Short communication

Mycoplasma ovipneumoniae can predispose bighorn sheep to fatal Mannheimia haemolytica pneumonia

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ABSTRACT

Mycoplasma ovipneumoniae has been isolated from the lungs of pneumonic bighorn sheep (BHS). However experimental reproduction of fatal pneumonia in BHS with M. ovipneumoniae was not successful. Therefore the specific role, if any, of M. ovipneumoniae in BHS pneumonia is unclear. The objective of this study was to determine whether M. ovipneumoniae alone causes fatal pneumonia in BHS, or predisposes them to infection by Mannheimia haemolytica. We chose M. haemolytica for this study because of its isolation from pneumonic BHS, and its consistent ability to cause fatal pneumonia under experimental conditions. Since in vitro culture could attenuate virulence of M. ovipneumoniae, we used ceftiofur-treated lung homogenates from pneumonic BHS lambs or nasopharyngeal washings from M. ovipneumoniae-positive domestic sheep (DS) as the source of M. ovipneumoniae. Two adult BHS were inoculated intranasally with lung homogenates while two others received nasopharyngeal washings from DS. All BHS developed clinical signs of respiratory infection, but only one BHS died. The dead BHS had carried leukotoxin-positive M. haemolytica in the nasopharynx before the onset of this study. It is likely that M. ovipneumoniae colonization predisposed this BHS to fatal infection with the M. haemolytica already present in this animal. The remaining three BHS developed pneumonia and died 1–5 days following intranasal inoculation with M. haemolytica. On necropsy, lungs of all four BHS showed lesions characteristic of bronchopneumonia. M. haemolytica and M. ovipneumoniae were isolated from the lungs. These results suggest that M. ovipneumoniae alone may not cause fatal pneumonia in BHS, but can predispose them to fatal pneumonia due to M. haemolytica infection.

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1. Introduction

Mannheimia (Pasteurella) haemolytica has been isolated from the lungs of bighorn sheep (BHS, Ovis canadensis) that died of pneumonia (Miller, 2001). Experimental infection with this organism has confirmed its ability to cause fatal bronchopneumonia in BHS (Foreyt et al., 1994; Dassanayake et al., 2009). M. haemolytica has long been identified as a commensal bacterium of the upper respiratory tract of ruminants (Dunbar et al., 1990; Weiser et al., 2009). Active viral infection and stress factors have been identified as predisposing factors for pneumonia caused by M. haemolytica in cattle (Rehmtulla and Thompson, 1981). However,

the role of predisposing factors in *M. haemolytica*-caused pneumonia in BHS has not been investigated.

Mycoplasma ovipneumoniae and *Mycoplasma arginini* have been isolated from the upper respiratory tract of young and adult domestic sheep (DS, *Ovis aries*; Brogden et al., 1988). *M. ovipneumoniae* (but not *M. arginini*) causes atypical pneumonia especially in DS lambs; however, experimental reproduction of pneumonia with *M. ovipneumoniae* in DS lambs has been inconsistent (Buddle et al., 1984; Ruffin, 2001). Unlike in DS, only non-pathogenic *M. arginini* has been isolated from healthy BHS (Woolf et al., 1970). In our recent study (Besser et al., 2008), *M. ovipneumoniae* was detected in the bronchoalveolar lavage fluid from pneumonic BHS lambs by culture and 16S rRNA species-specific PCR. *M. ovipneumoniae*-specific 16S sequences and antibodies were detected in lung tissues and serum respectively, of bronchopneumonic BHS, but not from BHS dying of other causes. However, experimental inoculation of *M. ovipneumoniae* failed to cause fatal pneumonia in BHS lambs (Besser et al., 2008). Therefore, the objective of this study was to elucidate the role of *M. ovipneumoniae* in BHS pneumonia.

2. Materials and methods

2.1. Bacterial strains and growth conditions

M. haemolytica serotype A1 strain 89010807N (Murphy et al., 1995) and serotype A2 strain WSU-1 (Foreyt et al., 1994) were grown in BHI agar supplemented with 5% sheep blood and BHI broth as previously described (Dassanayake et al., 2009).

2.2. Bighorn sheep challenge studies

Nasal and pharyngeal swabs and blood samples were collected from all the animals before bacterial inoculation, and were submitted to Washington Animal Disease Diagnostic Laboratory (WADDL) at Washington State University for detection of *M. ovipneumoniae*, *M. haemolytica* or other *Pasteurella* sp, and any known respiratory viruses. Serology was performed to detect antibodies against respiratory viruses, *M. ovipneumoniae* and *M. haemolytica* leukotoxin (LktA).

The animals were inoculated as detailed in Table 1. The inoculum was administered intranasally using an atomizer. *M. ovipneumoniae* inoculum for Groups I and II were obtained from two sources: (1) lung homogenates from pneumonic BHS (three lambs and one adult; Besser et al., 2008); (2) nasopharyngeal washings from DS (four ewes) that were *M. ovipneumoniae*-positive by culture and PCR assay. These preparations were filtered using a 0.22 μm filter (to remove any bacteria), and the filtrates were submitted to WADDL to detect respiratory viruses. The unfiltered nasopharyngeal washings and lung homogenates were treated with the antibiotic ceftiofur (64 μg/ml, 37 °C for 1 h), expected to reduce or eliminate *M. haemolytica* and other ceftiofur-susceptible bacteria, but not *M. ovipneumoniae*. BHS were inoculated either with the filtrate, ceftiofur-treated *M. ovipneumoniae*-positive lung homogenates, nasopharyngeal washings or *M. haemolytica* as detailed in Table 1.

The animals in each group were observed daily and scored for the signs of pneumonia including anorexia, lethargy, cough, dyspnoea and nasal discharge. The animals that died before the end of the observation period were necropsied immediately, and appropriate tissues were collected for bacteriological, viral and histopathological examinations. The animals in the control Group (III) were euthanized 3 weeks post-challenge, necropsied and tissue samples collected. The lungs were carefully examined for pneumonic lesions. The degree of involvement of right and left lungs was noted as percent pneumonic scores. Pleuritis was noted as present or absent. Bacterial and viral isolations were attempted using routine methods at WADDL. *M. haemolytica* isolates were serotyped by agglutination test using anti-serotype A1 and A2 specific sera.

2.3. PCR detection of M. ovipneumoniae and M. haemolytica leukotoxin

M. ovipneumoniae-specific 16S rRNA PCR was performed as previously described (McAuliffe et al., 2003; Besser et al., 2008). Leukotoxin A gene (*lktA*) of *M. haemolytica* and *Bibersteinia trehalosi* was amplified by PCR using *lktA*F (5′-TCAAGAAGAGCTGGCAAC-3′) and *lktA*R (5′-AGTGAGGGCAACTAAACC-3′) primers in a final volume

Table 1
Inoculation timeline of *M. ovipneumoniae* and *M. haemolytica* to BHS.

Group	BHS	Day	Inoculum[a]
I	OR26	0	Filtrate (0.22 μm) from *M. ovipneumoniae*-positive pneumonic BHS (three lambs and one adult) lung homogenates in PBS (10 ml)
	R124		
		28	*M. ovipneumoniae*-positive, pneumonic BHS (three lambs and one adult) lung homogenates in PBS, unfiltered but treated with ceftiofur (10 ml)
		42	Same treatment as on day 28
		70	*M. haemolytica* serotype A1 (1×10^6 CFU in 5 ml RPMI)
II	Y45	0	Filtrate (0.22 μm) from nasal washings (PBS) from four *M. ovipneumoniae*-positive DS (10 ml)
	R123		
		28	Nasal washings (PBS) from four *M. ovipneumoniae*-positive DS, unfiltered, but treated with ceftiofur (10 ml)
		42	Same treatment as on day 28
		70	*M. haemolytica* serotype A2 (1×10^6 CFU in 5 ml RPMI)
III	Y30	0	RPMI (5 ml)
	Y39		

[a] Administered intranasally using an atomizer.

of 50 μl with GoTaq® PCR SuperMix (Promega Inc., Madison, WI) under standard conditions.

2.4. Serology

Anti-LktA-neutralizing antibodies from BHS serum samples were detected by MTT dye reduction cytotoxicity assay as previously described (Gentry and Srikumaran, 1991). Indirect hemagglutination assay for *M. ovipneumoniae* was performed by WADDL using *M. ovipneumoniae* antigen-sensitized and non-sensitized erythrocytes with serially diluted serum samples as described previously (Besser et al., 2008). Serum neutralization assays were performed by WADDL to determine antibody titers for respiratory viruses including BRSV, BVDV, BHV-1, and PI-3.

2.5. Histopathology

Histopathology was performed by WADDL. Lung lesions were described by noting the character of the inflammatory infiltrate, degree of necrosis, presence or absence of abscessation and bacterial colonies (Besser et al., 2008; Dassanayake et al., 2009).

3. Results

3.1. The microbial profile of the nasopharynx of the BHS

All the animals were culture- and PCR-negative for *M. ovipneumoniae*, although all BHS were positive for non-pathogenic *M. arginini* (Table 2). As expected, none of the animals had demonstrable *M. ovipneumoniae* antibody titers (Table 3). All the animals were culture-positive for *B. trehalosi* but were negative for *lktA* by PCR (Table 2). Several animals had antibody titers to RSV (R123, R124, Y45) and PI-3 (OR26, Y45, R123, R124) (Table 3). However, all were culture-negative for respiratory viruses (Table 2). All the animals except two (R123, R124) were negative for *M. haemolytica* (Table 2). Of the two that were positive, one had *lktA*-positive *M. haemolytica* (R124) while the other one had *lktA*-negative *M. haemolytica* (R123; Table 2). All the animals had insignificant levels of anti-LktA antibodies (Table 3). We could not perform serological assays for the control animals' sera due to the poor quality of the sera.

3.2. M. ovipneumoniae fails to induce fatal pneumonia in BHS

Lung homogenates from pneumonic BHS lambs and nasopharyngeal washings from *M. ovipneumoniae*-positive DS ewes were used as the source of *M. ovipneumoniae* for inoculation of four BHS (Table 1). When animals were inoculated with the filtrates, none of the BHS developed any signs of respiratory viral infection, during the 4-week observation period, demonstrating the absence of any BHS respiratory viral pathogens in the inoculum. These preparations were negative for any viruses by culture as well. None of the animals developed any signs of pneumonia following intranasal

Table 2
Bacterial and viral pathogens isolated from or detected in nasopharynx and lungs of bighorn sheep before and after *M. ovipneumoniae* and *M. haemolytica* challenges.

Groups	animal	M.h Pre	M.h Post	M.ovi Pre	M.ovi Post	M.arg Pre	M.arg Post	B.t Pre	B.t Post	BRSV Pre	BRSV Post	PI-3 Pre	PI-3 Post	BVDV Pre	BVDV Post	BHV-1 Pre	BHV-1 Post
I	OR26	−	+[a,c]	−	+	+	+	+[b]	+[b]	−	+[e]	−	−	−	−	−	−
	R124	+[a]	+[a]	−	+	+	+	+[b]	+[b]	−	−	−	−	−	−	−	−
II	R123	+[b]	+[a,d]	−	+	+	+	+[b]	+[b]	−	+[e]	−	−	−	−	−	−
	Y45	−	+[a,d]	−	+	+	+	+[b]	+[b]	−	−	−	−	−	−	−	−
III	Y30	−	−	−	−	+	+	+[b]	+[b]	−	−	−	−	−	−	−	−
	Y39	−	−	−	−	+	+	+[b]	+[b]	−	−	−	−	−	−	−	−

M.h – *M. haemolytica*; M.ovi – *M. ovipneumoniae*; M.arg – *M. arginini*; B.t – *B. trehalosi*; BRSV – bovine respiratory syncytial virus; BVDV – bovine viral diarrhea virus; BHV-1 – bovine herpes virus-1; PI-3 – parainfluenza-3; Pre – before the challenge; Post – at necropsy.
[a] *lktA*-positive.
[b] *lktA*-negative.
[c] *M. haemolytica* serotype A1.
[d] *M. haemolytica* serotype A2.
[e] Detected by ELISA.

Table 3
Serum antibody titers for *Pasteurellaceae* leukotoxin A, *M. ovipneumoniae* and respiratory viruses before and after *M. ovipneumoniae* exposures and *M. haemolytica* challenges.

Groups	Animal	LktA Pre	LktA Post	M.ovi Pre	M.ovi Post	BRSV Pre	BRSV Post	PI-3 Pre	PI-3 Post
I	OR26	10	10	0	>2560	0	0	128	128
	R124	80	500	0	1280	32	128	32	64
II	R123	160	10	0	>2560	4	0	128	128
	Y45	40	100	0	>2560	0	8	64	32

LktA – *Pasteurellaceae* leukotoxin A (*M. haemolytica* and *B. trehalosi*). M.ovi – *M. ovipneumoniae*. BRSV – bovine respiratory syncytial virus. PI-3 – parainfluenza-3. Pre – before the challenge. Post – at necropsy.

challenge with ceftiofur-treated BHS pneumonic lung homogenates or DS nasopharyngeal washings although all four BHS became culture- and PCR-positive for *M. ovipneumoniae* 2 weeks post-challenge. Therefore, the animals were re-challenged with the same inoculum (Table 1). During the next few days, all the BHS developed signs of *M. ovipneumoniae* infection including lethargy, reduced appetite, intermittent cough, nasal discharge, and head shaking. One animal from Group I (R124) died of pneumonia on day 47. However, it should be noted that this animal had been consistently positive for *lktA*-positive *M. haemolytica* since day 1 of the experiment. Anti-LktA, anti-*M. ovipneumoniae* and BRSV titers gradually increased (Table 3). However, BRSV was not isolated by culture, from any of the samples collected. Post-mortem examination of R124 revealed acute bronchopneumonia in the right lung with severe consolidation over 50% of the ventral portion of all lobes. Histologically, the lungs had severe filling of alveoli and many bronchioles with neutrophils and variable amounts of fibrin and edema residue. All the samples taken from the lesional tissue were heavily positive for *M. haemolytica*, *B. trehalosi* and *M. ovipneumoniae*. All the isolates of *M. haemolytica* but not *B. trehalosi* were PCR-positive for *lktA*. R124 also showed neutralizing titers for BRSV and PI-3, but not for any other viruses (Table 3).

3.3. Inoculation of M. haemolytica causes pneumonia and death of M. ovipneumoniae-inoculated BHS

M. haemolytica serotype A1 and A2 were intranasally inoculated into the remaining BHS in Group I and two BHS in Group II, respectively, on day 70 as shown in Table 1. Both R123 and Y45 died 1 day post-inoculation with *M. haemolytica*. The gross- and histopathology of the lungs of both these animals were similar. The right and left lungs showed 30–50% consolidation (Fig. 1A). There was some fibrin deposition on the pleural surface of the left cranial lobe, bronchi and pericardium suggesting that the death of the animals was due to acute broncho/pleuropneumonia characteristic of *M. haemolytica* infection. The lungs had regional filling of alveoli with neutrophils, fibrin, and erythrocytes (Fig. 1C). All the samples collected from the animals, including the middle ears, were positive for *M. haemolytica* serotype A2, *B. trehalosi*, *Pasteurella multocida* (*toxA*-negative) and *M. ovipneumoniae*. All the isolates of *M. haemolytica*, but not *B. trehalosi*, were positive for *lktA* by PCR (Table 2). LktA-neutralizing titers of R123 changed only slightly over time except at the time of death when the titers became low (Table 3). Lkt neutralization titers of Y45 remained unchanged. *M. ovipneumoniae* titers of both R123 and Y45 increased from undetectable titers prior to experimental challenge to high titers (>1:2560) after the second *M. ovipneumoniae* challenge until the time of death.
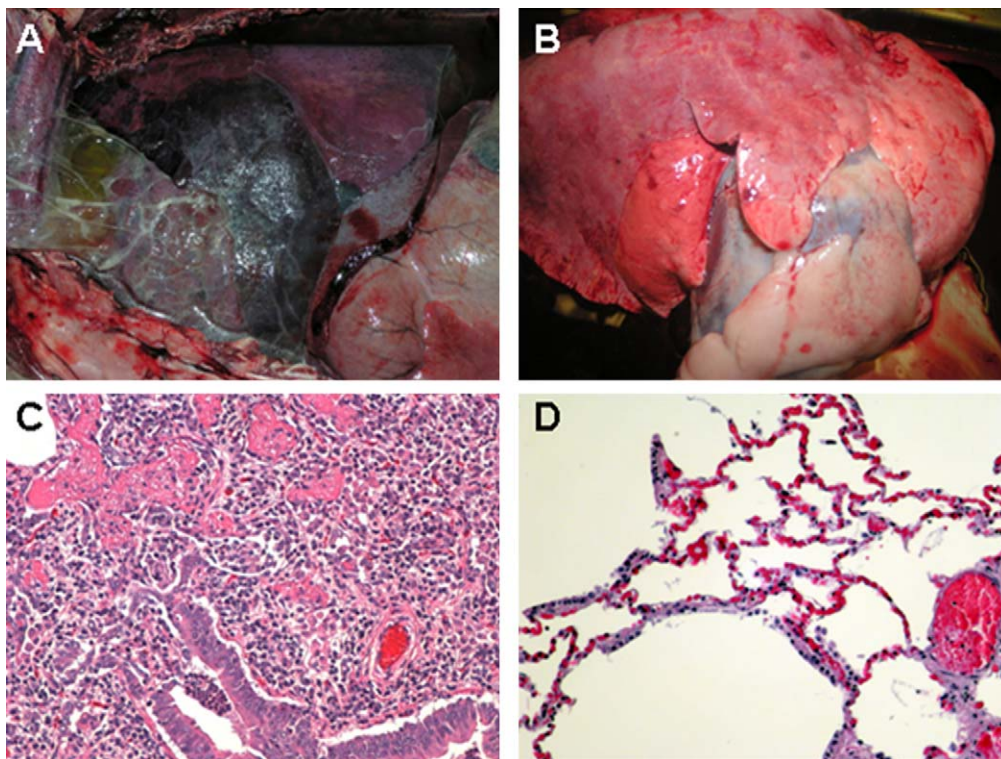

Fig. 1. Lung lesions of BHS inoculated with *M. ovipneumoniae* followed by *M. haemolytica*. BHS were inoculated with two doses of *M. ovipneumoniae* (2 weeks apart) followed by one dose of *M. haemolytica* 4 weeks after the second *M. ovipneumoniae* inoculation. (A) Gross pathology of the lungs of BHS (R123) inoculated with *M. ovipneumoniae* followed by *M. haemolytica* A2. (B) Gross pathology of the lungs of a control BHS (Y39) administered sterile RPMI. (C) Histopathology of lungs of R123. (D) Histopathology of lungs of Y39. (C, D: hematoxylin and eosin staining, original magnification = 100×).

Except for low neutralizing titers (1:4) for BRSV on two occasions and stable PI-3 titers (1:128), R123 did not show neutralization titers against other respiratory viruses tested (Table 3). Y45 had a BRSV positive titer of 1:8 at the time of death and fairly consistent PI-3 positive titers (1:32–64) during the study period. Although nasal swabs and lung tissues were positive for BRSV antigen in R123 by ELISA, we could not isolate any viruses in cell cultures (Table 2).

The animal OR26 died 5 days following *M. haemolytica* serotype A1 inoculation. The gross- and histopathologic lesions in this animal were similar to those of R123 and Y45. As expected, *M. haemolytica* A1, *M. ovipneumoniae*, *B. trehalosi* (*lktA*-negative), and *P. multocida* (*toxA*-negative) were isolated from numerous samples. This animal showed very low LktA-neutralizing antibody titer during the course of the study. *M. ovipneumoniae* titers increased from undetectable titers prior to challenge, to high titers (>1:2560) following second *M. ovipneumoniae* inoculation. Anti-PI-3 titer remained unchanged at 1:128, but no antibodies against other respiratory viruses were detected throughout the study period. OR26 also showed positive BRSV results by ELISA on lung tissues. No viruses were isolated in cell cultures. As expected, the lungs of the two BHS in the control group (Y30 and Y39) showed no evidence of pneumonia (Fig. 1B and D).

4. Discussion

M. ovipneumoniae has been isolated from wild BHS lambs and adults in naturally occurring pneumonia in previous studies (Besser et al., 2008). However, *M. ovipneumoniae* isolated from these animals failed to induce sustained clinical illness in two BHS lambs given multiple intranasal inoculations despite successful oropharyngeal colonization. Virulence attenuation of *M. ovipneumoniae* occurs during laboratory culture (Gilmour et al., 1979; Jones et al., 1982) which could be responsible for the failure to reproduce clinical disease. Therefore we reasoned that the use of lung homogenates from pneumonic BHS or nasopharyngeal washings from *M. ovipneumoniae*-positive DS should avoid this possibility of attenuation and better assess the etiologic role of this agent.

Although all BHS inoculated with unfiltered, but ceftiofur-treated, lung homogenates and nasopharyngeal washings developed clinical signs of *M. ovipneumoniae* infection, only one died prior to the time of intranasal *M. haemolytica* challenge. Therefore, based on this study, we propose that *M. ovipneumoniae* alone is not adequate for the induction of fatal bronchopneumonia in BHS, which is in agreement with the findings of our previous *M. ovipneumoniae* challenge studies with BHS lambs (Besser et al., 2008). Our finding that three out of three BHS developed bronchopneumonia and died 1–5 days post-inoculation with *M. haemolytica* clearly indicates that *M. haemolytica* is the pathogen that causes fatal pneumonia in BHS challenged under our experimental protocol. The difference in the interval between the inoculation and death of the BHS in Group II (1 day) and Group I (5 days) is very likely due to the difference in virulence between *M. haemolytica* serotype A2 and serotype A1 that was used to inoculate the BHS. Our earlier studies have indicated that

the serotype A2 is more virulent than serotype A1 (unpublished observation). The death of one BHS in Group I after inoculation with *M. ovipneumoniae*, but before inoculation with *M. haemolytica*, was very likely due to the presence of *lktA*-positive *M. haemolytica* in the nasopharynx of this BHS right from the onset of this study. The pneumonic lesions of the lungs were indicative of *M. haemolytica*-caused pneumonia. The death of all three BHS following intranasal inoculation with *M. haemolytica* suggests that *M. ovipneumoniae* acted as a primary pathogen, reducing the resistance of BHS to the *M. haemolytica* challenges predisposing these animals to relatively rapid development of fatal pneumonia due to *M. haemolytica* infection. It is likely that *M. ovipneumoniae*-induced loss of mucociliary defense of the respiratory tract (Niang et al., 1998) facilitated rapid proliferation and descent of *M. haemolytica* into the lower respiratory tract and induction of fatal bronchopneumonia. However, in a previous study by us intra-tracheal inoculation of *M. haemolytica* (1×10^9 CFU of a serotype A1 strain) resulted in the death of all four BHS within 48 h (Dassanayake et al., 2009). Furthermore, in a recent experimental challenge study by us, intranasal inoculation of a strain of *M. haemolytica* (1×10^6 CFU of serotype A2) caused the death of three out of four BHS within 48 h (unpublished observation), which questions the necessity for a predisposing agent such as *M. ovipneumoniae* to render the mucociliary apparatus dysfunctional in order for *M. haemolytica* to cause fatal bronchopneumonia, at least in that experimental challenge model.

Antibodies specific for RSV and PI-3 have been detected in several BHS herds (Elliott et al., 1994; Spraker et al., 1986). Although RSV was not isolated by culture from any of the BHS in this study, lung tissue from two of them (R123 and OR26) were positive for RSV by ELISA. The RSV titers of two animals (R124 and Y45) increased (from 1:32 to 1:128, and from undetectable to 1:8, respectively) during the experiment. Therefore, we cannot rule out the possibility that RSV also was involved in the induction of pneumonia in these animals. Studies are currently underway to elucidate the role of RSV and PI-3 in the etiology of pneumonia in BHS.

In summary, our findings indicate that *M. ovipneumoniae* by itself did not cause fatal pneumonia in BHS used in this study. However, it did predispose them to fatal pneumonia caused by *M. haemolytica*. We propose that low virulent strains, but not high virulent strains, of *M. haemolytica* may require a predisposing agent such as *M. ovipneumoniae* for the induction of fatal bronchopneumonia in BHS.

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