Prevalence and genotype of *Mycoplasma bovis* in beef cattle after arrival at a feedlot

Fernanda Castillo-Alcala, DVM, DVSc; Kenneth G. Bateman, DVM, MSc; Hugh Y. Cai, DVM, DVSc; Courtney R. Schott, BSc; Lois Parker, BSc; Mary Ellen Clark, BSc; Patricia McRaild, BSc; Rebecca M. McDowall; Robert A. Foster, DVM, PhD; Marie Archambault, DVM, PhD; Jeff L. Caswell, DVM, PhD

**Objective**—To determine the prevalence of *Mycoplasma bovis* infection in the lungs of cattle at various times after arrival at a feedlot, to measure the relationship between clinical disease status and the concentration and genotype of *M bovis* within the lungs, and to investigate changes in the genotype of *M bovis* over time.

**Sample**—Bronchoalveolar lavage fluid (BALF) from 328 healthy or pneumonic beef cattle and 20 *M bovis* isolates obtained from postmortem samples.

**Procedures**—The concentration of *M bovis* in BALF was determined via real-time PCR assays, and *M bovis* isolates from BALF were genotyped via amplified fragment length polymorphism (AFLP) analysis.

**Results**—Prevalence of *M bovis* in BALF was 1 of 60 (1.7%) at arrival to a feedlot and 26 of 36 (72.2%) and 36 of 42 (85.7%) at ≤15 days and 55 days after arrival, respectively. Neither the concentration nor the AFLP type of *M bovis* in BALF was correlated with clinical disease status. The *M bovis* AFLP type differed between early and later sampling periods in 14 of 17 cattle.

**Conclusions and Clinical Relevance**—The findings implied spread of *M bovis* among calves and suggested that host factors and copathogens may determine disease outcomes in infected calves. Chronic pulmonary infection with *M bovis* may represent a dynamic situation of bacterial clearance and reinfection with strains of different AFLP type, rather than continuous infection with a single clone. These findings impact our understanding of why cattle with chronic pneumonia and polyarthritis syndrome inadequately respond to antimicrobial treatment. (Am J Vet Res 2012;73:1932–1943)

*Mycoplasma bovis* is a major cause of pneumonia and death in beef cattle across North America, but a lack of understanding of how the disease develops has limited the ability of researchers and clinicians to develop effective control measures.1–3 Retrospective analysis of archival material has revealed that characteristic lesions were overlooked or attributed to other pathogens.1 However, there appears to be an increase in prevalence of disease attributable to this organism in North American feedlots,4,5 and the emergence of *M bovis* infection in Ireland following increased importation of cattle from other areas of Europe illustrates the contagious nature of the disease.5–7

Study of the pathogenesis and impact of *M bovis* in feedlot beef cattle is complicated by the high prevalence of infection in healthy feedlot cattle and the difficulty in clinically differentiating pneumonia attributable to infection with *M bovis* from other forms of bacterial pneumonia, unless concurrent polyarthritis is present.2 Thus, identifying *M bovis* in nasal swab specimens or BALF or identifying specific antibodies against *M bovis* in serum does not necessarily imply that *M bovis* is the cause of respiratory tract disease. Examination of lung tissue from affected animals is considered diagnostic.

**ABBREVIATIONS**

| **AFLP** | Amplified fragment length polymorphism |
| **BALF** | Bronchoalveolar lavage fluid |
| **PFGE** | Pulsed-field gel electrophoresis |

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From the Departments of Pathobiology (Castillo-Alcala, Schott, Clark, Foster, Caswell) and Population Medicine (Bateman), and the Animal Health Laboratory (Cai, Parker, McRaild, McDowall), Ontario Veterinary College, University of Guelph, Guelph, N1G 2W1 ON, Canada; and the Département de Pathologie et Microbiologie, Faculté de Medicine Vétérinaire, Université de Montréal, St Hyacinthe, J2S 2M2 QC, Canada (Archambault). Dr. Castillo-Alcala’s present address is Department of Pathobiology, School of Veterinary Medicine, Ross University, St Kitts, West Indies. Supported by the Ontario Cattlemen’s Association, the Beef Cattle Research Council of Canada, and the Natural Sciences and Engineering Research Council of Canada (CRDPJ 356983–07). Presented in part as a poster presentation at the International Conference on Bovine Mycoplasmosis, Saskatoon, SK, Canada, July 2009, and the Conference for Research Workers in Animal Disease, Chicago, December 2010. The authors thank Bob Bechtel, Patrick Boerlin, and Shu Chen for technical assistance. Address correspondence to Dr. Caswell (jcaswell@uoguelph.ca).
cally useful, providing *M. bovis* antigen can be localized to the characteristic lesions of caseonecrotic bronchopneumonia. This lesion is characterized by a cranioventral distribution, similar to that for other forms of bronchopneumonia, but is unique because of the multiple coalescing round friable foci of caseous necrosis within the consolidated areas. The importance of *M. bovis* as a cause of other forms of bronchopneumonia in feedlot cattle remains uncertain, but its role in caseonecrotic bronchopneumonia is compelling.

Two hypotheses to explain the aforementioned findings are that certain strains of *M. bovis* are more virulent than other strains or that other host factors (eg, presence of pneumonia caused by *Mannheimia haemolytica* or other pathogens) allow *M. bovis* to colonize the lungs and perpetuate the disease. The study reported here was conducted to address these hypotheses by investigating whether the genotype and concentration of *M. bovis* in the lungs are factors that determine disease outcome. We also investigated the prevalence of pulmonary infection with *M. bovis* at various times after arrival of cattle in a feedlot, the spread of *M. bovis* genotypes within the feedlot cattle, and changes in *M. bovis* genotype in individual cattle over time.

**Materials and Methods**

**Animals**—The study was conducted in a single commercial feedlot located in Baden, ON, Canada. Six-month-old mixed-breed heifer calves (n = 328) with a mean ± SEM body weight of 249 ± 1 kg (range, 179 to 328 kg) were obtained from 4 farms located in western Canada (North Battleford, SK, and Saskatoon, SK). The calves were commingled, allocated into 2 shipment groups, and transported to the feedlot in November. The first shipment of 219 calves was processed 48 to 72 hours after arrival at the feedlot. The second shipment of 109 calves arrived in the feedlot 5 days after the first shipment and was processed 48 hours after arrival.

During processing, each calf was weighed, received a single dose of long-acting oxytetracycline (20 mg/kg, IV), and was vaccinated with a modified-live virus vaccine against bovine herpesvirus-1, bovine viral diarrhea virus types 1 and 2, bovine parainfluenza virus-3, and bovine respiratory syncytial virus. All calves received ivermectin (500 µg/kg as a pour-on treatment). For the duration of the study, calves were housed in pens consisting of 100 to 150 calves; calves were fed barley silage, barley, and a supplement-type product (7.2 kg of dry matter/d) and provided ad libitum access to water. Owner consent was obtained for inclusion of the calves in the study. The study was approved by the University of Guelph Animal Care Committee.

**Study design**—At the time of arrival at the feedlot, 60 healthy calves (group 1) were selected (40 from the first shipment and 20 from the second shipment, which represented approximately every fifth calf). Body weight was recorded, and samples of blood and BALF were collected. These calves were managed under the same conditions as the remainder of the cattle in the feedlot for the duration of the study. No samples were collected at this time from the remaining 268 healthy calves.

The feedlot operator monitored all 328 calves daily for clinical signs of respiratory tract disease. Acute respiratory tract disease was diagnosed when a calf was lethargic, was reluctant to approach the feeder while feed was being provided, appeared to lack rumen fill, had labored respiration or an increased respiratory rate, and had no evidence that the illness was attributable to nonrespiratory disease. In addition to these clinical signs, rectal temperature ≥ 40°C was required for the diagnosis of acute respiratory tract disease. Once a calf was identified with acute respiratory tract disease, a healthy control calf from the same pen was arbitrarily selected. Samples of blood and BALF were obtained from both of the calves (calves with acute respiratory tract disease [group 2A] and healthy control calves [group 2B]). Rectal temperature and body weight were recorded for the calves. Samples were obtained during the first 13 days after group 2A and 2B calves arrived at the feedlot (early sample collection period). Of the 60 calves of group 1, 10 became ill and 9 were used as healthy control calves. Of the 268 remaining calves, 33 became ill and 32 were used as healthy control calves. Thus, there were 43 samples obtained from group 2A calves and 41 samples obtained from group 2B calves.

After samples were collected, calves with respiratory tract disease received a single dose of tilmicosin (10 mg/kg, SC) and were identified with a red ear tag. No treatment was administered to the healthy control calves after sample collection. Immediately after sample collection and treatment, sick calves and healthy control calves were returned to their original pens. Calves that relapsed or that did not respond to initial treatment received a single dose of florfenicol (40 mg/kg, SC); however, no samples were collected at the time of florfenicol administration.

Fifty-five days after the first shipment of calves arrived at the feedlot, all calves of both shipments were weighed and examined and samples were collected from selected calves (late sample collection period). No calves had clinical signs specifically attributable to chronic respiratory tract disease (eg, chronic cough, respiratory distress, or arthritis). Calves that may have had chronic pneumonia were selected via the following criteria: calves that had the lowest weight gains of the whole group (group 3A [n = 10 calves]), calves from which samples were obtained at the time of arrival and during acute respiratory tract disease and that had the lowest weight gains within this cohort (group 3B [10]), calves from which samples were not collected at the time of arrival but from which samples were collected during acute respiratory tract disease and that had the lowest weight gains within this cohort (group 3C [11]), and healthy calves from which samples had been collected at the time of arrival and concurrently with calves that had acute respiratory tract disease (group 3D [11]). Samples were collected from all 42 selected calves during a 2-day period. Samples of blood and BALF were collected from each calf, and the rectal temperature and body weight were recorded.

**Sample collection and analysis**—Blood samples (21 mL/sample) were collected via jugular venipuncture and placed into 2 types of tubes. Tubes without anticoagulant were kept warm until the blood clotted and
then were chilled at environmental temperature; serum was harvested approximately 2 to 4 hours after sample collection. Tubes containing EDTA were kept chilled on wet ice and submitted to the Animal Health Laboratory at the University of Guelph for a CBC.

Bronchoalveolar lavage was performed with a sterilized flexible bronchoscope, as previously described. Approximately 40% to 60% of the instilled lavage fluid was retrieved. The bronchoscope was washed with PBS solution and 70% alcohol between uses in subsequent calves. The bacteria isolated from BALF was not related to the sampling sequence in another study; we had similar results for the present study (data not shown), which suggested that there was no between-calf contamination of BALF. The BALF samples were kept chilled on wet ice until they were processed approximately 2 to 4 hours after collection. Total cell counts from whole BALF were obtained with an automated cell counter. Cytocentrifuge slides were prepared in a routine manner, and differential counts were obtained.

**Bacterial and mycoplasmal culture**—Two 1.5-mL aliquots of whole BALF were collected from the bottom of the cloudiest aliquot (which was expected to contain the most cell clumps and flocculent debris) and thus maximize the likelihood that it would yield an isolate of *M. bovis* and submitted to the Animal Health Laboratory at the University of Guelph for bacterial and mycoplasmal culture. This sample was sent for culture on the day of sample collection after being chilled on ice for approximately 2 to 4 hours after collection for the calves from which samples were obtained at the time of arrival at the feedlot (group 1 [n = 60]) and for samples obtained from calves during the late sample collection period (group 3A, 3B, 3C, and 3D [42]). The BALF collected during the early sample collection period was cultured on the same day of collection if the calf was part of the group from which samples had been collected at the time of arrival; otherwise, the sample was frozen at −80°C and cultured at a later date. Cultures were performed for 36 BALF samples from 34 calves in the early sample collection period, which represented 22 of 43 samples from calves with acute respiratory tract disease (group 2A) and 14 of 41 samples from healthy control calves (group 2B). These included samples from all 19 calves from which samples were collected both at the time of arrival (group 1) and during the early sample collection period (group 2A or 2B) as well as a random subset of 16 calves from which samples had not been previously collected. Samples were collected from 2 of these calves both as healthy control calves (group 2B) and later as calves with respiratory tract disease (group 2A). We did not attempt to isolate *M. bovis* from the nasal cavity because there appears to be an inconsistent relationship between nasal and pulmonary infection attributable to *M. bovis* and other bacterial pathogens and because we were primarily interested in the relationship between pulmonary infection and clinical evidence of pneumonia. The remaining aliquots of BALF collected from each calf were pooled, filtered through sterile gauze, frozen at −80°C, and used for real-time PCR assays and additional mycoplasmal cultures.

For bacterial isolation, BALF was inoculated directly onto 5% Columbia sheep blood agar and MacConkey agar. Blood agar plates were incubated at 35°C in 6% CO₂ for 48 hours; MacConkey agar plates were incubated at 35°C in room air for 48 hours. Plates were examined at 24 and 48 hours after inoculation. Isolates recovered were subcultured and further identified via colony morphology, presence of hemolysis, Gram stain characteristics, and results of biochemical tests.

For isolation of mycoplasmas, BALF was cultured on modified Hayflick medium containing 15% horse serum. Approximately 20 μL of BALF was plated directly onto the agar and incubated at 35°C in 5% CO₂. Plates were examined via a 40X stereomicroscope every 48 hours for *Mycoplasma* growth. Colonies were identified to the species level via an indirect fluorescent antibody test. Serologic testing for *M. bovis* was performed with the passive hemagglutination test; a titer ≥ 1:80 was considered a positive result.

A total of 92 BALF samples were retrospectively selected for *M. bovis* real-time PCR assay to analyze an equivalent number of repeated samples from the same calves at the various time points. Whereas aliquots of whole BALF were collected for mycoplasmal culture from the bottom of the cloudiest aliquot in an attempt to maximize the likelihood of obtaining an *M. bovis* isolate, aliquots of pooled and gauze-filtered BALF were tested via real-time PCR assay to obtain a more representative estimate of the *M. bovis* concentration in lung fluid. The DNA was extracted from BALF samples with a DNA extraction kit and an extraction instrument, which were used in accordance with the manufacturer’s instructions. *Mycoplasma* bovis Donetta spiked in BALF was used as a positive extraction-control sample, and no inhibition of the PCR assay by BALF was detected. The primers, probes, and PCR conditions were those described elsewhere. The estimated detection limit for the real-time PCR assay, determined on the basis of serial dilution of extracted DNA, was 770 CFUs/mL.

**AFLP analysis**—All of the 62 *M. bovis* isolates derived from BALF samples obtained from 43 calves were analyzed via AFLP typing. Additionally, isolates were selected from a previous necropsy-based study from 9 cattle that had gross and histologic lesions of caseonecrotic bronchopneumonia typical of that caused by *M. bovis*, 9 cattle that died of causes unrelated to respiratory tract disease (rumen tympany, myocarditis, trauma, or lesions suggestive of septicemia) and had no bronchopneumonia or had bronchopneumonia lesions that affected < 10% of the lungs, 1 animal with acute fibrinous bronchopneumonia, and 1 animal with chronic supplicative bronchopneumonia without caseous necrosis. All isolates were purified during 3 passages with a single-colony technique. All purified *M. bovis* isolates were grown in Hayflick broth with 15% horse serum and incubated aerobiologically at 35°C for 48 hours before DNA extraction for use in AFLP typing. *Mycoplasma* cells were collected from 5 mL of culture material by centrifugation at 13,000 × g for 10 minutes. Genomic DNA was extracted from the cell pellet in accordance with the manufacturer’s protocol for gram-positive bacteria.

Procedures used for AFLP analysis were as described elsewhere. Briefly, genomic DNA was digested with the restriction enzymes BglII and MfeI, and...
the resulting DNA fragments were ligated to specific adapters. The BglII-Mfj fragments tagged with specific adapters were then amplified via PCR techniques that involved the use of BglII-0 (labeled with 6-carboxyfluorescein) and Mfj-0 primers. Sequences of the adapters and primers and the PCR conditions were as described elsewhere. Amplified DNA fragments were separated on an analyzer. An internal size standard was used for each sample. Fragment data were analyzed for size and intensity normalization by use of software. All electropherograms were visually inspected to ensure data quality. Fragments in the range from 60 to 600 bp were included in the analysis. Further data analysis for determining the relationship of various samples was performed with commercial software. Files generated via the first software program were inputted into the second software program, and the fragments were compared via a band-matching function. Similarities among the profiles of each sample were calculated via a binary dice coefficient. A dendrogram was constructed via the unweighted pair group method by use of arithmetic averages. Cluster grouping was based on 95% similarity of the profiles.

Additional AFLP analyses were conducted on M. bovis isolates obtained from 3 calves. These calves were selected because, for each calf, the AFLP type of the M. bovis isolate from the early sample collection period (5, 7, and 8 days after arrival) differed from that in the late sample collection period (55 days after arrival). For these calves, mycoplasmal cultures were prepared from frozen BALF collected during the early and late sample collection periods, then 24 additional M. bovis isolates (4 isolates/calf/time point) were randomly selected from the culture plates. Each isolate was purified via 3 passages in vitro, and the AFLP types were determined as described previously.

Finally, the effect of in vitro passage on AFLP results was investigated. The same aforementioned 3 isolates from the early sample collection period were passaged every 2 days by transferring several colonies to a fresh agar plate. After 56 days, the AFLP types of the original and the passaged isolates were determined as described previously.

Statistical analysis—Data were analyzed for normality via the D’Agostino and Pearson omnibus normality test. Data that did not pass the normality test were logarithmically transformed and reanalyzed for a Gaussian distribution. Normally distributed data were analyzed via an unpaired 2-tailed Student t test (body weights) or a 1-way ANOVA followed by the Tukey multiple comparisons test (total cell counts in BALF and lymphocyte counts in blood). Data that were not normally distributed (rectal temperature, differential cell counts in BALF, lymphocyte-to-neutrophil ratio, neutrophil counts, and M. bovis concentration in BALF) were analyzed with the Mann-Whitney U test or Kruskal-Wallis test with Dunn multiple comparisons as the post hoc test. Statistical software was used for the analysis. Values of P < 0.05 were considered significant. Descriptive statistics were used for data from bacterial and mycoplasmal cultures, M. bovis serologic testing, and AFLP analysis. Data were expressed as mean ± SEM unless otherwise indicated.

Results

Animals—A total of 186 samples were collected from 130 of the 328 calves in the study population (Table 1). Calves that developed acute respiratory tract disease (group 2A) were identified at a mean ± SD of 6.1 ± 2.3 days after arrival at the feedlot. At 55 days after arrival, when all calves appeared healthy, mean ± SEM body weight of the 43 calves that had developed respiratory tract disease (group 2A) was significantly (P = 0.04) lower than that of the other calves in the feedlot (295.3 ± 4.6 kg vs 305.8 ± 1.8 kg).

Mean ± SEM rectal temperature of calves with acute respiratory tract disease (group 2A; 40.8 ± 0.1°C) was significantly (P < 0.001; Mann-Whitney U test) higher, compared with that of the healthy control calves (group 2B; 39.7 ± 0.1°C). Significant differences were not detected between calves with acute respiratory tract disease and healthy control calves (groups 2A and 2B) with respect to total cell counts in BALF, percentages of neutrophils or macrophages in BALF, or neutrophil or lymphocyte counts in blood. Total cell counts in BALF of calves in group 1 were significantly lower than the total cell counts of the calves in the other groups. The mean percentage of neutrophils in BALF during the early sample collection period was significantly (P < 0.001) high-

<table>
<thead>
<tr>
<th>Sample collection</th>
<th>Group</th>
<th>No. of samples</th>
<th>Disease status</th>
</tr>
</thead>
<tbody>
<tr>
<td>At arrival</td>
<td>1</td>
<td>60</td>
<td>Healthy</td>
</tr>
<tr>
<td>Early sample</td>
<td>2A</td>
<td>43</td>
<td>Acute respiratory tract disease</td>
</tr>
<tr>
<td>collection period</td>
<td>2B</td>
<td>41</td>
<td>Healthy</td>
</tr>
<tr>
<td>Late sample</td>
<td>3A</td>
<td>10</td>
<td>Healthy; lowest weight gain of entire group</td>
</tr>
<tr>
<td>collection period</td>
<td>3B</td>
<td>10</td>
<td>Healthy; lowest weight gain of calves from groups 1 and 2A from which samples were collected</td>
</tr>
<tr>
<td></td>
<td>3C</td>
<td>11</td>
<td>Healthy; lowest weight gain of calves from group 2A from which samples were collected</td>
</tr>
<tr>
<td></td>
<td>3D</td>
<td>11</td>
<td>Healthy; typical weight gain of calves from group 1 or 2B from which samples were collected</td>
</tr>
<tr>
<td>Total</td>
<td>NA</td>
<td>186</td>
<td>NA</td>
</tr>
</tbody>
</table>

*At arrival samples were collected during processing of calves performed within 48 or 72 hours after arrival at the feedlot. The early collection period was ≤ 15 days after arrival at the feedlot, and the late collection period was 55 days after arrival at the feedlot. NA = Not applicable.
Bacterial and mycoplasmal culture results for 138 BALF samples collected from beef calves at various times after arrival at a feedlot.*

Table 2—Bacterial and mycoplasmal culture results for 138 BALF samples collected from beef calves at various times after arrival at a feedlot.

Table 3—Relationship between serum antibody titer against M. bovis at the time of arrival at a feedlot and the prevalence of clinical respiratory tract disease during the subsequent 15 days of the early sample collection period for the calves of group 1.

Figure 1—Concentration of Mycoplasma bovis (as determined via real-time PCR assay) in BALF samples obtained from calves at the time of arrival at a feedlot (group 1 [n = 60]), calves with acute respiratory tract disease (group 2A [43]), or healthy control calves (group 2B [41]) during the early sample collection period (≤15 days after arrival) or obtained from calves during the late sample collection period (65 days after arrival; group 3 [42]). Mycoplasma bovis was not cultured from all BALF samples. Values < 103 CFUs/mL were less than the limit of detection of the assay. Notice that the 4 samples with the highest concentration of M. bovis were from calves with acute respiratory tract disease; however, there was no significant (P > 0.05) difference between groups 2A and 2B.

Mycoplasma bovis serologic testing—The overall seroprevalence for M. bovis was 20 of 60 (33.3%) for group 1, 25 of 34 (73.5%) for groups 2A and 2B, and 42 of 42 (100%) for groups 3A, 3B, 3C, and 3D. The seroprevalence for M. bovis in calves with acute respiratory tract disease (group 2A) and in healthy control calves (group 2B) was 16 of 20 (80.0%) and 9 of 14 (64.3%), respectively, and these values did not differ significantly (P = 0.43; Fisher exact test). The number of calves with titers against M. bovis ≥ 1:1,280 was 10 of 60 (16.7%) for group 1, 16 of 34 (47.1%) for groups 2A and 2B, and 11 of 42 (26.2%) for groups 3A, 3B, 3C, and 3D. Titters against M. bovis at the time of arrival did not differ significantly (P = 0.89; χ² test) between calves that subsequently developed disease and those that remained healthy during the early sample collection period (Table 3).

Real-time PCR assay for M. bovis—A total of 92 BALF samples were tested with the real-time PCR assay in parallel with culture. Of the 59 M. bovis culture-pos-
itive BALF samples, 32 (54.2%) had positive results for the real-time PCR assay. All of the 33 *M* bovis culture-negative BALF samples had negative results for the real-time PCR assay.

*Mycoplasma* bovis concentration in BALF ranged from 1.8 x 10^9 to 1.03 x 10^9 CFUs/mL. Although the highest *M* bovis concentrations were found in BALF obtained from calves with acute respiratory tract disease (group 2A), there were no significant (P = 0.14) differences in *M* bovis concentrations among groups (Figure 1).

A large number of BALF samples (27/59) were culture-positive for *M* bovis and were not detected via the real-time PCR assay. Therefore, 7 of these samples were cultured again and retested with the real-time PCR assay. Because only frozen, gauze-filtered BALF samples were available, both culture and real-time PCR assays were conducted on these samples (whereas the original cultures were performed on sediment-enriched aliquots of BALF). Only 3 of 7 samples were culture-positive for *M* bovis, and none were detected on real-time PCR assay. However, 2 of 7 samples had clearly evident melting curves for the real-time PCR assay, with a melting peak of 63°C that was indicative of the presence of *M* bovis DNA; however, the samples did not have a positive amplification curve and thus were considered to be below the limit of detection.

### AFLP analysis

A total of 62 *M* bovis isolates from the present study and 20 isolates from the necropsy-based study were typed with AFLP techniques (Figure 2; Table 4). Amplification of BglII-MfeI templates with the AFLP primers yielded a mean of 65 fragments (range, 40 to 171 fragments) in the size range of 50 to 600 bp. Four isolates had lower homology (< 73%) when compared with the rest of the group and were designated as types D, E, and F. The remaining isolates formed 3 clusters with > 80% homology and were designated as types A, B, and C. These broader clusters were divided into 13 subgroups (eg, A1, A2, and A3) on the basis of the observed grouping of the data and > 90% homology within each subgroup.

*Mycoplasma* bovis isolates obtained from group 1 (at arrival) and groups 2A and 2B (early sample collection period [≤ 15 days after arrival]) had limited diversity, with only types A2, B2, and C2 represented (Table 4). In contrast, isolates obtained from groups 3A, 3B, 3C, and 3D (late collection period [55 days after arrival]) represented 8 AFLP types, and 23 of 36 (63.9%) isolates had AFLP types not represented in the early isolates. Isolates from the early sample collection period, there were nearly identical numbers of each *M* bovis AFLP type obtained from calves with acute respiratory tract disease (group 2A) and healthy control calves (group 2B). Similarly, we did not detect differences in AFLP types among groups 3A, 3B, 3C, and 3D (data not shown), which indicated that there were no differences between calves that never had respiratory tract disease and calves that previously had acute respiratory tract disease or that had the lowest weight gains.

The AFLP types were determined for an additional 20 *M* bovis isolates from a necropsy-based study; those isolates were cultured from lungs with well-characterized pathological and bacteriologic findings. None of the AFLP types represented in the early samples from the present study (A2, B2, and C2) were represented in the isolates from the necropsy-based study. In contrast, isolates from a necropsy-based study adhered to the format of PM-N1, where PM indicates an isolate obtained during postmortem examination; N, P, or O indicate calves with lungs that had no or minimal pneumonia, caseous necrotic bronchopneumonia, or other forms of pneumonia, respectively; and 1 refers to the isolate number within each group.
Table 4—Results of AFLP typing of 82 isolates of *M. bovis* cultured from feedlot cattle on the basis of study group.*

<table>
<thead>
<tr>
<th>AFLP type</th>
<th>Group 1</th>
<th>Group 2A</th>
<th>Group 2B</th>
<th>Group 3A–3D</th>
<th>No pneumonia</th>
<th>Caseonecrotic pneumonia</th>
<th>Other pneumonia</th>
<th>Total</th>
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<tbody>
<tr>
<td>A1</td>
<td>—</td>
<td>—</td>
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<td>—</td>
<td>3</td>
<td>7</td>
<td>1</td>
<td>17</td>
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<td>A2</td>
<td>1</td>
<td>6</td>
<td>6</td>
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<td>—</td>
<td>—</td>
<td>—</td>
<td>14</td>
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<tr>
<td>A3</td>
<td>—</td>
<td>—</td>
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<td>3</td>
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<td>—</td>
<td>3</td>
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<td>A4</td>
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<td>—</td>
<td>1</td>
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<td>B1</td>
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<td>8</td>
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<td>C3</td>
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<td>—</td>
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<td>—</td>
<td>—</td>
<td>—</td>
<td>1</td>
<td>—</td>
<td>1</td>
</tr>
<tr>
<td>F</td>
<td>—</td>
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<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Total</td>
<td>1</td>
<td>12</td>
<td>13</td>
<td>36</td>
<td>9</td>
<td>9</td>
<td>2</td>
<td>82</td>
</tr>
</tbody>
</table>

Data represent the number of isolates of each AFLP type for the 82 isolates tested.
— Not isolated.

See Table 1 for remainder of key.

Table 5—Results for AFLP typing of *M. bovis* isolated from the same calf at different sample collection times.*

<table>
<thead>
<tr>
<th>Early sample collection period</th>
<th>Late sample collection period</th>
<th>Interval (No. of days)†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calf No.</td>
<td>Group AFLP type</td>
<td>Group AFLP type</td>
</tr>
<tr>
<td>21</td>
<td>2B, 2A C2</td>
<td>3B B1</td>
</tr>
<tr>
<td>20§</td>
<td>2A A2</td>
<td>3B B1</td>
</tr>
<tr>
<td>23</td>
<td>2B A2</td>
<td>3B B2</td>
</tr>
<tr>
<td>27</td>
<td>2A A2</td>
<td>3B B2</td>
</tr>
<tr>
<td>28</td>
<td>2B B2</td>
<td>3D B1</td>
</tr>
<tr>
<td>32</td>
<td>2A A2</td>
<td>3B B2</td>
</tr>
<tr>
<td>36</td>
<td>2B B2</td>
<td>3B B2</td>
</tr>
<tr>
<td>39§</td>
<td>2A C2</td>
<td>3B A2</td>
</tr>
<tr>
<td>51</td>
<td>2B A2</td>
<td>3D B1</td>
</tr>
<tr>
<td>55</td>
<td>2B A2</td>
<td>3D B2</td>
</tr>
<tr>
<td>59</td>
<td>2B A2</td>
<td>3D B1</td>
</tr>
<tr>
<td>74§</td>
<td>2B, 2A B2</td>
<td>3C B1</td>
</tr>
<tr>
<td>93</td>
<td>2B B2</td>
<td>3D A1</td>
</tr>
<tr>
<td>99§</td>
<td>2A B2</td>
<td>3C B2</td>
</tr>
<tr>
<td>116</td>
<td>2A B2</td>
<td>3C F</td>
</tr>
<tr>
<td>117</td>
<td>2A B2</td>
<td>3C B2</td>
</tr>
<tr>
<td>118</td>
<td>2A B2</td>
<td>3C B2</td>
</tr>
</tbody>
</table>

†Number of days between collection of samples during the early and late sample collection periods. 15 samples were originally collected from this calf as a healthy control calf (group 2B) and subsequently were collected again 1 or 3 days later because the calf developed acute respiratory tract disease (group 2A). 3 Calf selected for further analysis of the change in genotype. Calf did not have a change in AFLP type of *M. bovis* isolated from the lungs.

See Table 1 for remainder of key.

chronologically similar *M. bovis* isolates (obtained within 1 or 3 days of each other) were of the same AFLP type, whereas the isolate during the late sample collection period was of a different type. For the 15 calves with 2 available isolates obtained 44 to 50 days apart, the AFLP types of the isolates were the same for 3 of 15 calves and differed for 12 of 15 calves (Table 5).

To investigate the reasons for these differences in AFLP types from samples of the same calves at different times, the AFLP types were determined for additional *M. bovis* isolates isolated from frozen BALF of 3 calves (4 isolates/calf at each of the early and late sample collection periods; total of 24 additional isolates). For 2 of these calves (Nos. 20 and 39), all isolates obtained from the same sample were of the same AFLP type, but AFLP type differed between sample collection periods. Specifically, there was high homology among the 4 isolates obtained during the early (mean ± SEM, 96.0 ± 3.1% and 98.1 ± 1.4%) or late (98.3 ± 0.9% and 99.1 ± 0.5%) sample collection periods, but low homology between isolates obtained during the early versus late sample collection periods (74.3 ± 1.9% vs 68.4 ± 4.2%). For the third calf (No. 74), the 4 isolates from the early sample collection period had high homology (mean ± SEM, 99.1 ± 0.5%), as did 3 of the 4 isolates from the late sample collection period (mean, 99.0 ± 0.6%). However, the fourth isolate from the late sample collection period had low homology when compared with the other isolates from the late sample collection period (mean ± SEM, 75.2 ± 1.6%) and high homology with the isolates obtained during the early sample collection period (mean, 98.6 ± 0.6%). Thus, for these 3 calves, isolates obtained during the early sample collection period were similar for a single calf but differed from those obtained during the late sample collection period, except for 1 isolate (Table 6).

To address the possibility that a clone of *M. bovis* might change its AFLP type over time, 3 of the aforementioned isolates were passaged in vitro for 56 days. Two of the original isolates were closely related (92.9% homology), whereas the third isolate was more distantly related (68.4% and 68.8% homology with the other
These patterns were maintained when comparing the 3 passages of the original isolates. Passaged isolates were homologous with the corresponding original isolate (homologies of 99.3%, 93.3%, and 99.5%).

Discussion

The objective of the present study was to determine temporal changes in the prevalence of \textit{M. bovis} infection in feedlot beef cattle and to investigate whether changes in \textit{M. bovis} concentration and genotype may influence the disease outcome in cattle with naturally occurring respiratory tract disease. For the conditions of the present study, AFLP type and \textit{M. bovis} concentration in BALF did not correlate with disease status in feedlot calves with naturally occurring respiratory tract disease. Furthermore, the data indicated a low prevalence of pulmonary infection with \textit{M. bovis} in cattle at arrival to the feedlot, which increased markedly in the subsequent weeks. An unexpected finding was that the AFLP type of \textit{M. bovis} within a chronically infected calf was not stable, and this chronic \textit{M. bovis} infection may represent clearance of one strain and reinfection with another strain or DNA recombination among different strains.

A single commercial feedlot operation located in Ontario was chosen as the site for the study, which was expected to reduce the chance that environmental factors were the basis for the observed differences in \textit{M. bovis} genetic variability and disease manifestation. A limitation of this approach was that this setting represented a unique epidemiological scenario. Nevertheless, this setting was typical of management practices in North American feedlot operations where incoming calves have been recently weaned, commingled in sale yards, transported to a feedlot, and treated metaphylactically with antimicrobials. In the present study, respiratory tract disease developed within the first 15 days after arrival at the feedlot, which is consistent with results for a number of studies conducted in feedlots with similar management.

In the present study, an experienced feedlot operator monitored the calves daily for clinical signs of respiratory tract disease (reluctance to approach the feeder, reduced alertness, thin appearance, labored breathing, and increased respiratory rate). These are subjective diagnostic criteria and may have led to variations when assigning calves to clinical disease categories over the course of the study as well as variation in classification of calves as diseased versus healthy between this study and other studies. However, the fact that all clinical assessments were performed by the same observer minimized the variation of the findings within the present study, and the fact that these were the same diagnostic criteria commonly used in commercial feedlots suggested that the findings may be comparable to those of the general population of North American feedlots.

In the early sample collection period, healthy control calves were defined on the basis of a lack of clinical signs. However, 13 of 41 (31.7%) of these control calves had an increased rectal temperature (up to 40.5°C), and the percentage of neutrophils in BALF was similar in healthy control calves and in calves with acute respiratory tract disease. This lack of a relationship between clinical disease status and leukocyte counts in BALF or blood has been described. Thus, it is likely that some calves selected as healthy control calves were in fact affected by subclinical inflammation of the respiratory tract.

### Table 6—Percentage homology in AFLP profiles between multiple isolates of \textit{M. bovis} cultured from samples obtained from each of 3 calves during the early and late collection periods.

<table>
<thead>
<tr>
<th>Calf No.</th>
<th>Isolate No.</th>
<th>Within early collection period</th>
<th>Within late collection period</th>
<th>Between early and late collection periods</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>2-E 3-E 4-E</td>
<td>2-L 3-L 4-L</td>
<td>1-L 2-L 3-L 4-L</td>
</tr>
<tr>
<td>20</td>
<td>1-E</td>
<td>99 95 91</td>
<td>98 98 99</td>
<td>98 98 99</td>
</tr>
<tr>
<td></td>
<td>2-E</td>
<td>— 98 95</td>
<td>— 100 98</td>
<td>71 73 73</td>
</tr>
<tr>
<td></td>
<td>3-E</td>
<td>95 — 99</td>
<td>98 — 98</td>
<td>74 73 74</td>
</tr>
<tr>
<td></td>
<td>Mean</td>
<td>96.0</td>
<td>98.3</td>
<td>74.3</td>
</tr>
<tr>
<td>39</td>
<td>1-E</td>
<td>99 97 96</td>
<td>100 98 99</td>
<td>98 98 99</td>
</tr>
<tr>
<td></td>
<td>2-E</td>
<td>— 99 98</td>
<td>99 99 99</td>
<td>98 99 73</td>
</tr>
<tr>
<td></td>
<td>3-E</td>
<td>97 — 99</td>
<td>99 — 100</td>
<td>71 71 75</td>
</tr>
<tr>
<td></td>
<td>Mean</td>
<td>98.1</td>
<td>99.1</td>
<td>88.4</td>
</tr>
<tr>
<td>74</td>
<td>1-E</td>
<td>100 98 99</td>
<td>76 100 98</td>
<td>98 99 75</td>
</tr>
<tr>
<td></td>
<td>2-E</td>
<td>— 99 100</td>
<td>76 73 73</td>
<td>77 99 76</td>
</tr>
<tr>
<td></td>
<td>3-E</td>
<td>98 — 99</td>
<td>100 — 99</td>
<td>79 98 78</td>
</tr>
<tr>
<td></td>
<td>Mean</td>
<td>99.1</td>
<td>87.1</td>
<td>81.9</td>
</tr>
<tr>
<td></td>
<td>Mean with 2-L excluded</td>
<td>99.1</td>
<td>99.0</td>
<td>76.4</td>
</tr>
</tbody>
</table>
We did not define disease status on the basis of laboratory findings, and doing so would have increased the apparent prevalence of respiratory tract disease, depending on the intensiveness of the laboratory assessment. However, the objective of the study was not to identify all calves with any degree of pulmonary inflammation but instead to identify those calves with respiratory tract disease of sufficient clinical severity that antimicrobial treatment would be necessary. Although the healthy control calves had some evidence of respiratory tract inflammation, the lack of any deaths despite the lack of antimicrobial treatment implied that these calves were classified correctly.

One objective of the present study was to assess the prevalence of *M. bovis* infection in the lungs of calves at the time of arrival at the feedlot and to determine changes in prevalence over time. The prevalence for *M. bovis* at the time of arrival at the feedlot as determined via culture and serologic testing was low but subsequently increased during the following weeks. These findings concur with serologic studies in which seroprevalence was low at the time of arrival and increased during the first few weeks after arrival. In the present study, the prevalence for *M. bovis* determined by serologic testing was slightly higher than the prevalence determined by culture, but both prevalences followed a similar increasing pattern over time. These findings suggested that infected cattle may spread the organism to other cattle when commingled in sale barns, during shipping, and after arrival at feedlots.

We found no difference in the prevalence of *M. bovis* infection or seroprevalence in calves with respiratory tract disease and healthy control calves from which samples were collected in parallel during the early sample collection period. These findings are consistent with those reported in other studies. During the late sample collection period, all calves were seropositive for *M. bovis*, and most had positive culture results, which indicated prior exposure as well as active pulmonary infection. These data may provide useful epidemiological information because reported prevalences of *M. bovis* have been based primarily on postmortem results.

It is also necessary to consider that pathogens other than *M. bovis* may have contributed to respiratory tract disease in the population of calves. Most BALF samples yielded mixed bacterial cultures with other common respiratory tract pathogens such as *M. haemolytica*, *Pasteurella* spp, and *H. somni*. At the end of the present study, *M. haemolytica* was not isolated from any of the calves. Similarly, *Pasteurella* spp and *H. somni* were commonly isolated from BALF of healthy calves and from calves with respiratory tract disease. These Pasteurellaceae were presumably contributing to the respiratory tract disease in this group of calves, which is consistent with the observation that in cattle, most or all pneumonias attributable to infection with *M. bovis* represent mixed infections. The culture prevalence of *H. somni* increased considerably at the end of the study, and as mentioned previously, no clinical signs of respiratory tract disease were evident at that time. Although the culture prevalence of *M. bovis* during the late sample collection period was high, it was not possible to definitively determine the role of this pathogen in the respiratory tract disease of these calves.

Attempts have been made to decipher the mechanisms of pathogenicity and virulence factors of *M. bovis*. However, despite data indicating a high prevalence of *M. bovis* infection in the lungs of diseased and healthy feedlot cattle, there is little information on the factors that determine whether pulmonary infection with *M. bovis* leads to clinical disease. The determining factors may include the number of *M. bovis* organisms in the lungs, differences in virulence between strains of *M. bovis*, concurrent infection with bovine viral diarrhea virus or other viruses, or the presence of preexisting bacterial bronchopneumonia. The concentration of *M. bovis* in BALF samples was highly variable, but no differences in concentration were found between calves with respiratory tract disease and healthy control calves in the early sample collection period. Similarly, there was no difference in the *M. bovis* concentration in BALF when samples collected during the early and late sample collection periods were compared. Although bacterial numbers in BALF may not perfectly mirror those in lung tissue because BALF samples are not easily obtained from consolidated areas of pneumonic lungs, findings of the present study implied that neither the presence nor the concentration of *M. bovis* in the lungs determined which infected calves would develop clinical disease.

*Mycoplasma bovis* was detected less frequently with the real-time PCR assay than with culture. This may indicate a lower sensitivity of the PCR assay but may also have reflected differences in the samples analyzed. Mycoplasmal and bacterial culture was performed on BALF samples containing particulates and sediment to maximize the likelihood of obtaining an isolate of *M. bovis*, whereas real-time PCR assay was performed on gauze-filtered BALF. Retesting of samples revealed that *M. bovis* was isolated from only 3 of 7 filtered BALF samples that had negative results for real-time PCR assay but for which the corresponding sediment-enriched BALF samples were *M. bovis* culture-positive. These findings indicated that the higher prevalence of *M. bovis* infection determined on the basis of culture versus real-time PCR assay may have resulted from a lower sensitivity of the PCR assay and the fact that sediment-enriched BALF was used for culture. Regardless, the discrepancy between culture and real-time PCR assay was not expected to affect the major objective of the study because the purpose for using the culture was to maximize the likelihood of obtaining an isolate of *M. bovis* for genotyping, whereas the purpose for using the real-time PCR assay was to estimate the concentration of *M. bovis* in the lung epithelial lining fluid obtained in the BALF samples.

A major objective was to determine whether variation in strain genotype may be associated with disease severity in calves with *M. bovis* infections. We used AFLP typing because this technique has been validated for typing *Mycoplasma* spp, has good discriminatory power, and is reproducible. For the 62 *M. bovis* isolates analyzed, we obtained 13 distinct AFLP profiles that represented 3 main clusters. This genetic heterogeneity of *M. bovis* isolates was similar to that reported...
between AFLP profiles and the disease status of the calves from which the isolates were recovered for the early or late sample collection periods. In fact, AFLP types for calves with and without clinically apparent respiratory tract disease (groups 2A and 2B, respectively) were almost perfectly matched.

Results for the necropsy-based study extended these findings via analysis of M bovis isolates from 43 calves. The calves with lesions of caseonecrotic bronchopneumonia, which is considered to be characteristic of pneumonia attributable to M bovis infection, had a relatively limited diversity of M bovis AFLP profiles because all 9 calves were represented by AFLP types A1 and C1. These 2 AFLP types were also found in 3 of 9 and 2 of 9 calves that had no pneumonia; thus, there was no clear association between the M bovis AFLP profiles and the presence or absence of caseonecrotic bronchopneumonia.

The AFLP typing measures broad genetic differences between bacterial isolates and is not likely to detect differences with regard to the presence of specific virulence factors. Thus, we could not rule out the possibility that certain isolates of M bovis may have specific virulence factors that influence disease outcomes in infected calves, but the findings of the present study do not support the hypothesis that genetic differences between M bovis isolates are important factors that determine whether infection remains subclinical or leads to severe disease.

In addition to the considerable heterogeneity of AFLP profiles in this single feedlot, analysis of the results suggested that different M bovis AFLP profiles may predominate in different situations. In this study, only 3 of 13 AFLP profiles were identified in samples obtained at arrival and during the early sample collection period, whereas these 3 as well as 6 other AFLP profiles were identified in samples collected during the late sample collection period. Mycoplasmal cultures were attempted on 136 samples from 83 of 328 calves in the population, and AFLP typing was conducted on the resulting 62 isolates from 43 calves. Thus, it is possible that additional AFLP types may have been identified at these various time points if samples had been obtained from more calves. Nonetheless, these findings implied spread and changing patterns of different M bovis strains within the feedlot over the course of the feeding period. It is worthy of mention that there was no commonality in M bovis AFLP types from the necropsy-based study compared with those for live calves in the early sample collection period, whereas there was more overlap between AFLP types for the necropsy study and the live calves in the late sample collection period. The implications of this finding are unknown and may simply reflect the different populations of calves represented by these 2 groups of samples.

Mycoplasma bovis isolates were available from both the early and late sample collection periods for 17 calves, and the M bovis AFLP profiles differed between these times for 14 of 17 (82.4%) calves. This finding differs from that of a recent study in which isolates obtained 4 weeks after an episode of respiratory tract disease had no change in M bovis genotype as determined on the basis of PFGE analysis. This is consistent with reported discrepancies between AFLP analysis and PFGE analysis, perhaps because of the number of fragments analyzed (mean of 65 fragments in the present AFLP analysis, compared with 6 to 10 fragments in the PFGE analysis in other studies or differences in the restriction enzymes used (BglII and MfeI vs KpnI, MfiI, and Smal), although the discrepancy may also reflect differences in the interval between sample collections (44 to 50 days in the present study vs 3 weeks) and differences in the nature of the disease in Canadian beef feedlots and French veal feedlots.

We considered the following 3 possible explanations for the reason that different samples from the same calf had differences in M bovis AFLP profiles: recombination of the M bovis genome might change the AFLP profile over time, concurrent infection with multiple strains might artifactualy lead to identification of different AFLP profiles at the different times, or a calf might eliminate 1 strain of M bovis but become reinfected with another strain. Samples were collected from 2 calves that were healthy control calves; however, additional samples were collected 1 or 3 days later because both calves developed acute respiratory tract disease. The fact that the same AFLP type was identified at both sampling times for these 2 calves suggests low diversity of M bovis genotypes or a dominant clone at a given time, which therefore favors the latter possibility of a change in the strains that infected the calves at different time points. To further evaluate these possibilities, 4 M bovis isolates were prepared from 1 BALF sample obtained at each of 2 sample collection times for 3 calves. These results indicated limited diversity of M bovis AFLP profiles in a single BALF sample, which implied that a single BALF sample generally contains only 1 M bovis strain. However, for 1 BALF sample from the late sample collection period, 3 of 4 isolates were homologous and differed from isolates of the early sample collection period, whereas the fourth M bovis isolate was homologous with isolates of the early sample collection period. We interpreted this finding to represent a transition in which the early M bovis strain was not yet eliminated but a new strain was colonizing the lungs. Thus, although it is possible for > 1 M bovis strain to colonize a single BALF sample, analysis of the data indicated that this was not a frequent event. Although we cannot rule out the possibility that different areas of the lungs may be infected with different M bovis strains, the findings suggested that elimination of 1 M bovis strain and reinfection with a second strain is a more likely explanation.

Finally, we considered the possibility that the AFLP type might change over time in a single clone of M bovis, such as by genomic recombination, although this is not likely to induce the magnitude of genetic change required to substantially alter the AFLP profile. Three isolates of M bovis were passaged in vitro every 2 days for 56 days, which was comparable to the duration of the present study. The pairs of M bovis isolates and subcultures had 93.3% to 99.5% homology in AFLP profiles before and after passage, which indicated that there was no change in the AFLP type. Similar findings were re-
ported in a recent analysis of *M. bovis* conducted via re-
striction fragment length polymorphism analysis.30 Although genetic change may have been greater if a broth
culture of mixed *M. bovis* strains were used to allow genetic recombin- 

The findings of the present study may substantially affect the understanding of pneumonia attributable to
*M. bovis* infection in feedlot cattle. This disease is char-
acterized by its chronicity, as reflected by the name chronic pneumonia and polyarthritis syndrome.31,32 It has been presumed that this syndrome represents chronic infection with a single strain of *M. bovis*, which 

The present study indicated that the prevalence of pulmonary infection with *M. bovis* in this population of
feedlot beef cattle was low at the time of arrival to the feedlot but increased considerably during the first 15 days after arrival, and pulmonary infection with *M. bovis* was detected in most calves at 55 days after arrival. The presence of clinically diagnosed acute respiratory tract disease was not associated with the concentration of *M. bovis* in BALF (as determined via real-time PCR assay), with the *M. bovis* genotype (as determined by AFLP typing), or with the serum antibody titer against *M. bovis* at the time of arrival to the feedlot or at the time of clinical diagnosis. These results suggest that host factors such as the presence of pneumonia or infection with copathogens may determine the disease outcome in calves infected with *M. bovis*. The results indicate changes in the genotype of *M. bovis* isolated from calves at different time points that were not attributable to the presence of multiple bacterial genotypes in a single BALF sample or to the ability of a single bacterial clone to substantially alter its AFLP profile. These findings suggest that chronic pul- 

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