

Behavioral and physiologic changes in Holstein steers experimentally infected with *Mannheimia haemolytica*

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OBJECTIVE

To evaluate changes in behavior and surfactant protein (SP) A and D concentrations in serum and bronchoalveolar lavage fluid (BALF) samples of calves experimentally infected with *Mannheimia haemolytica*.

ANIMALS

Twelve 4- to 5-month-old Holstein steers.

PROCEDURES

Calves were divided into 2 treatment groups and instrumented with a data logger to collect behavioral data. After 10 days of acclimation, calves were experimentally inoculated with 3×10^9 CFUs to 5×10^9 CFUs of *M haemolytica* suspended in approximately 5 mL of PBS solution (infected calves; $n = 6$) or 5 mL of PBS solution without *M haemolytica* (control calves; 6) through a catheter into the right accessory lung lobe. Calves were clinically evaluated twice daily for 7 days after inoculation. Blood and BALF samples were collected from all calves at predetermined times for determination of serum and BALF SP-A and SP-D concentrations. Serum and BALF concentrations of SP-A and SP-D and behavioral data were evaluated over time and between treatment groups.

RESULTS

Compared with control calves, infected calves spent more time lying in general and more time lying on the right side during the 24 hours and 6 days after inoculation, respectively. Mean rectal temperature for infected calves (41.3°C) was significantly greater than that for control calves (39.2°C) 12 hours after inoculation. Mean respiratory rate for infected calves (52.5 breaths/min) was significantly greater than that for control calves (45.4 breaths/min) throughout the observation period.

CONCLUSIONS AND CLINICAL RELEVANCE

Results indicated continuous behavioral monitoring may improve detection of calves with respiratory tract disease. (*Am J Vet Res* 2017;78:1056–1064)

Bovine respiratory disease is the most common cause of illness and death in cattle feedlots in the United States.¹ Results of the USDA National Animal Monitoring System 2011 feedlot survey¹ indicate that 16.2% of feedlot cattle develop clinical signs of BRD and 87.5% of those cattle are treated with antimicrobials. As of January 2011, it was estimated that there were 14.0 million cattle in US feedlots²; thus, approximately 2 million cattle in feedlots develop BRD, of which 1.8 million receive antimicrobial treatment. In 2011, the mean cost of BRD treatment was \$23.60/treated animal,¹ which was almost twice that reported in 1999 (\$12.59/treated animal).³ Therefore, the total cost of treating feedlot cattle with clinical BRD may approach \$40 million annually, although that number is likely an underestimation because

mild and subclinical cases of BRD were not included in the estimated BRD treatment cost.¹ Additionally, meat quality and yield for cattle treated for BRD are less than those for cattle not treated for BRD.⁴ In the United States, BRD is also the most common disease of weaned dairy heifers, affecting approximately 11.2% of that population, and the second most common disease of preweaned dairy heifers, affecting approximately 18.1% of that population.⁵ This further indicates the overall impact of BRD on the US cattle production.

Early detection and treatment of cattle with BRD is important to ensure positive clinical outcomes.⁶ Visual assessment of calves from a distance and the appraisal of cattle with subtle clinical signs often initiate the diagnosis and treatment of BRD. However, cattle that are ill instinctively alter their behavior so as to appear fit or healthy, especially when interacting with humans.⁷ The sensitivity (61.8%) and specificity (62.8%) of visual assessment and clinical illness scoring for the detection of calves with lung lesions are fairly low.^{8–10} Consequently, alternative strategies

ABBREVIATIONS

BALF Bronchoalveolar lavage fluid
BRD Bovine respiratory disease
CIS Clinical illness score
RR Respiratory rate
SP Surfactant protein

to improve detection of cattle in the early stages of BRD need to be considered.

Surfactant proteins are promising biomarkers for the assessment of pulmonary inflammation. The protein fraction of pulmonary surfactant consists of 4 proteins, SP-A, SP-B, SP-C, and SP-D. Surfactant proteins B and C are hydrophobic in nature and function primarily to reduce surface tension at the air-liquid interface. Surfactant proteins A and D are predominantly involved in innate defense by assisting resident phagocytic cells in the clearance of pathogens. Surfactant proteins A and D are upregulated during infection and can enter the systemic circulation via an increase in lymphatic drainage or leakage through damaged alveolar basement membranes.^{11,12} The SP-D concentration increases from baseline concentrations in the lungs of calves¹² and lambs¹³ experimentally infected with *Mannheimia haemolytica* and horses¹⁴ experimentally infected with *Streptococcus zooepidemicus*.

All mammals commonly develop behavioral changes such as lethargy and inappetence along with physiologic changes,¹⁵⁻¹⁷ and those behavioral changes can be detected by behavioral monitoring.¹⁸⁻²⁰ For example, calves experimentally infected with *M haemolytica* spend more time lying down than uninfected calves.²⁰ Thus, objective measurement of activity and lying time could aid in the detection of calves with BRD.

Alternative strategies that facilitate the discernment of calves with subclinical or mild BRD are necessary so the cattle industry can address judicious use of antimicrobials. Although results of multiple studies^{13,14,20,21} suggest that monitoring physiologic biomarkers such as SP-A or SP-B and lying behavior could be useful for identifying calves with BRD, information regarding the relationship between changes in SP concentrations and lying behavior in calves with BRD is lacking. The purpose of the study reported here was to evaluate changes in behavior and SP-A and SP-D concentrations in BALF and serum samples of calves experimentally infected with *M haemolytica*.

Materials and Methods

Animals

All study procedures were approved by the University of Tennessee Institutional Animal Care and Use Committee. Twelve 4- to 5-month-old Holstein steers (calves) from the University of Tennessee Little River Dairy Unit in Walland, Tenn, were enrolled in the study. Prior to enrollment, calves were determined to be immunologically naïve to *M haemolytica* on the basis of the absence of *M haemolytica* growth on bacterial cultures of nasopharyngeal swab specimens and the absence of serum antibodies against *M haemolytica* as determined by a whole cell-lysate ELISA.

Calves were moved from a group pasture to research pens and allowed to acclimate to the new housing conditions for 10 days prior to initiation of

study procedures. On the third day of acclimation, calves were divided into 2 groups (strata) on the basis of body weight, then calves within each stratum were randomly allocated by means of a software-based random number generator^a to 1 of 2 treatment groups, a sham-inoculated control group (control group; n = 6), and a group experimentally infected with *M haemolytica* (infected group; 6). This ensured that the mean body weight was similar between the 2 treatment groups. Calves were separated into 2 covered pens (approx 20 X 20 m) on the basis of treatment group (ie, all 6 calves within a treatment group were housed together). There was a 10-m separation between the 2 pens, and each pen had independent feed and water sources. Each calf had ad libitum access to hay in a feeder and water and was provided approximately 2.7 kg of a complete starter-grower ration formulated for dairy calves twice daily in a trough that was accessible through headlocks for the duration of the acclimation and study periods. The pelleted ration contained 18% crude protein and 2% crude fat. Calves were monitored for disease development twice daily throughout the acclimation period.

Strict biosecurity measures were implemented throughout the acclimation and study periods to minimize cross-contamination between the 2 treatment groups. A boot wash was maintained at the exit of each pen. Study and animal care personnel donned clean personal protective equipment (disposable coveralls and gloves) prior to entry into either pen, and a separate supply of personal protective equipment was maintained for each group. Calves in the control group were always observed, fed, and sampled before the calves in the infected group. Likewise, the control pen was always cleaned before the infected pen.

Experimental model

Mannheimia haemolytica was prepared for inoculation as described.²² Briefly, lyophilized *M haemolytica* serotype 1 was obtained from another researcher^b and reconstituted in sterile water. It was cultured on brain-heart infusion agar supplemented with 5% bovine blood and incubated in a CO₂ incubator at 37°C for 18 to 22 hours. Bacterial colonies were selected and suspended in brain-heart infusion broth and incubated at 37°C for 7 hours. The broth cultures were centrifuged at 1,600 X g for 15 minutes to pellet the bacteria. The bacterial pellet was washed 3 times with sterile PBS solution, then resuspended in PBS solution to achieve a suspension containing approximately 1 X 10⁹ CFUs of *M haemolytica*/mL. The bacterial concentration was determined by measurement of the optical density of the suspension at a wavelength of 650 nm and plotting the result against a standard curve of CFUs versus optical density. Plate counts were conducted on a sample of each prepared suspension to confirm the bacterial concentration.

All calves were inoculated with the assigned treatment on day 0. Each calf was restrained in a standing position in a headlock. Two halters were used to elevate and stabilize the calf's head. The area

around the external nares was cleaned, and a 5.9-mm endoscope with a 2-mm biopsy channel was introduced into the right nasal passage. The endoscope was passed through the nasopharyngeal region and laryngeal folds into the trachea and progressed until the tracheal bronchus was identified. A sterile tracheal wash catheter (length, 190 cm; diameter 1.8 mm) was passed through the endoscopic biopsy channel and into the right accessory lung lobe in a manner similar to that described in another study.²³ Calves in the infected group were inoculated with approximately 3×10^9 CFUs to 5×10^9 CFUs of *M haemolytica* suspended in approximately 5 mL of PBS solution through the catheter directly into the right accessory lung lobe. The inoculum was followed by 60 mL of PBS solution to ensure that the entire infective dose of *M haemolytica* was administered. Calves in the control group were inoculated in the same manner except the *M haemolytica* inoculum was replaced with 5 mL of PBS solution. A new sterile catheter was used for each calf.

Sample collection

From each calf, blood (approx 40 mL) was collected via jugular venipuncture directly into serum separator blood collection tubes or blood collection tubes containing potassium EDTA as an anticoagulant immediately prior to inoculation (day 0) and daily thereafter through day 7. All blood samples were centrifuged at $3,000 \times g$ for 10 minutes at 4°C . The resulting serum or plasma was harvested and stored as 2-mL aliquots in cryovials at -80°C until analyzed.

Bronchoalveolar lavage fluid samples were obtained via endoscopic lavage of the tracheal bronchus and right accessory lung lobe immediately prior to inoculation and on days 1, 3, 5, and 7. Briefly, a sterile tracheal wash catheter was advanced into the tracheal bronchus, and up to 120 mL of sterile saline (0.9% NaCl) solution was injected through the catheter and then immediately aspirated until a minimum of 5 mL of BALF was recovered. Samples were transferred into blood collection tubes containing potassium EDTA, then separated into 2-mL aliquots and stored in cryovials at -80°C until analyzed.

SP analysis

Concentrations of SP-A and SP-D were determined in each serum and BALF sample by use of commercially available ELISA kits^c that were specific for bovine SP-A and SP-D. Each assay was validated by the manufacturer and performed in accordance with the manufacturer's instructions.

Behavioral and clinical observations

Two days prior to inoculation, each calf was instrumented with a data logger^d to collect behavioral activity data as described and validated.¹⁹ Briefly, the data logger was wrapped and attached to the lateral aspect of the left metatarsus of each calf with a cohesive bandage.^e Data loggers were removed from calves

in the control group after blood and BALF sample collection on day 7 and from calves in the infected group after euthanasia.

Calves were observed twice daily beginning at approximately 6:30 AM and 6:30 PM throughout the acclimation (days -10 to -1) and study (days 0 to 7) periods by 5 trained and experienced clinical observers. Because of the biosecurity measures implemented, the clinical observers could not remain unaware of (be blinded to) treatment group assignment for individual calves. Calves in the control group were assessed before the calves in the infected group at each observation. At each observation, each calf was assigned a CIS (**Appendix**) on a scale of 1 to 4 that was modified from a scoring system described by Perino and Apley.²⁴ Any calf that was assigned a CIS of 4 (ie, moribund) was immediately euthanized and necropsied. Calves were assigned CISs while unrestrained prior to sample collection with the observers outside the pens. After the CISs were recorded, the allotted starter-grower ration was placed in the feeding trough to entice the calves to put their heads through the self-locking headlocks so they could be restrained for sample collection and measurement of RR and rectal temperature. Each calf was also individually weighed daily during the morning observation.

Postmortem analysis

Calves that were assigned a CIS of 4 at any time during the study period and calves remaining in the infected group following sample collection on day 7 were euthanized with a barbiturate overdose and necropsied as soon as possible thereafter. A veterinary internal medicine specialist (MC) experienced in lung lesion scoring evaluated the lungs of all calves in situ. A scoring system adapted from that described by Fajt et al²⁵ was used to assign a lung consolidation score to each calf. Briefly, the total lung consolidation score = $(0.06 \times \text{proportion of consolidation in right caudal apical lung lobe}/100) + (0.063 \times \text{proportion of consolidation in right cranial apical lung lobe}/100) + (0.053 \times \text{proportion of consolidation in left cranial apical lung lobe}/100) + (0.049 \times \text{proportion of consolidation in left caudal apical lung lobe}/100) + (0.319 \times \text{proportion of consolidation in left diaphragmatic lung lobe}/100) + (0.043 \times \text{proportion of consolidation in intermediate lung lobe}/100) + (0.352 \times \text{proportion of consolidation in right diaphragmatic lung lobe}/100) + (0.061 \times \text{proportion of consolidation in accessory lung lobe}/100)$. Lung tissue specimens were then collected for aerobic bacterial culture and histologic evaluation to verify the presence of the *M haemolytica* challenge strain in active lesions and to confirm that those lesions had histologic evidence of inflammation and disruption of alveolar architecture characteristic of *M haemolytica* infection.

Data analysis

Behavioral data were summarized as described.²⁶ Variables calculated included number and duration

of lying bouts and time spent lying on the right and left sides (laterality) as well as collectively on days 1 through 6. Behavioral data were not evaluated on days 0 and 7 because calves underwent extra manipulation or handling on those days (calves were experimentally inoculated on day 0 and either were euthanized [calves in the infected group] or had the data logger removed [control calves] on day 7), which might have affected the behavioral data. Dependent variables evaluated included total lying time, rectal temperature, RR, and SP-A and SP-D concentrations in serum and BALF. The data distributions for those variables were assessed for normality by the Shapiro-Wilk test. A mixed linear regression model was created for each dependent variable. Each model included fixed effects for treatment group, study day, and the interaction between treatment group and study day and a random effect for calf within treatment. Calf was the experimental unit, and observations were repeated by day. Similar mixed models were created to assess the effect of laterality on the dependent variables, with additional fixed effects for laterality and the interactions between laterality and treatment group and between laterality and study day. A linear regression model was also used to assess the association between the maximum rectal temperature and lying time on day 1 (ie, during the first 24 hours after inoculation).

For each calf, the maximum rectal temperature and CIS were recorded during the 24 hours immediately after inoculation (day 1). The mean baseline lying time for each calf was calculated on the basis of behavioral data collected for days -2 and -1 (ie, the 48-hour period before inoculation). For each calf, the change in lying time at maximum rectal temperature and CIS was calculated by subtracting the baseline lying time from the lying time for day 1. A 1-way ANOVA was used to assess the respective effects of maximum rectal temperature and CIS on the change in lying time. All analyses were conducted with statistical software,^f and values of $P < 0.05$ were considered significant.

Table 1—Least squares mean \pm SE SP-A and SP-D concentrations in serum and BALF samples obtained from twelve 4- to 5-month-old Holstein steers immediately before (day 0) and at various times after the calves were experimentally inoculated with 3×10^9 CFUs to 5×10^9 CFUs of *Mannheimia haemolytica* (infected group; $n = 6$) or a sham inoculum (5 mL of PBS solution; control group; 6).

Variable	Treatment group	Duration after inoculation (d)							
		0	1	2	3	4	5	6	7
Serum SP-A (ng/mL)	Infected	5.5 \pm 1.4	8.4 \pm 4.1*	3.8 \pm 1.4	1.7 \pm 1.4*	8.2 \pm 1.4*	11.1 \pm 1.4*	7.3 \pm 1.4	8.1 \pm 1.4
	Control	6.8 \pm 1.4	8.9 \pm 1.4*	5.0 \pm 1.4	3.7 \pm 1.4*	9.5 \pm 1.4*	11.7 \pm 1.4*	9.0 \pm 1.4	7.9 \pm 1.4
Serum SP-D (ng/mL)	Infected	0.22 \pm 4.6	0.22 \pm 4.6	0.0 \pm 4.6	12.4 \pm 4.6*	0.0 \pm 0.0	32.3 \pm 4.6*	0.22 \pm 4.6	0.22 \pm 4.6
	Control	1.8 \pm 4.6	1.8 \pm 4.6	0.0 \pm 4.6	20.9 \pm 4.6*	5.7 \pm 4.6	30.2 \pm 4.6*	1.1 \pm 4.6	1.4 \pm 4.6
BALF SP-A (ng/mL)	Infected	36.0 \pm 2.6	37.5 \pm 2.6	—	36.4 \pm 2.6	—	36.8 \pm 2.6	—	41.6 \pm 2.6
	Control	36.4 \pm 2.6	33.8 \pm 2.6	—	33.8 \pm 2.6	—	34.0 \pm 2.6	—	38.0 \pm 2.6
BALF SP-D (ng/mL)	Infected	21.0 \pm 6.6	54.1 \pm 6.6*	—	23.7 \pm 6.6	—	32.8 \pm 6.6	—	15.6 \pm 6.6
	Control	18.6 \pm 6.6	44.0 \pm 6.6*	—	15.9 \pm 6.6	—	17.9 \pm 6.6	—	15.4 \pm 6.6

Blood samples were collected from each calf on a daily basis from days 0 through 7, whereas BALF samples were collected only on days 0, 1, 3, 5, and 7.

*Within a variable, value differs significantly ($P < 0.05$) from the corresponding value on day 0.

— = Not determined.

Results

Calves

All calves in the control group remained clinically normal (CIS = 1) and survived the 7-day study period. None of the calves in the infected group was assigned a CIS > 3 at any observation; therefore, all calves in the infected group were euthanized following sample collection on day 7. During the observation 12 hours after experimental inoculation, 5 of the 6 calves in the infected group were assigned a CIS of 2 and the remaining calf was assigned a CIS of 3, whereas all 6 calves in the control group were assigned a CIS of 1.

SP-A and SP-B concentrations

The mean SP-A concentrations in serum ($P = 0.40$) and BALF ($P = 0.38$) did not differ significantly between calves in the infected and control groups (Table 1). Likewise, the mean SP-D concentrations in serum ($P = 0.41$) and BALF ($P = 0.13$) did not differ significantly between the calves in the infected and control groups. The interaction between treatment group and study day was not significantly associated with the SP-A or SP-D concentration in serum or BALF. However, study day was significantly associated with serum SP-A ($P < 0.001$) and SP-D ($P < 0.001$) concentrations and BALF SP-D concentration ($P < 0.001$) but not BALF SP-A concentration ($P = 0.15$).

Behavior

The interaction between treatment group and study day was significantly ($P < 0.001$) associated with lying time when laterality was not included in the model. The mean \pm SE lying time for calves in the infected group (16.4 \pm 0.5 hours) was significantly greater than that for the calves of the control group (14.4 \pm 0.5 hours) during the first 24 hours after experimental inoculation (Figure 1). That was the only time when the mean lying time differed between the infected and control groups. For the duration of the study, the mean \pm SE lying time was 880.0 \pm 47.8 min/d (range, 786 to 984 min/d [13.1 to 16.4 h/d]).

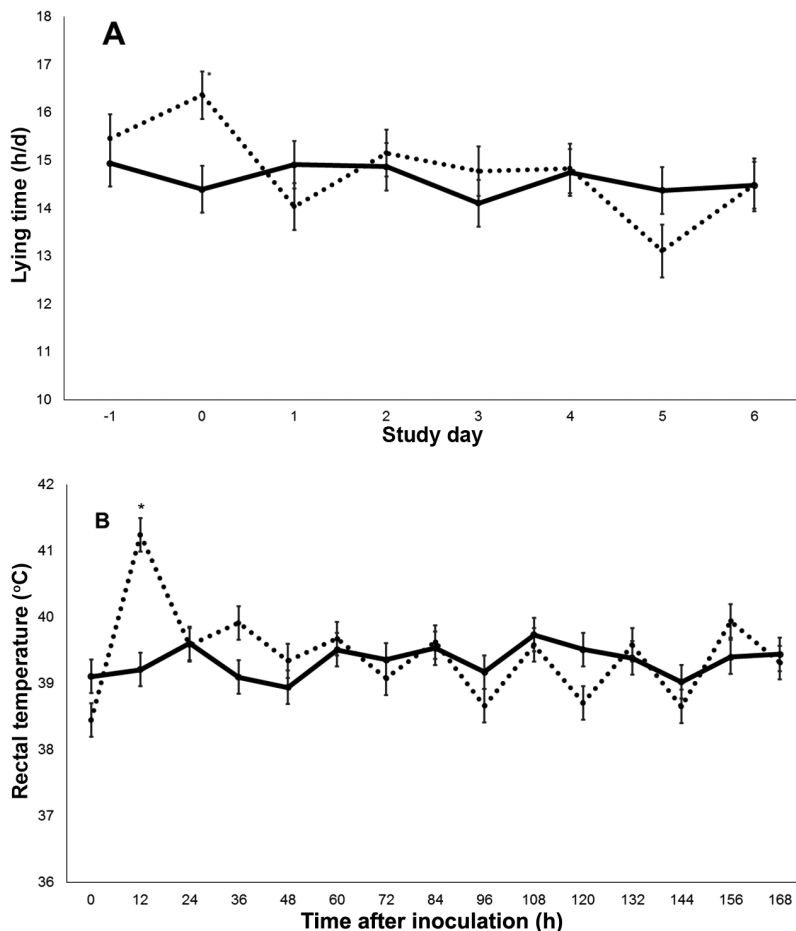


Figure 1—Least squares mean lying time (A) and rectal temperature (B) over time for twelve 4- to 5-month-old Holstein steers that were experimentally inoculated with 3×10^9 CFUs to 5×10^9 CFUs of *Mannheimia haemolytica* (infected group; dotted line; $n = 6$) or a sham inoculum (5 mL of PBS solution; control group; solid line; 6) on day 0 (hour 0). Brackets delimit the SE. *Within an observation, values differ significantly ($P < 0.01$) between the infected and control groups.

for the calves in the infected group and 873.4 ± 47.8 min/d (range, 846 to 984 min/d [14.1 to 14.9 h/d]) for the calves in the control group.

When laterality (right or left side) was included in the model for lying time, there was a significant interaction between laterality and treatment group. Calves in the infected group spent significantly ($P = 0.01$) more time lying on the right side (7.9 h/d) than on the left side (6.8 h/d) throughout days 1 through 6 (**Figure 2**), whereas the mean time calves in the control group spent lying on the right and left sides did not differ significantly ($P = 0.22$). However, treatment group ($P = 0.89$), study day ($P = 0.59$), the interaction between treatment group and study day ($P = 0.49$), and the interaction between laterality and study day ($P = 0.24$) were not significantly associated with lying time in that model.

Clinical observations

Respiratory rate was significantly associated with treatment group ($P = 0.03$) but not study day ($P = 0.06$)

or the interaction between treatment group and study day ($P = 0.16$). The mean \pm SE RR for calves in the infected group (52.5 ± 2.0 breaths/min) was significantly ($P = 0.03$) greater than that for calves in the control group (45.4 ± 2.0 breaths/min) throughout the study period.

Rectal temperature was significantly affected by study day ($P < 0.001$) and the interaction between treatment group and study day ($P < 0.001$) but not treatment group ($P = 0.24$). At 12 hours after experimental inoculation, the mean \pm SE rectal temperature for the calves in the infected group ($41.3 \pm 0.3^\circ\text{C}$) was significantly ($P < 0.001$) greater than that for the calves of the control group ($39.2 \pm 0.3^\circ\text{C}$; **Figure 1**). Maximum rectal temperature was not associated with the change in lying time during the first 24 hours after experimental inoculation ($R^2 = 0.01$; $P = 0.13$).

The CISs did not differ significantly between calves in the infected and control groups after day 1, and the maximum CIS was recorded on day 1 for all calves. On day 1 when the maximum CIS was recorded, there was a positive association between lying time and CIS; however, those interactions were not significant. The mean \pm SE change in lying time was -72.5 ± 42.2 minutes for all 6 calves in the control group that had a maximum CIS of 1 and -52.1 ± 34.3 minutes for the 5 calves in the infected group that had a maximum CIS of 2. The change in lying time was 43.5 minutes for the remaining calf in the infected group that had a maximum CIS of 3. Negative values for the change in lying time indicated that calves spent less time lying in the 24 hours after inoculation than at baseline (ie, mean lying time for the 48 hours prior to inoculation), whereas positive values for change in lying time indicated that calves spent more time lying in the 24 hours after inoculation than at baseline.

Postmortem findings

All 6 calves in the infected group had gross lung lesions consistent with bronchopneumonia such as generalized congestion. Acute focal pneumonic lesions, which were characterized by discrete areas with mild to moderate fibrinous tissue consolidation and edema, were commonly observed in the right cranial lung lobes. Gross evidence of *M haemolytica* infection was not detected in other areas of the lung. The mean \pm SE lung consolidation score was $7.32 \pm 0.39\%$ (median, 7.69%; range, 6.12% to 8.33%).

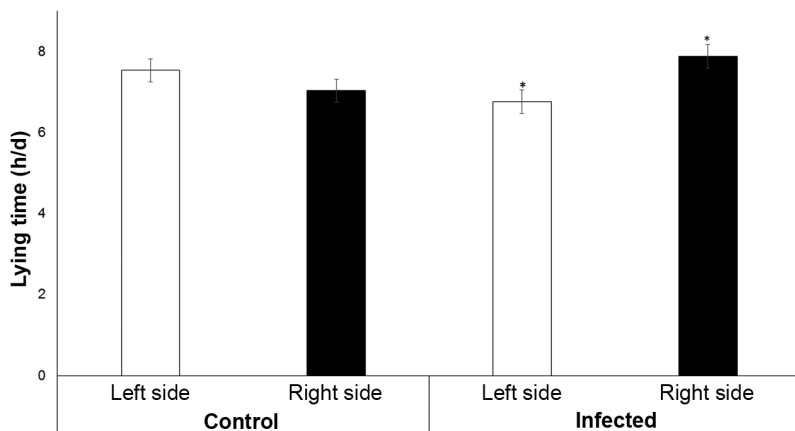


Figure 2—Least squares mean time the calves of Figure 1 spent lying on their right and left sides (laterality) on a daily basis for days 1 through 6. *Within a treatment group, values differs significantly ($P = 0.01$) between the 2 sides. See Figure 1 for remainder of key.

Histologic examination of lung tissue specimens obtained from the margins of active lesions revealed tissue infiltration by neutrophils and macrophages, the deposition of proteinaceous debris in affected alveoli, extensive edema and widening of interstitial spaces adjacent to affected alveoli, and damage to type I pneumocytes and underlying basement membranes. *Mannheimia haemolytica* was cultured from each of 3 lung tissue specimens collected from the margins of active lesions in all calves of the infected group.

Discussion

In the present study, calves experimentally inoculated with *M haemolytica* (infected calves) spent more time lying down and had significantly greater rectal temperatures during the first 12 to 24 hours after inoculation, compared with control calves that were sham inoculated with PBS solution. The mean RR and time spent lying on the right side for infected calves were also significantly greater than that for control calves throughout the study period (days 0 through 7). These findings suggested that continuous behavioral monitoring may improve disease detection in calves.

In the present study, concentrations of SP-A and SP-D in serum and BALF did not differ significantly between infected and control calves at any time during the study period. However, the concentrations of both SP-A and SP-D in BALF were consistently higher than those in serum for both infected and control calves. This suggested that instillation of the experimental inoculant (regardless of whether that inoculant was *M haemolytica* or PBS solution) directly into the right accessory lung lobe resulted in local induction of SP-A and SP-D, although confounding effects of the experimental model and the calves used might have attenuated that response relative to the response induced by natural infection. The SP-D

concentration in lung tissue is abnormally increased in lambs¹³ experimentally infected with *M haemolytica* and horses experimentally infected with *S zooepidemicus*.¹⁴ In horses, serum SP-D concentrations are positively associated with disease severity.¹⁴ In the present study, the only period that CIS (a measure of disease severity) differed significantly between the infected and control calves was during the first 12 hours after experimental inoculation (day 0), and it is possible that the severity of disease induced in the infected calves relative to the reaction induced by the sham inoculation in the control calves was insufficient to induce a significant increase in SP-A or SP-D concentrations. Alternatively, the milder than expected disease severity observed for the infected calves

might have been caused by preparation procedures for the *M haemolytica* inoculum that resulted in an ineffectual challenge organism or an inherent disease resistance among the study calves owing to inaccurate identification of their *M haemolytica* immune status (eg, infected calves were not truly naïve to *M haemolytica*). Although the experimental inoculation successfully induced *M haemolytica* infection (as evidenced by culture of the organism from lung tissue specimens obtained from the margins of active lesions) in all calves of the infected group, that infection may not have been adequate to induce sufficient damage to the lung parenchyma and allow excessive leakage of SP-A and SP-D into BALF or serum or cause severe clinical disease.

Similar to findings of other studies,^{20,23} the calves experimentally inoculated with *M haemolytica* in the present study spent significantly more time lying down during the first 24 hours after inoculation (mean \pm SE lying time on day 1, 16.4 ± 0.5 hours) than did control calves that received a sham inoculation (mean \pm SE lying time on day 1, 14.4 ± 0.5 hours). By comparison, in a study²⁰ by Theurer et al, calves experimentally infected with *M haemolytica* spent about 55% (approx 13.2 hours) of the first day after inoculation lying down, whereas the control calves spent only 40% (approx 9.6 hours) of that day lying down. Unlike the present study in which the mean lying time differed significantly between the infected and control calves only during the first 24 hours after inoculation, the *M haemolytica*-infected calves of that study²⁰ spent significantly more time lying down than control calves on multiple days during the 9-day observation period. The median total lung consolidation score for the *M haemolytica*-infected calves of that study²⁰ (6.8%) was similar to that for the infected calves of this study (7.69%), which suggested that the disease severity was similar for *M haemolytica*-infected calves of both studies. However, contrary

to the present study, the Theurer et al study²⁰ was conducted during a period of high ambient environmental temperatures. The mean daily lying time of beef heifers decreases following transportation during high ambient temperatures,²⁷ which suggests that daily lying time decreases as stress increases. In the present study, the mean daily lying time ranged from 13.1 to 16.4 hours for the infected calves and 14.1 to 14.9 hours for the control calves throughout the duration of the observation period, which was substantially longer than the mean daily lying time for the *M baemolytica*-infected (range, 9.6 to 13.2 hours) and control (range, 6.0 to 9.6 hours) calves of the Theurer et al study.²⁰ It is likely that the high ambient temperatures to which the calves of that study²⁰ were exposed contributed to the apparent decrease in daily lying time and created additional stress for the *M baemolytica*-infected calves, which in conjunction with BRD further exaggerated the decrease in daily lying time.

Although results of the present study and the Theurer et al study²⁰ indicated that the time spent lying down for calves experimentally inoculated with *M baemolytica* increased during the first 24 hours after inoculation, findings of a study²⁸ by Borderas et al indicate that lying behavior did not change for calves experimentally inoculated with a low dose of bacterial endotoxin. However, in the Borderas et al study,²⁸ the behavior of calves was monitored for only the 2 hours before and after peak body temperature was achieved following inoculation, whereas the behavior of the calves of the present study and Theurer et al study²⁰ was monitored continuously throughout the observation period. Thus, the apparently contradictory effect of experimental inoculation on lying time may simply reflect differences among studies in the collection of behavioral data. Diseased calves are expected to become lethargic, regardless of whether the disease occurs naturally or is experimentally induced²⁹; therefore, it is likely that the calves of the Borderas et al study²⁸ had similar behavioral changes as the calves of the present study and the Theurer et al study,²⁰ but behavioral changes (eg, increase in lying time) only become noticeable when data are collected for a fairly long time (ie, days instead of hours).

The infected calves of the present study spent more time lying on their right sides than on their left sides throughout the observation period, whereas the control calves did not have any evidence of laterality when lying down. Interestingly, the *M baemolytica* inoculum was instilled into the right accessory lung lobe, and calves developed lesions only in the right lung lobes. Mature dairy cows spend approximately the same amount of time lying on their right and left sides,³⁰ although that balance in laterality occasionally varies during incidences of discomfort such as late gestation³¹ or following rumen cannulation.³² It is possible that the development of pathologic changes in the right lung lobes caused the infected calves

discomfort that was partially alleviated by lying on their right sides, similar to the response to discomfort observed in mature cows.³⁰ To our knowledge, the present study was the first to evaluate lying laterality in calves, particularly in relation to experimentally induced BRD.

Although housing the infected and control groups in separate pens and the adherence to strict biosecurity measures prevented the control calves from becoming infected with *M baemolytica*, it totally confounded treatment with pen. However, during evaluation of behavioral data, no outliers indicative of abnormally excitable behaviors were detected. The calves of this study were housed together in a pasture before being enrolled in the study and were provided a 10-day acclimation period before experimental inoculation to adjust to the treatment pens. Results of another study³³ indicate that a 3-day acclimation period is sufficient for dairy calves to reestablish social hierarchy and return to normal behavior after being comingled. Moreover, the overall mean daily lying time did not differ between the calves of the infected and control groups. Thus, we believe that the calves of this study were well acclimated to their pen mates, and the behavioral differences detected were not the result of a pen-specific effect aside from treatment.

The diagnosis of BRD solely on the basis of clinical observation, even by trained observers, can lack precision³⁴ and accuracy.^{6,35} In the present study, maximum rectal temperature was recorded 12 hours after inoculation for all calves in both treatment groups. At that time, all 6 calves in the control group were assigned a CIS of 1 (clinically normal), 5 of the 6 calves in the infected group were assigned a CIS of 2 (mild disease), and the remaining calf in the infected group was assigned a CIS of 3 (moderate disease). Because of the biosecurity measures implemented for the study, it was not possible to blind observers to the treatment group of individual calves; therefore, the CIS assignment might have been biased. However, the behavioral data collected during the first 24 hours after inoculation indicated that the calves in the infected group were not as active as the calves in the control group, which indicated that the infected calves may have been lethargic with other signs of illness consistent with the CISs assigned during that period.

The rectal temperature and RR of calves with BRD are expected to increase.^{28,36} In the present study, the mean rectal temperature for the infected calves was significantly greater than that for the control calves 12 hours after inoculation, and the mean RR for the infected calves was significantly greater than that for the control calves throughout the postinoculation observation period. In the previously described Theurer et al study,²⁰ all calves experimentally inoculated with *M baemolytica* developed lung lesions, and the mean rectal temperature for those calves was significantly greater than that for the control calves on days 0 (inoculation) and 1; however, the mean RR did not

differ between the 2 groups. In another study,²³ the RR of calves following experimental inoculation with *M haemolytica* was increased from that prior to inoculation, with significant increases detected on days 5 through 8 after inoculation. In the present study, RR was associated with treatment group only; it was not associated with study day or the interaction between treatment group and study day. This indicated that, although RR varied by treatment, it remained fairly stable throughout the postinoculation period.

In both the present study and the Theurer et al study,²⁰ the CIS, rectal temperature, and lying time of *M haemolytica*-infected calves were significantly greater than those of healthy control calves during the 24 hours immediately following experimental inoculation. A decrease in activity and increase in CIS were likewise observed in calves following experimental inoculation with *M haemolytica* in another study.²³ However, the relationship between CIS and lying time was not evaluated in either of those other 2 studies.^{20,23} A slight positive linear relationship was detected between the CIS and change in lying time after experimental inoculation for the calves of the present study. It is possible the lethargy or other clinical signs of BRD subjectively quantified by the CIS reflected an increase in postinoculation lying time, particularly for the infected calf that was assigned a CIS of 3.

Results of a crossover study²⁷ conducted to evaluate the effect of transportation during periods of high ambient temperature on physiologic and behavioral indices of beef heifers indicate that the rectal temperature was decreased and the lying time was increased for heifers during the first 24 hours after transportation, compared with those for the same heifers when they were not transported. All cattle of that study²⁷ were clinically normal without any clinical signs of BRD, and the transportation and control (nontransportation) phases were conducted when the ambient temperature was > 32.2°C. The mean peak rectal temperature ($\geq 40.0^\circ\text{C}$) was recorded 8 hours after the start of each phase.²⁷ The rectal temperature and lying time for calves with experimentally induced BRD also increase during the 24 hours after transportation.²⁰ Although mean rectal temperature and lying time increased during the first 24 hours after experimental inoculation for the infected calves of the present study, the change in lying time was not significantly associated with rectal temperature.

All infected calves of the present study had gross lung lesions that were characteristic of mild *M haemolytica* infection. The lung consolidation score for the infected calves of this study (mean \pm SE, 7.32 \pm 0.39%; median, 7.69%; range, 6.12% to 8.33%) was similar to that for calves of the Theurer et al study²⁰ (median 6.8%; range, 5.04% to 9.71%) but less than that for calves with BRD that were not treated with an antimicrobial in another study²⁵ (mean, 18.1%). Both of those other studies^{20,25} used the same lung consolidation scoring system used in the present

study. Collectively, these results suggested that the *M haemolytica* experimental models used for the present study and the Theurer et al study²⁰ induced pulmonary infection of similar magnitude that was milder than that induced by the *M haemolytica* experimental model used in the other study.²⁵

Results of the present study indicated that mild BRD infections, as determined by lung consolidation score, may not cause detectable changes in SP-A and SP-D concentrations in serum and BALF samples. However, calves experimentally infected with *M haemolytica* had an increase in lying time and shift in lying laterality during the first 24 hours and 6 days after inoculation, respectively, which can be detected by use of continuous monitoring systems. Thus, behavioral data, when combined with other clinical evaluations, may improve early detection and treatment of calves with BRD. Treatment of calves in the early stages of BRD should improve treatment success and animal welfare.

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Footnotes

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Appendix

Description of scoring system used to assign CISs to twelve 4- to 5-month-old Holstein steers that were experimentally inoculated with 3×10^9 CFUs to 5×10^9 CFUs of *Mannheimia haemolytica* (infected group; n = 6) or a sham inoculum (5 mL of PBS solution; control group; 6).

CIS	Description	Clinical appearance
1	Clinically normal	No abnormalities noted.
2	Slightly ill	Signs of mild depression and gaunt with or without a cough.
3	Moderately ill	Signs of severe depression, labored breathing, presence of ocular or nasal discharge with or without a cough.
4	Severely ill	Moribund; little response to touch.

Each calf was assigned a CIS twice daily at approximately 6:30 AM and 6:30 PM for the 10 days prior to and 7 days after experimental inoculation (day 0).