Clinical, behavioral, and pulmonary changes in calves following inoculation with *Mycoplasma bovis*

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**Objective**—To characterize clinical and behavioral changes in calves following inoculation with *Mycoplasma bovis* and evaluate relationships between those changes and pulmonary disease.

**Animals**—22 healthy Holstein steers.

**Procedures**—20 calves were inoculated intranasally with $< 10^8$ CFU or $> 10^9$ CFU of *M. bovis*. Calves were assigned a clinical illness score (CIS) on a scale of 1 through 4 twice daily on the basis of severity of cough, labored breathing, and lethargy. For each calf, distance traveled and time spent near the waterer, feed bunk, or shelter were determined via a remote location monitoring device. Calves were euthanized and necropsied 22 days after inoculation.

**Results**—13 calves became clinically ill after challenge inoculation; 3 calves were euthanized within 20 days. Among all calves, consolidation was evident in 0% to 79.9% of the lungs; extent of lung consolidation did not differ between the challenge dose groups. Distance traveled and percentages of time spent in proximity to the feed bunk and shelter were associated with CIS; calves with more severe disease traveled less distance and spent less time at the feed bunk and more time in the shelter. Distance traveled by calves was negatively associated with extent of lung consolidation ($< 10^8$ or $> 10^9$ CFU); this effect was modified by trial day.

**Conclusions and Clinical Relevance**—Following inoculation with *M. bovis*, calf behavior patterns were associated with both CIS and severity of pulmonary disease. Use of behavior monitoring systems may aid in recognition of respiratory tract disease in calves. (Am J Vet Res 2012;73:490–497)
Observations of cattle behavior and attitude are commonly used to identify cattle that require treatment or further evaluation. This approach relies on subjective evaluations performed by cattle health-care providers, and limited information exists on associations between CIS and objective measurements of cattle behavior. Data obtained on cattle behavior by use of remote monitoring devices indicate that cattle affected with BRD have a change in frequency and duration of feeding and watering behaviors, compared with those behaviors in healthy cattle. Differences in feeding behavior of dairy calves have also been detected in a study conducted to evaluate behavior after inoculation with a low dose of bacterial endotoxin. The comparison of subjective clinical assessments with objective measures of cattle behavior and pulmonary disease could provide knowledge needed to improve current methods for the identification of cattle with BRD.

The objective of the study reported here was to determine behavioral and clinical changes in calves after intranasal inoculation with M. bovis and evaluate the relationship between these changes and pulmonary disease. The intent was to provide data to refine the disease challenge technique and aid in selection of appropriate methods to monitor, identify, and manage cattle with clinical BRD.

Materials and Methods

Calves and selection procedures—Male Holstein calves (5 to 9 weeks old) from a single source in Nebraska were identified for pretreatment screening tests for potential inclusion in the study. Calves were screened for M. bovis infection via mycoplasmal culture of serum samples and nasal swab specimens and for antibodies against M. bovis via ELISA of serum samples. For each calf, the ventral meatus of a nostril was swabbed with a nasal swab. The nasal swab was then placed in Hayflick complete medium and transported on ice to the diagnostic microbiology laboratory. The nasal swab specimens were incubated at 37°C for 48 hours before being inoculated onto Mycoplasma agar plates. The agar plates were then incubated at 37°C in a 5% CO₂ incubator and observed daily for mycoplasmal colony formation for 10 days.

From each calf, 6 mL of blood was collected by jugular venipuncture to obtain serum for M. bovis serologic testing. Blood samples were placed on ice and transported to the laboratory. Samples were then centrifuged, and the serum was removed and frozen at approximately −80°C until analyzed. Serum antibodies against M. bovis were detected by the use of a commercial ELISA kit that had been validated for use in cattle. The kit included a 96-well microtitration plate with an M. bovis recombinant protein expressed by Escherichia coli in half of the wells. To reduce false-positive test results, each well was paired with another well containing a negative control antigen. Positive and negative plate controls provided in the test kit were also used. Serum samples were diluted in buffer, and aliquots were placed into individual wells of the microtitration plate. The plate was incubated at 21°C (range, 18°C to 24°C) for 1 hour, then washed 3 times with an automatic plate washer. Conjugate was added to each well of the plate, and the plate was incubated for another hour at 21°C (range, 18°C to 24°C) and then washed 3 times with an automatic plate washer. Chromogen was added to each well, and the plate was incubated at 21°C (range, 18°C to 24°C) for 10 minutes. A stop solution was then added to each well, and the ODs were measured by the use of a plate reader and a 450-nm filter. The value for each negative control well was subtracted from the corresponding serum OD value, and the result was divided by the corrected value for the positive control for that plate. That value was then multiplied by 100 to obtain the sample-to-positive signal percentage. For the particular kit used to test the serum samples in the present study, a sample was considered positive when the OD was ≥1.373, in accordance with the manufacturer’s recommendations.

Calves for which results of mycoplasmal culture of nasal swab specimens were negative and ELISA OD readings were the lowest were selected for inclusion in the study. Twenty-two calves were transported from the farm of origin to the research facility at Kansas State University. This included 20 calves needed for the study and 2 calves for use as substitutes in case any calf developed clinical illness during the 10-day observation period prior to M. bovis inoculation.

Calves were fed a complete starter grain ration for dairy calves and grass hay ad libitum throughout the study period. The starter ration contained 18.0% crude protein, 3.2% crude fat, 1.0% calcium, and 0.5% phosphorus. During the first week after arrival at the research facility, calves were fed 1.1 kg of starter ration/calf/d, which was gradually increased to 1.4 kg/calf/d for the remainder of the study. The starter ration was fed in a bunk equipped with an overhead rack for placement of hay that was provided ad libitum. Calves were housed in a 25.6 × 11.9-m pen with unrestricted access to an automatic waterer and a covered, open-faced shelter.

All study procedures were conducted in accordance with a protocol approved by the Kansas State University Institutional Animal Care and Use Committee. Calves were humanely handled during the research project, and observations were conducted twice daily (in the morning and afternoon) for signs of clinical illness. In accordance with the protocol, any calf that became severely ill or moribund at any point during the study was immediately euthanized.

Behavioral and clinical observations—Six days prior to M. bovis inoculation, a commercial remote location monitoring device attached to a standard ear tag button was applied to an ear of each calf to record calf behavior and activity during the study. The device transmitted and received ultrawideband radio pulses within a containment area (pen). The device transmitted information about the calf’s location within the pen to a wireless sensor that relayed the information to a computer, where it was stored for analysis. Information from the location monitoring system was recorded continuously throughout the trial. The system recorded the X (length), Y (width), and Z (height) coordinates of the ear device approximately every 5 seconds. The pen was mapped by use of the same system to generate specific areas of interest, including the automatic waterer, feed...
bunk, and an open-faced shelter on the north side of the pen (Figure 1).

At the end of the study, a commercial software program was used to record each calf’s location at each 5-second interval on the basis of its X and Y coordinates relative to the known X and Y coordinates of the waterer, feed bunk, or shelter. The location was then dichotomously (yes or no) classified as being within a 0.3-m radius of the waterer, feed bunk, or shelter. The time (in seconds) spent by each calf at each location (waterer, feed bunk, shelter, or other area of the pen) was summed and aggregated by hour within each study day (8 AM to 8 AM). The distance that a calf traveled between adjacent coordinate readings was calculated by use of the Pythagorean theorem as follows:

\[
\sqrt{(X_1 - X_2)^2 + (Y_1 - Y_2)^2}
\]

where \(X_1\) is the X coordinate at a given time, \(X_2\) is the X coordinate at the subsequent time, \(Y_1\) is the Y coordinate at a given time, and \(Y_2\) is the Y coordinate at the subsequent time. The total distance traveled (in meters) was aggregated by calf and study day for analyses.

Calves were observed twice daily for 10 days prior to \(M\) bovis inoculation by 1 veterinarian (LLK), who was trained in the detection of clinical illness and BRD. At each observation, each calf was assigned a CIS on the basis of its health characteristics. The CIS ranged from 1 through 4 with the following criteria used for each score: 1 = normal behavior, 2 = slight illness (mild lethargy or cough), 3 = moderate illness (severe lethargy, labored breathing, or cough), and 4 = severe illness (calf was moribund or had little response to humans). During the observation period prior to \(M\) bovis inoculation, calves assigned a CIS ≥ 2 underwent complete physical examinations, which included measurement of rectal temperature. Calves with CIS ≥ 2 and rectal temperature ≥ 40°C prior to \(M\) bovis inoculation were removed from the study and received appropriate treatment. For 22 days after \(M\) bovis inoculation, each calf was observed twice daily and assigned a CIS at each observation by the same trained veterinarian (LLK). A calf that was assigned a CIS of 4 at any time during the study was immediately euthanized, and a necropsy was performed on that same day.

\(M\) bovis inoculation—Calves received 1 of 2 \(M\) bovis inoculation doses; random assignment of dose was performed by use of commercial software. Calves were inoculated with a low dose (< 10^8 CFU/dose [\(n = 10\)]) or high dose (> 10^9 CFU/dose [10]) of \(M\) bovis administered intranasally on 2 consecutive days (days 0 and 1).

Throughout the study, calves were housed together in a pen and isolated from direct contact with other animals. During inoculation, personnel wore disposable gloves, protective coveralls, and a respirator face mask. All personnel who were in daily contact with the calves wore coveralls and boots that were designated only for use with the study calves in the study facility. Personnel walked through a transition room and a footbath before entering and leaving the facility. All disposable protective equipment worn by personnel was used once and then discarded in accordance with guidelines for biohazard disposal.

Necropsy and pathological changes—Calves were euthanized and necropsied if they became severely ill (CIS ≥ 4) at any time during the study or at 22 days after \(M\) bovis inoculation. Euthanasia (by use of a penetrating captive bolt) was conducted in accordance with the AVMA euthanasia guidelines.

A necropsy, including gross examination of all major organ systems, was performed on each calf. The lungs were removed from each calf for subjective lesion scoring and mycoplasmal culture. Lung lesions were scored by use of a standardized system on the basis of the percentage of consolidation in each lung lobe. The total percentage of consolidated lung tissue was calculated by multiplying the percentage of consolidation in each lung lobe by the proportion of the total lung that each lobe represented. The lobe values were totaled and multiplied by 100 to provide the reported lesion score. Histologic evaluation of representative specimens from each lung was performed. Lung specimens were...
were euthanized and necropsied on day 22. The percentage of time that calves spent at a specific location in the pen (waterer, feed bunk, shelter, or other area of the pen) during a study day was evaluated by use of a mixed generalized linear model that included the CIS for the subsequent morning and random effects to account for repeated measures on individual calves and specific study days. For subsequent comparisons with behavior outcomes, calves were dichotomized as having minimal or moderate disease (ie, < 10% or ≥ 10% of consolidated lung tissue). This 10% cutpoint was chosen on the basis of the investigators’ clinical experience. The percentage of time each calf spent at a specific pen location over the entire study period was estimated by a pathologist (DAM) and were

Table 1—Mean, median, and range values for the percentage of consolidated lung tissue in each lung lobe of 20 Holstein calves that had < 10% lung consolidation (n = 13) or ≥ 10% lung consolidation (7) as determined during necropsy.

<table>
<thead>
<tr>
<th>Lung lobe</th>
<th>Calves with &lt;10% total lung consolidation</th>
<th>Calves with ≥10% total lung consolidation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>Median</td>
</tr>
<tr>
<td>Left cranial apical</td>
<td>1.5</td>
<td>0.0</td>
</tr>
<tr>
<td>Left caudal apical</td>
<td>2.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Left diaphragmatic</td>
<td>1.2</td>
<td>0.0</td>
</tr>
<tr>
<td>Right cranial apical</td>
<td>10.5</td>
<td>3.0</td>
</tr>
<tr>
<td>Right caudal apical</td>
<td>0.8</td>
<td>0.0</td>
</tr>
<tr>
<td>Right cardiac</td>
<td>3.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Right diaphragmatic</td>
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<td>0.0</td>
</tr>
<tr>
<td>Right intermediate</td>
<td>6.6</td>
<td>0.0</td>
</tr>
<tr>
<td>Total</td>
<td>2.5</td>
<td>1.9</td>
</tr>
</tbody>
</table>

Calves were inoculated intranasally on 2 consecutive days with a low dose (< 10⁶ CFU/dose; n = 10) and high dose (> 10⁹ CFU/dose; 10) of Mycoplasma bovis and were euthanized and necropsied when they became severely ill (3) or at 22 days after initial inoculation (17).
summarized (Table 1). Total percentage of consolidation in the lungs ranged from 0% to 79.9% (mean $\pm$ SD, 14.4 $\pm$ 21.1%) and did not differ significantly ($P = 0.29$) between calves inoculated with the high dose (9.2 $\pm$ 13.6%) or low dose (19.5 $\pm$ 26.5%) of $M$ bovis. The distribution of lung tissue consolidation was similar in each challenge inoculation group, but there was a large variation in the extent of lung tissue consolidation among calves within each challenge inoculation group. The number of calves that had $M$ bovis cultured from lung specimens did not differ significantly ($P = 0.65$) between the low-dose (7/10) and high-dose (5/10) inoculation groups.

Associations between behavioral and clinical observations—In each model for evaluation of potential associations between CIS and behavior as measured by the amount of time that calves spent in specific locations, the effect of study day was significant, but no significant interactions between study day and CIS were identified. Clinical illness scores were associated with the percentage of time calves spent at the feed bunk ($P = 0.005$), shelter ($P = 0.002$), or other areas of the pen ($P = 0.03$) and distance traveled ($P < 0.001$; Figure 3). Compared with calves with a CIS of 1 or 2, calves with a CIS of 3 spent less time at the feed bunk and more time in the shelter during the 24 hours prior to assignment of the CIS. Calves with a CIS of 3 also traveled significantly less distance during the 24 hours prior to assignment of the CIS, compared with the distance traveled by calves with a CIS of 1 or 2.

The challenge inoculation dose was not associated with the probability of a calf becoming clinically ill at least once during the study period, distance traveled by a calf, or amount of time spent by a calf at specific locations within the pen. Calves that were clinically ill (CIS > 1) at least once during the study had a significantly ($P = 0.01$) higher mean $\pm$ SD percentage (21.3 $\pm$ 23.7%) of consolidated lung tissue, compared with the percentage (1.5 $\pm$ 1.4%) of consolidated lung tissue in calves that were never identified as clinically ill. However, there were 2 calves that received the high-dose inoculation that had no lung consolidation; one had a CIS of 2 assigned 2 times, and the other had a CIS of 2 assigned 3 times.

Evaluation of the distance traveled by calves per day revealed a significant interaction between extent of consolidation in the lungs and day of the study. The distance traveled per day for calves with $\geq 10\%$ consolidated lung tissue did not differ from that for calves with
The amount of time that calves spent at the feed bunk was not associated with the extent of lung consolidation but was associated \( (P < 0.001) \) with day of the study. Evaluation of the time that calves spent at the waterer revealed no interaction between extent of lung consolidation and study day; however, the extent of lung consolidation and study day were each associated with the percentage of time that calves spent at the waterer. Calves with \( \geq 10\% \) consolidated lung tissue spent less time \( (7.2 \text{ min/d}; P = 0.05) \) at the waterer, compared with the time \( (8.6 \text{ min/d}; P = 0.05) \) spent at the waterer by calves with \(< 10\% \) consolidated lung tissue. Also, calves spent less time at the waterer on days 10 and 11 than on other days of the study.

### Discussion

In the study reported here, the disease challenge technique resulted in the development of clinical illness and lung lesions compatible with \( M\ bovis \) infection in most calves. The onset of clinical signs of disease (as determined by CIS) was \( 4 \) to \( 6 \) days after the challenge inoculation, and most calves developed signs of clinical illness, at least transiently, during the study period. There was large variation in the extent of lung consolidation among individual calves, but there was no significant difference in the mean percentage of lung consolidation between the inoculation dose groups. On the basis of measurements obtained by use of the remote location monitoring devices, changes in behavior were associated with calves that had moderate illness (CIS, 3). The percentage of lung consolidation \( (< \text{ or } \geq 10\%) \) in a calf was associated with CIS and the time that the calf spent at specific locations within the pen. This finding indicated the usefulness of these measurement techniques for discriminating between calves with moderate and mild illness caused by BRD.

The disease challenge technique used in the study reported here resulted in clinical signs of illness and lung consolidation that differed among calves, but the overall extent of lung consolidation was similar to findings of previous studies\(^{17,18} \) in which investigators used \( Mannheimia\ haemolytica \) as a challenge agent. Clinical signs of illness began \( 4 \) days after challenge inoculation, and although the incubation period has not been determined for \( M\ bovis \), the onset of illness in the present study was consistent with that of other studies.\(^{8,9} \) Not all calves became clinically ill after inoculation with \( M\ bovis \), and calves that were not assigned a CIS \( > 1 \) had a lower percentage of consolidated lung tissue, compared with the percentage of consolidated lung tissue in calves that were assigned a CIS \( > 1 \) at least once. Although the CIS system used was subjective and was not a perfect predictor of lung consolidation, none of the calves that maintained a CIS of 1 throughout the study had \( > 4\% \) consolidated lung tissue. Conversely, there were 2 calves with a CIS of 2 that had no lung consolidation. In the present study, repeated twice-daily observations of calf attitude by use of a CIS system were effective in the discrimination of calves with mild, moderate, or severe illness after challenge inoculation with \( M\ bovis \).

Calf behavior was continually monitored throughout the study by use of remote location monitoring devices that provided information on the amount of time that calves spent at each specific location of the pen.
The usefulness of the measurement of time spent by calves at specific locations within the pen was evaluated through comparison of CIS assigned in the morning and calf behavior during the 24-hour period prior to assignment of that CIS. Calves with a CIS of 1 or 2 did not significantly differ in patterns of behavior; however, calves with a CIS of 3 traveled less distance and spent less time at the feed bunk and more time in the shelter. These changes in calf behavior were expected because anorexia and lethargy are common indicators of clinical illness. A CIS of 3 was associated with moderate disease; therefore, objective measures of behavior may be useful for identifying moderate BRD in calves.

The distance traveled during a given study day depended on the extent of lung consolidation detected in each calf during necropsy. Knowledge of the extent of lung consolidation at the end of the study allowed evaluation of changes in behavior associated with lesion development. In the present study, calves were categorized into 2 groups: one group included calves with minimal (<10% consolidated lung tissue) disease, and the other included calves with moderate (≥10% consolidated lung tissue) disease. Thus, the study results should be interpreted with care because behavior may change with different gradations of lung consolidation. Calves with ≥10% consolidated lung tissue travelled less distance daily after day 4 of the study, the time that coincided with the onset of clinical signs. As the study progressed, the distance traveled daily by calves with ≥10% consolidated lung tissue continued to decrease, compared with the distance traveled daily by calves with <10% consolidated lung tissue. This finding indicated that calves with more severe disease were less active; this was similar to results of another study in which calves took fewer steps after induction of pneumonia in inoculation with M haemolytica. The decrease in distance traveled for calves with ≥10% consolidated lung tissue in the present study supports the hypothesis that sick cattle decrease unnecessary activities (eg, walking) to focus on maintaining primary needs.

The percentage of time that calves spent in the shelter or other locations in the pen was associated with study day as well as with the extent of lung consolidation. The fact that calf behavior differed by day was expected, and other researchers have found that morbid cattle spend less time at the feed bunk, compared with the amount of time spent at the feed bunk by healthy cattle. Although not all calves in the present study developed the same extent of lung consolidation, all were inoculated and the decreased time spent at the feed bunk may have represented both subclinical and clinical illness in the study calves.

In the present study, calves spent less time at the waterer on days 10 to 12 of the study, which coincided with the period during which there was an increased proportion of calves assigned a CIS of 3. Another study found that morbid calves had increased drinking activity 4 to 5 days after entry into a feedlot. Direct comparisons of findings of the present study with those of that study are not possible because in the present study, changes in calf drinking behavior were detected relative to inoculation with M bovis, whereas in the other study, changes in call drinking behavior were detected relative to call arrival at a feedlot. However, in both studies, there appeared to be an association between drinking behavior and clinical illness. Results should be interpreted cautiously because there was no control group in either study and external environmental factors, such as weather, could have influenced the results. Thus, changes in calf behavior after inoculation with M bovis warrant further research.

The present study confirmed that the M bovis pneumonia challenge technique used was effective in the induction of clinical illness and pathological changes in the lungs of inoculated calves. Calf behavior changed after M bovis inoculation and was detected by subjective CIS and objective measurements of call movement within the pen by use of a remote location monitoring device. Calves that were assigned a CIS >1 at any point in the study had a greater extent of lung consolidation, compared with the lung consolidation in calves assigned a CIS of 1. Results from the present study indicated that information obtained by the use of remote location monitoring devices was effective for the identification of calves with moderate BRD.

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