Viability Of Airborne *Pasteurella* Spp.

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Abstract: Pasteurella spp. are commensal organisms in bighorn sheep that have been frequently associated with pneumonia die-offs. The method of transmission in bighorn sheep generally has been assumed to be by direct (nose-to-nose) contact. An observational study was conducted to determine the effects of two wind tunnel distances (short and long), two bacterial doses (high and low), and two seasons (summer and winter) on the airborne bacterial viability of three strains of *Pasteurella* bacteria. One strain from each of the species *P. haemolytica, P. trehalosi* and *P. multocida* was nebulized into a wind tunnel. Selective media plates were suspended at the tunnel exit to collect viable organisms. The experiments were done in triplicate. The final multiple linear regression model suggests that the *P. multocida* strain was significantly more likely to survive aerosol transmission than either the *P. haemolytica or P. trehalosi* strain (P = 0.0043). A majority of the variation (74%) in airborne viability could be explained by the *P. multocida* strain alone. Although not statistically significant at the P = 0.05 level, there was evidence that the number of viable colonies recovered was higher in summer conditions (P = 0.1396), and temperature dependent (P = 0.1036). At an initial high bacterial dose (1 x 10^6 cfu), the predicted number of *P. multocida* bacteria remaining viable over short (6.1 m) and long (18.3 m) distances during summer was n = 2820 (0.28 %) and n = 1620 (0.16 %), respectively, and during winter was n = 1800 (0.18%) and n = 600 (0.06%), respectively. At the initial high bacterial dose for the *P. haemolytica* strain, the predicted number of bacteria remaining viable over short and long distances during summer was n = 2370 (0.24%) and n = 1170 (0.12%), respectively, and during winter was n = 1350 (0.14%) and n = 150 (0.02%), respectively. Results for the *P. trehalosi* strain did not differ significantly from the results for the *P. haemolytica* strain. These findings suggest a potential exists for *Pasteurella* spp. to be transmitted between animals without direct contact.

Key Words: bighorn sheep, *Pasteurella* spp., aerosol transmission, wind tunnel, *Pasteurella* viability

Pasteurella-related pneumonia epizootics continue to be a major factor in the decline of bighorn sheep populations (Onderka and Wishart 1984, Spraker et al. 1984, Coggins 1988, Cassirer et al. 1996). The predominant mechanism for transmission of *Pasteurella* spp. among ungulates is generally assumed to be by direct (nose-to-nose) contact (Carter and De Alwis 1980, Chanter and Rutter 1980, Frank 1980, Gilmour and Gilmour 1980). Other mechanisms for transmission of *Pasteurella* spp. have been identified including exposure to contaminated water for waterfowl (Rhoades and Rimler 1980), and aerosol transmission in livestock (Gilmour and Gilmour 1980, Dinter and Muller 1984) and rabbits (Manning et al.

1980). Possible similar mechanisms of transmission have not been ruled out in bighorn sheep.

In 1995-96, a die-off of major proportions appears to have systematically worked its way through bighorn sheep herds residing on either side of the Snake River near and in the Hells Canyon National Recreation Area, ID, OR and WA. Initially, 72 Rocky Mountain bighorn sheep were captured from the Black Butte, WA herd and transported to captivity for further study at the Idaho Department of Fish and Game, Wildlife Health Laboratory (WHL) (Cassirer et al. 1996). Despite carefully implemented controls at the WHL to prevent human foments between the visiting Black Butte herd and resident WHL bighorn sheep, within three weeks, resident bighorn sheep began showing signs of respiratory disease. Extremely windy weather conditions with a prevailing wind direction in favor of aerosol transmission from the Black Butte herd to the resident captive herd were noted. The epidemiology of this epizootic (on the range and in captivity), and others, has raised the question of whether aerosol transmission may play a role in some bighorn sheep die-offs. The following are findings of an observational study at the WHL using a wind tunnel system to study the effects of wind tunnel distance, bacterial dose, and season on the potential for airborne transmission of three selected strains of *Pasteurella* bacteria.

MATERIALS AND METHODS

Wind Tunnel Design.

The wind tunnel was constructed of one or three 6.1 m lengths of PVC pipe, each with a 30.2 cm diameter. A 120 V squirrel cage fan was secured completely over the entrance of the pipe, to generate a fixed wind speed of approx. 11.3 m/s, as measured by a Turbo Meter Wind Speed Indicator. A glass nebulizer was suspended at the tunnel entrance, centered on top at a distance of 25 cm from the wind source. A 1/3 hp, 115 V electric air pump (General Electric) was connected to the nebulizer with 0.63 cm diameter surgical tubing and run at 8 psi. At the tunnel exit, a 10 cm selective media plate (CBAA, Ward et al. 1986) was centered and suspended to collect viable *Pasteurella* spp.

Wind Tunnel Experiment.

Humidity and temperature were recorded before each trial using a Weksler sling psychrometer. A series of two experiments were run each day, in triplicate. At the beginning of each experiment day, a control run was carried out by suspending a selective CBAA media plate at the exit end of the tunnel and running the fan for five min before removal. Next, a fresh CBAA plate was placed at the exit end of the tunnel, and pipetting 3 ml bacterial broth into a marked, weighed nebulizer set at the entrance end. The fan and air pump were run for five minutes; the pump was shut off while the fan was run an additional minute. The CBAA plate was exchanged for a fresh control CBAA plate, the fan run for an additional five min, and the nebulizer removed for a post-trial weight to determine the volume of inoculum vaporized. All CBAA plates were placed in a 35 C incubator with 10% added CO_2 immediately after the experiment was completed, and examined at 24 hr and 48 hr for viable *Pasteurella* colonies. Representative colonies were selected and identified using the essential tests of Jaworski et al. (1998).

Wind tunnel distances of 6.1 m and 18.3 m were used for summer trials, and a single distance of 18.3 m was used for winter trials. Three trials were run using a

dilute bacterial dose based on colony forming units (cfu) (1 x 10^4cfu), followed by three trials at a more concentrated bacterial dose (1 x 10^6 cfu) on a given day at the WHL. Initial bacterial doses were chosen based on previous publications (Gilmour et al 1975; Gilmour et al. 1984). The day following a wind tunnel trial, the tunnel was sampled with a sterile swab around the entrance and exit, and the swabs were cultured on a 5% Columbia blood agar plate (CBA) media and CBAA media to determine the number of viable *Pasteurella* spp., as described above.

Bacteriology

The three *Pasteurella* strains chosen for this study were a *P. haemolytica*, biovariant 1 strain (CVTC #94-1427), a *P. trehalosi*, biovariant 2 strain (CVTC #89-269-L), and a *P. multocida multocida* A strain (CVTC #96-162). Each strain was isolated in pure culture from lung samples from three Rocky Mountain bighorn sheep carcasses (*Ovis canadensis canadensis*) submitted during the seven year period from 1989-1996 to the University of Idaho, Caine Veterinary Teaching Center (CVTC) for *Pasteurella* culture.

Bacterial cultures for each aerosol trial were prepared by inoculating brain heart infusion (BHI) broth with one of the three *Pasteurella* spp. strains and incubated overnight at 35 C in an incubator with 10% added CO_2. The next morning the percent transmittance (%T) for each inoculate was determined at a wavelength of 610 nm and adjusted to 75 %T, to approximate 1 x 10^8 colony forming units (cfu) per ml (Blau et al. 1987). Ten-fold serial dilutions (10^8-10^2) were plated on CBAA media plates. The following day, *Pasteurella* colonies on each dilution series plate were counted to determine bacterial concentration (cfu/ml), and the identities of representative colonies from the dilution series were confirmed using the essential tests of Jaworski et al. (1998).

Statistics

Data analysis was initially conducted using a Student's T test, followed by re-evaluation using a multiple linear regression model. The reference group for this analysis was the percent of viable *P. haemolytica* recovered from an initial 1 x 10^4 (cfu) dose at a distance of 6.1 m during winter at 0° C and 0% humidity. Significance for tests was determined using a P value of $P < 0.05$. Statistics were performed using Statview.

RESULTS

Summer temperatures ranged from 22–32 C, with humidity at 50-55% for trials at 6.1 m distance and 28-34% for trials at 18.3 m distance. Winter temperatures ranged from –1.0 – 4.5 C, with humidity at 55-82%. Control media plates used before and after each experimental trial were always culture negative for *Pasteurella* spp. Biochemical identification for each strain of *Pasteurella* spp. recovered on media plates during each trial resulted in a monoculture of the identical *Pasteurella* strain used (data not shown).

The two sample T Test for initial dose and distance resulted in a trend toward a higher initial dose for the long distance experiments ($P < 0.12$) (Table. 1). To avoid the potential of confounding of this reaction, a multiple linear regression model was generated with an interaction term (FarInit) of distance (Far) and initial dose (Initial) (Table 2).

Table 1. Two-sample T Tests for initial dose vs distance

DISTANCE	MEAN DOSE
6.1 M	1 X 10^6
18.3 M	5.5 X 10^6

P < 0.12

Table 2. Linear regression model of % viable bacteria recovered

Predictor variables	Coefficient	95% CI	P value
Constant	0.135	-0.037, 0.307	0.1320
P. multocida	0.045	0.016, 0.074	0.0043
P. trehalosi	-0.002	-0.029, 0.026	-0.9003
Summer	0.102	-0.033, 0.235	0.1396
Humidity 10	0.007	-0.011, 0.024	0.4770
Temperature 10	-0.018	-0.038, -0.003	-0.1036
Initial	-3.48×10^{8}	-0.000, 0.000	0.0000
Far	-0.119	0.166, -0.072	0.0000
FarInit	3.49×10^{8}	-0.000, 0.000	0.0000

Definitions of Independent Variables for the Regression Model:

P. multocida = A variable coded 1 if the organism was *P. multocida* and 0 if it was not

P. trehalosi = A variable coded 1 if the organism was *P. trehalosi* and 0 if it was not

Summer = A variable coded 1 if the current season was summer and 0 if it was winter

Humidity10 = A variable representing the effect of a 10% change in humidity

Temperature10 = A variable representing the effect of a 10 C change in temperature

Initial = The initial dose of the bacteria of interest in colony forming units

Far = A variable coded 1 if the distance was 18.3 m and 0 if it was 6.1 m

Results from the multiple linear regression model showed that the *P. multocida* strain used in this experiment was significantly more likely to survive aerosol transmission than the *P. haemolytica* or *P. trehalosi* strains ($P = 0.0043$). There were non-significant trends for season (summer) ($P = 0.1396$) and increasing temperatures ($P = 0.1036$) for the viability of airborne *Pasteurella* strains used in this study.

The square of the correlation coefficient for the final regression model, R^2, was approximately 0.76, indicating that 76% of the variation in airborne viability could be explained by the variables in this model, with approximately 74% explained by the *P. multocida* strain alone.

DISCUSSION

This observational study demonstrated that small numbers, colony forming units (cfu), of selected strains of *Pasteurella* bacteria, particularly the *P. multocida* strain, remained viable after traveling distances of up to 18.3 m through a wind tunnel, at a fixed wind speed of 11.3 m/s both in summer and winter climates in Caldwell, Idaho. Analysis of the dataset using two-sample T tests indicated a potential for confounding from a possible relationship between initial dose and distance. To avoid such a case, further analysis was conducted using a multiple linear regression model to partition out the initial dose and distance factors. The model can be used to predict the number of viable bacteria recovered from the windtunnel study (Figure 1, Table 3). For example, from an initial dose of 1×10^{6} cfu of the *P. multocida* strain, 2.8×10^{3} (0 – 6160) (0.28 %) are predicted to survive aerosol transmission of 6.1 m in the summer, while from an initial dose of 1×10^{6} cfu of the *P. haemolytica* strain, 1.5×10^{1} (0, 2340) (0.02 %) are predicted to

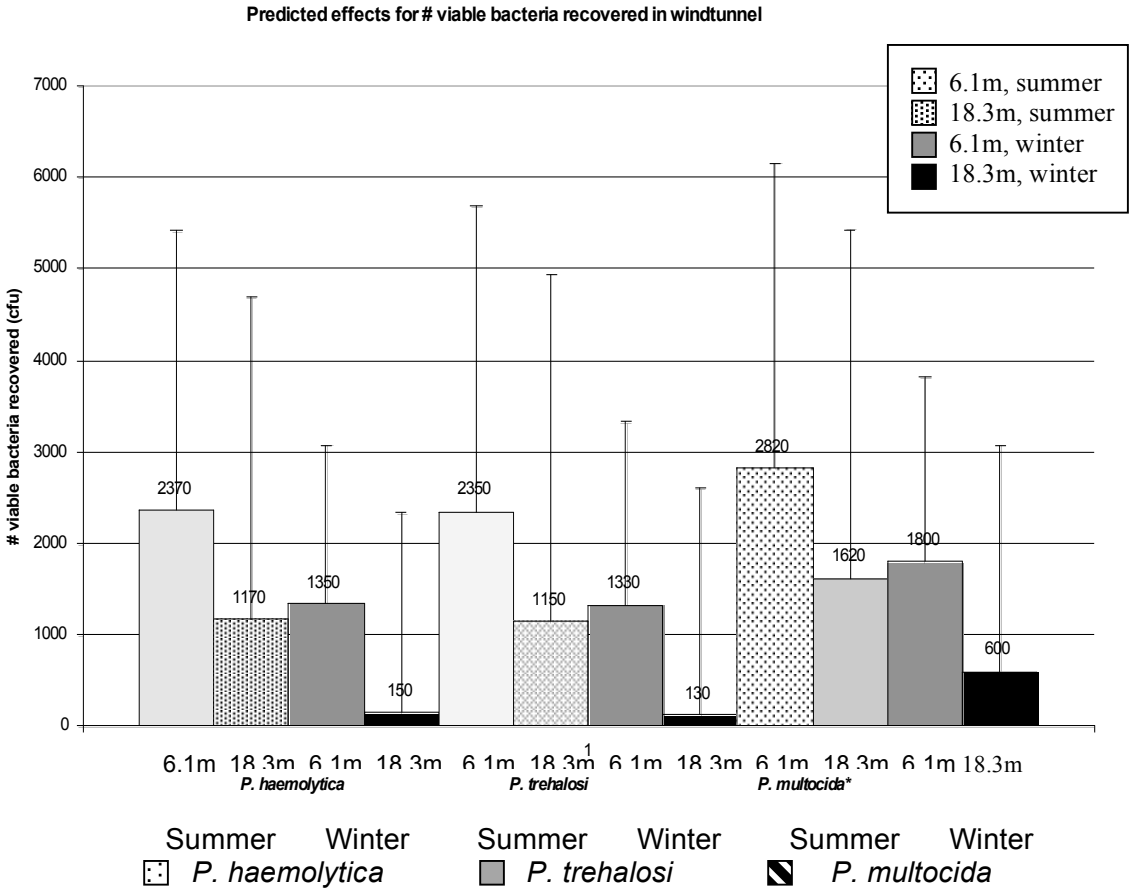

Figure 1. Graph of predicted effects for number of viable bacteria recovered from the wind tunnel at an initial high dose (1 x 10^6 cfu). *indicates statistical significance (P = .0043) for the *Pasteurella* strain *P. multocida*.

survive aerosol transmission of 18.3 m in the winter.

Of great interest is the finding that the square of the correlation coefficient, R2, indicated that three quarters of the variation in airborne viability could be explained by the strain of *Pasteurella* spp alone. This provides preliminary evidence for the importance of Pasteurella strain to the success of airborne transmission, and much less so on other factors such as season, temperature and humidity.

The biological relevance of these results to bighorn sheep management may be gained from consideration of two previously published papers (Gilmour et al. 1975; Gilmour et al. 1984). In these two studies, Caesarean-derived, colostrum-deprived, specific pathogen free domestic lambs at 8 wk of age were used in experimental infection studies with an aerosol of *P. haemolytica*. In the first study (Gilmour et al. 1975), it was determined that experimental aerosol administration of a *P. haemolytica* strain at a dose of 1 x $10^{4.8}$ cfu resulted in pneumonia 7 days later in 4/9 experimental (non-vaccinated) lambs, indistinguishable from that described in the natural disease. In this study, an infectious dose of 1 x $10^{4.8}$ cfu resulted in

Table 3. Predicted effects for viable bacteria recovered at high dose (1 x 10^6cfu)

Bacterial strain	Season	Distance	Number viable bacteria recovered	% viable bacteria recovered	95% CI
P. haemolytica	Summer	6.1 m	2370	0.24%	0-5420
P. haemolytica	Summer	18.3 m	1170	0.12%	0-4690
P. haemolytica	Winter	6.1 m	1350	0.14%	0-3070
P. haemolytica	Winter	18.3 m	150	0.02%	0-2340
P. trehalosi	Summer	6.1 m	2350	0.24%	0-5680
P. trehalosi	Summer	18.3 m	1150	0.12%	0-4950
P. trehalosi	Winter	6.1 m	1330	0.13%	0-3330
P. trehalosi	Winter	18.3 m	130	0.01%	0-2600
P. multocida	Summer	6.1 m	2820	0.28%	0-6160
P. multocida	Summer	18.3 m	1620	0.16%	0-5430
P. multocida	Winter	6.1 m	1800	0.18%	0-3810
P. multocida	Winter	18.3 m	600	0.06%	0-3080

lamb mortality of 4/9 lambs on day 7 post-infection.

In the second aerosol study (Gilmour et al. 1984), it was determined that in lambs first infected with parainfluenza 3 (PI-3) virus, then 7 days later exposed to aerosols of a *P. haemolytica* strain at an initial dose as low as 5.5 x 10^2 cfu, 6/7 lambs developed pneumonia as determined by necropsy seven days post *P. haemolytica* infection. Under the conditions of the study, a PI-3 viral infection significantly reduced the infectious dose necessary for lethality in domestic sheep lambs. This study and others reviewed in Brogden et al. (1998) demonstrates the importance of predisposing factors, such as the PI-3 virus, to the health of sheep.

The resulting predicted number of viable *Pasteurella* organisms in our study was two to four fold less than that described in the Gilmour et al. (1975) study, and likely would not be considered a risk as an infectious dose. However, such a dose as identified in our study may result in colonization and growth of airborne *Pasteurella* spp. in the oro-pharyngeal passages of bighorn sheep, with a potential of developing into pneumonia. We propose that such a dose as identified in this study may serve as a clinically active dose. Further, results from the Gilmour et al (1984) paper clearly suggest that doses on the order identified in this study can result in immediate death, if exposure occurs concurrently with other risk factors such as viral infections.

Results from this observational study, especially when taken together with previous studies discussed above, lead us to suggest that further directed studies are warranted.

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