Nevertheless, the findings of this study suggest that Lkt-positive members of Pasteurellaceae and *M. ovipneumoniae* are certainly a deadly combination for BHS. Both of these organisms are transmitted by DS to BHS. Therefore until strategies to prevent the transmission of these organisms from DS to BHS and/or efficacious vaccines against these two pathogens are developed, it is prudent to enforce spatial separation of the two species.

In summary, nasopharyngeal treatment of *B. trehalosi* reduced the shedding of Lkt-positive *M. haemolytica*. DS that were not inoculated with *B. trehalosi* shed large numbers of *M. haemolytica*. *B. trehalosi*-inoculated DS shed fewer *M. haemolytica*. Yet, these DS transmitted
M. haemolytica to BHS. However, the exposure to low doses of M. haemolytica was sufficient to produce protective levels of Lkt-neutralizing antibodies. Further, these antibodies protected the BHS from the natural challenge with virulent M. haemolytica serotype 2. However, these BHS developed fatal pneumonia on commingling with DS that were positive for Lkt-positive M. haemolytica, Lkt-positive B. trehalosi and M. ovipneumoniae.

ACKNOWLEDGMENTS

This research was supported by funds from the Wild Sheep Foundation and its State Chapters, and US Forest service.
REFERENCES


13. Foreyt, W. J. 1994. Effects of controlled contact exposure between healthy bighorn sheep and llamas, domestic goats, mountain goats, cattle, domestic sheep, or mouflon
sheep. Proceedings of the Biennial Symposium of the Northern Wild Sheep and Goat


haemolytica leukotoxin affinity-purify the toxin from crude culture supernatants. Microb.
Pathog. 10:411–417.

in a bighorn sheep population coinciding with the appearance of a domestic sheep. Journal of
Wildlife Diseases 44: 388–403.

17. Gonzalez DJ., Haste NM., Hollands A., Fleming TC., Hamby M., Pogliano K., Nizet V.,
Dorrestein PC. 2011. Microbial competition between Bacillus subtilis and Staphylococcus

2000. Inactivation of Pasteurella (Mannheimia) haemolytica leukotoxin causes partial

3:69–82.

20. Lawrence, P.K., Shanthalingam S., Dissanayake R. P., Subramaniam R., Herndon C. N.,
K., and Srikumaran S. 2010. Transmission of Mannheimia haemolytica from domestic
sheep (*Ovis aries*) to bighorn sheep (*Ovis canadensis*): unequivocal demonstration with green fluorescent protein-tagged organisms. J Wildl Dis. 46:706-717


functions by normal sheep alveolar macrophages and their alteration by interaction with Mycoplasma ovipneumoniae. Veterinary Microbiology 58:31–43


Table 1: Commingling of DS and BHS: Experimental design.

<table>
<thead>
<tr>
<th>Animals and ID #</th>
<th>Day</th>
<th>Procedure</th>
</tr>
</thead>
<tbody>
<tr>
<td>DS</td>
<td>23, 41, 42, 83</td>
<td>-21 First treatment with ceftiofur</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-17 Second treatment with ceftiofur</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-13 Administration of Lkt-negative <em>B. trehalosi</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td>-3 Collection of samples</td>
</tr>
<tr>
<td>DS</td>
<td>23, 41, 42, 83</td>
<td>0 Commingling of 4DS with 4BHS, collection of samples</td>
</tr>
<tr>
<td>DS</td>
<td>50, 4, 43, 17</td>
<td>55 Collection of samples</td>
</tr>
<tr>
<td></td>
<td></td>
<td>106 Collection of samples</td>
</tr>
<tr>
<td>DS</td>
<td>24, 40</td>
<td>0 Commingling of 2 DS with 4BHS, collection of samples</td>
</tr>
<tr>
<td></td>
<td></td>
<td>48 Collection of samples</td>
</tr>
<tr>
<td>BHS</td>
<td>50, 4, 43, 17</td>
<td>55 Collection of samples</td>
</tr>
<tr>
<td></td>
<td></td>
<td>106 Collection of samples</td>
</tr>
<tr>
<td>DS</td>
<td>23, 41, 42, 83</td>
<td>-21 First treatment with ceftiofur</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-17 Second treatment with ceftiofur</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-13 Administration of Lkt-negative <em>B. trehalosi</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td>-3 Collection of samples</td>
</tr>
<tr>
<td>DS</td>
<td>86, 15</td>
<td>0 Commingling of 2DS with 2BHS, collection of samples</td>
</tr>
<tr>
<td>BHS</td>
<td>17, 50</td>
<td>0 Commingling of 2DS with 2BHS, collection of samples</td>
</tr>
</tbody>
</table>

DS 23, 41, 42, 83 were positive for Lkt+ *M. haemolytica* and *B. trehalosi*, but negative for *M. ovi*.

DS 24 and 40 were positive for Lkt+ *M. haemolytica* and Lkt+ *B. trehalosi*, but negative for *M. ovi*.

DS 86 and 15 were positive for Lkt+ *M. haemolytica*, *B. trehalosi*, and positive for *M. ovi*. 
**Table 2:** Lkt-neutralizing antibody titers of the commingled BHS. Titers are represented as the reciprocal dilution of the serum that provided 50% cytotoxicity inhibition.

^a Lkt-neutralizing antibody titer on day 82 at which the animal died of injury to the spinal cord.

<table>
<thead>
<tr>
<th>BHS</th>
<th>Phase 1</th>
<th>Phase 2</th>
<th>Phase 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 0</td>
<td>Day 14</td>
<td>Day 105</td>
</tr>
<tr>
<td># 50</td>
<td>20</td>
<td>40</td>
<td>800</td>
</tr>
<tr>
<td># 17</td>
<td>40</td>
<td>80</td>
<td>640</td>
</tr>
<tr>
<td># 43</td>
<td>80</td>
<td></td>
<td>1280^a</td>
</tr>
<tr>
<td># 4</td>
<td>40</td>
<td>160</td>
<td>dead</td>
</tr>
</tbody>
</table>
Table 3: Nasopharyngeal microbial profile of the commingled DS and BHS from day 105 to the end of the study. Bacterial counts of *M. haemolytica* and *B. trehalosi* are presented as the mean of the nasal and pharyngeal counts (Log<sub>10</sub> CFU/g of epithelial lining fluid) at indicated time points. During phase 2, both BHS and the DS were positive for lkt-positive *M. haemolytica* and *B. trehalosi*. Presence of the virulent *M. haemolytica* serotype 2 was confirmed by an agglutination assay. As expected, all the animals were negative for *M. ovipneumoniae*. Absence of *M. ovipneumoniae* was confirmed by the absence of sero-conversion against this organism. During phase 3, both BHS carried Lkt-positive *M. haemolytica* and no *M. ovipneumoniae* on Day 0 and was positive for the above mentioned organisms at death.

\[a\] - Presence of lkt+ *B. trehalosi*, confirmed by PCR and cytotoxicity assay.

<table>
<thead>
<tr>
<th>Sheep</th>
<th>Organism</th>
<th>Phase 2</th>
<th>Phase 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Day 105</td>
<td>Day 211</td>
</tr>
<tr>
<td>DS</td>
<td><em>M. haemolytica</em></td>
<td>2.28</td>
<td>4.18</td>
</tr>
<tr>
<td></td>
<td><em>B. trehalosi</em></td>
<td>3.45</td>
<td>4.72</td>
</tr>
<tr>
<td></td>
<td><em>M. ovipneumoniae</em></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>BHS</td>
<td><em>M. haemolytica</em></td>
<td>3.95</td>
<td>3.78</td>
</tr>
<tr>
<td></td>
<td><em>B. trehalosi</em></td>
<td>5.26</td>
<td>5.78</td>
</tr>
<tr>
<td></td>
<td><em>M. ovipneumoniae</em></td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
Fig 1: Intranasal inoculation of *B. trehalosi* reduces shedding of *M. haemolytica* by DS.

A. *B. trehalosi* successfully colonized the pharynx of the DS. B. Nasal shedding of leukotoxin-positive *M. haemolytica* was quantified by colony PCR. Compared to the pre-treatment state, there was a reduction in shedding of *M. haemolytica* for a total of 28 days post-treatment (14 days before and 14 days after day 0 of commingling). The histogram represents the experimental group mean CFU counts of bacteria present in one gram of epithelial lining fluid.
Fig 2: In spite of the reduced shedding by DS, BHS acquire leukotoxin-positive *M. haemolytica* from DS within 14 days of commingling. The figure represents Lkt-positive *M. haemolytica* in the nasal (A) and pharyngeal (B) swabs collected from the commingled BHS. The histogram represents the experimental group mean cfu counts of bacteria present in one gram of epithelial lining fluid.
Fig 3: Histopathology of lungs of the rams that died on day 47 and 84 confirms the absence of pneumonia in these animals. The lungs of both BHS (#4 [A] and #43 [B]) that died during phase one did not exhibit pneumonic lesions.
Fig 4: Histopathology of lungs of the rams that died on day 14 and 47 (phase III) confirms 
**bacterial pneumonia in these animals.** A) Severe filling of alveoli and small bronchioles with 
neutrophils, edema residue (arrow) in the lungs of #17. B) Severe filling of alveoli and small 
bronchioles with neutrophils, edema residue (arrow) with the addition of moderate lymphocyte 
infiltration surrounding a small bronchiole in the lungs of # 50.
CONCLUSION

Fatal pneumonia caused by *M. haemolytica* is an important disease responsible for the decline of BHS population in North America. Although, infection with high numbers of *M. haemolytica* consistently causes fatal pneumonia, this organism has not been frequently isolated by culture-based methods from the pneumonic lungs obtained from field cases. Our laboratory showed that *B. trehalosi* and *P. multocida*, the bacteria isolated more frequently from the pneumonic lung samples, inhibit the growth of *M. haemolytica* in vitro. However, there were cases in which *M. haemolytica* was not isolated even in the absence of *B. trehalosi* and *P. multocida* suggesting possible inhibition by other bacteria as well. Importantly, the time lapse between the death and sampling of the dead BHS in the wild provides an additional window of time for other bacteria to kill *M. haemolytica* in the pneumonic lungs. My studies have significantly enhanced the understanding of the growth inhibition of *M. haemolytica* by other pathogens. My studies have revealed that *E. coli* also inhibits the growth of *M. haemolytica* similar to *B. trehalosi* and *P. multocida* via a proximity-dependent mechanism. Co-culture of *M. haemolytica* with a diverse array of *B. trehalosi* strains suggested that the inhibition of *M. haemolytica* is conserved across the *B. trehalosi* strains, and that the inhibitory phenotype is chromosomally-encoded. Furthermore, *Streptococcus* spp. and *Staphylococcus* spp. did not have any effect on the growth of *M. haemolytica*. *Streptococcus* spp. and *Staphylococcus* spp. are commensal flora of the nasopharynx. Hence, isolation of these organisms from the pneumonic lungs could be incidental. It is very likely that the inhibition of growth of *M. haemolytica* by one or more bacteria compromises the isolation of this bona fide etiological agent of BHS pneumonia. My study emphasizes the importance of not making conclusions based on the
isolation of bacteria from the pneumonic BHS. Further studies directed at identification of the molecular mechanism/s of growth inhibition of *M. haemolytica* could pave the way for developing therapeutic agents that could specifically inhibit *M. haemolytica*, thereby minimizing the use of antibiotics.

Studies from our laboratory and that of others show that BHS often acquire Lkt-positive *M. haemolytica* from DS and subsequently develop fatal pneumonia. Further, *M. ovipneumoniae* has been shown to be a predisposing factor for pneumonia in BHS. My studies have shown that the DS that carry Lkt-positive *M. haemolytica*, when treated with *B. trehalosi* shed reduced numbers of *M. haemolytica* compared to untreated sheep and the effect lasts for almost a month post-treatment. Furthermore, healthy BHS that carry no *M. haemolytica* do not develop pneumonia when they are commingled with *B. trehalosi*-treated DS. However, the few *M. haemolytica* shed by DS are adequate to infect the commingled BHS. Interestingly, the BHS that acquire small doses of *M. haemolytica* develop high levels of Lkt-neutralizing antibodies. It is likely that the amount of *M. haemolytica* that was transmitted was not enough to induce pneumonia. The Lkt-neutralizing antibodies protect the BHS from the subsequent continuous exposure to *M. haemolytica*. In the final phase of this work, BHS developed fatal pneumonia when they are exposed to DS that carry Lkt-positive *M. haemolytica*, Lkt-positive *B. trehalosi* and *M. ovipneumoniae*. Most of the DS carry these pathogens as commensal organisms, it is important to develop strategies to either prevent shedding of Lkt-positive *M. haemolytica*, Lkt-positive *B. trehalosi* and *M. ovipneumoniae* by DS or develop protective vaccines against these organisms for use in BHS. Until then, it is prudent to spatially separate these two species.