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Relative virulence in bison and cattle of bison-associated genotypes of *Mycoplasma bovis*

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ABSTRACT

Mycoplasma bovis, a frequent contributor to polymicrobial respiratory disease in cattle, has recently emerged as a major health problem in North American bison. Strong circumstantial evidence suggests it can be the sole pathogen causing disease manifestations in outbreaks of mortality in bison, but direct evidence is lacking. The goal of this study was to compare clinical signs and lesions in bison and cattle experimentally infected with field isolates of *M. bovis* recovered from bison. Bison (n = 7) and cattle (n = 6), seronegative for anti-*M. bovis* IgG, were exposed intranasally to *M. bovis* and necropsied 4–6 weeks later. Blood and nasal swabs were collected on day 0 (before exposure), day 11 and at necropsy. Samples of lung, lymph node, liver and spleen were also collected at necropsy. The only clinical sign observed was an elevation in the core body temperature of bison during the first few weeks post-exposure. Grossly visible lesions were apparent at necropsy in the lungs of five bison and the lymph node of one bison, while none were evident in cattle. Histologic evaluation revealed moderate to severe pulmonary lesions in four bison but none in cattle. *M. bovis* was recovered from tissues demonstrating gross lesions and from the lymph nodes of one additional bison and two cattle. All animals seroconverted by the time of necropsy. These data provide the first direct evidence that *M. bovis* can be a sole or primary cause of respiratory disease in healthy bison, although the isolates used were unable to cause disease in healthy cattle.

1. Introduction

Mycoplasma bovis is a cause of mastitis, arthritis, otitis media and keratoconjunctivitis in cattle and is frequently identified as a contributor to the polymicrobial syndrome known as bovine respiratory disease complex. First recognized as a disease agent more than 55 years ago in a cow with mastitis (Hale et al., 1962) the bacterium subsequently spread from the United States to nearly all countries of the world and today imposes a considerable economic and animal health burden on cattle production.

Until this century *M. bovis* was not considered to be an infectious disease threat to North American bison. In the early 2000's anecdotal reports began to accumulate describing outbreaks in bison of polyarthritis and pneumonia with clinical signs and lesions reminiscent of mycoplasmosis in cattle but with exceptionally high morbidity and mortality. An additional feature distinct from the norm in cattle is that *M. bovis* is the only infectious agent consistently recovered from cases that were investigated (United States Department of Agriculture, 2013). Healthy cattle exposed to *M. bovis* may become chronic carriers but rarely develop disease in the absence of co-infecting pathogens or other stressors. Over the next several years, mycoplasmosis in bison spread widely throughout North America and was reported in ranched and free-ranging bison of all ages, with case fatality rates as high as 45% (Bras et al., 2017; Dyer et al., 2008, 2013; Janardhan et al., 2010; Register et al., 2013b). Comparison of isolates using multilocus sequence typing (MLST) demonstrated that those associated with disease in bison possess unique sequence types (STs) as compared to isolates causing disease in cattle (Register et al., 2015). The consistent isolation of *M. bovis* from affected tissues of bison and infrequent isolation of other bacterial or viral pathogens has led to a consensus among

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Table 1
M. bovis isolates used in this study[a].

Isolate	ST[b]	Geographic origin	Year of isolation
NADC1	1	Montana	2011
NADC15	1	North Dakota	2007
NADC16	2	Manitoba, Canada	2012
NADC30	2	South Dakota	2012

[a] All isolates were obtained from lung lesions of bison with pneumonia.
[b] MLST sequence type (ST) based on the method of Register et al. (Register et al., 2015).

veterinary and industry experts that *M. bovis* is a primary disease agent in bison (United States Department of Agriculture, 2013). While the accumulated evidence for this conclusion is compelling, thus far there has been no attempt to demonstrate that infection of bison with *M. bovis* alone leads to disease. The primary goal of this study was to determine whether clinical signs and lesions arise in healthy bison following intranasal exposure to *M. bovis* isolates recovered from bison with mycoplasmosis. A secondary goal was to ascertain whether bison isolates that are genetically distinct from those associated with bovine disease have the capacity to act as primary disease agents in healthy cattle.

2. Materials and methods

2.1. M. bovis isolates and growth conditions

Details pertaining to isolates of *M. bovis* used in this study, all obtained from lung lesions of bison with pneumonia, are found in Table 1. Seed stocks were derived from axenic cultures expanded from single, well-isolated colonies and were identified as *M. bovis* based on characteristic colony morphology and a species-specific PCR (Clothier et al., 2010). Isolates prepared for use as inoculum were grown for 18–24 hr at 37 °C in an atmosphere of 5% CO_2 in PPLO broth (BD Diagnostic Systems) supplemented with 10 g/l of yeast extract (BD Diagnostic Systems) and 20% horse serum (Life Technologies).

2.2. Preparation of inoculum

Little is understood regarding the basis for virulence of *M. bovis*, particularly as it relates to bison. Because there was no information available to guide the choice of a single isolate most appropriate for use as a challenge strain, we chose to create a cocktail of four independent isolates representing different herds and geographic locations and with the STs most commonly associated with outbreaks in bison (Table 1; Register et al., 2015).

Bacteria from broth cultures of each isolate were pelleted by centrifugation at 15,000 *x g* for 20 min, resuspended in 1/100 of the original volume of PPLO broth and gently passed several times through a 25-gauge needle. Aliquots were snap-frozen in a dry ice/EtOH bath and stored at -80 °C until use. An aliquot of each culture was thawed, serially diluted and plated on PPLO agar, in triplicate, to quantitate the number of cfu/ml. On day 0 of the study, appropriate volumes of each isolate were thawed and combined to achieve a concentration of 5×10^{10} cfu/ml per isolate, collectively amounting to 2×10^{11} cfu/aml. The bacterial suspension was drawn into 5 ml syringes, one for each animal, and kept on ice until use.

As noted below, a Nasal MAD^{TM} Intranasal Mucosal Atomization Device was used to aerosolize the inoculum for delivery to the nasal passages. This device creates a mist of droplets roughly 30–100 µm in size. Although the manufacturer suggested the pore size of the membrane used to generate droplets within the device was unlikely to be small enough to trap significant numbers of a bacterium the size of *M. bovis*, which easily passes through pores as small as 450 nm, we were unable obtain more detailed information. An additional consideration was whether the pressure that accumulates within the device during aerosolization might be sufficient to disrupt the bacterial cell membrane, perhaps significantly reducing the number of viable organisms in the aerosolized suspension. To evaluate these concerns, a test batch of inoculum was prepared as described above and the number of cfu/ml in atomized samples retrieved following delivery into a sterile, 50 ml polypropylene tube was compared to the number found in samples of the same suspensions retrieved just prior to drawing them into a syringe. While some reduction in concentration was noted (an average of 1.54×10^{11} cfu/ml in atomized suspensions as compared to 1.98×10^{11} cfu/ml in the starting material), the Nasal MAD^{TM} device was judged to be acceptable for the purposes of this study and was used as recommended by the manufacturer.

2.3. Animals and experimental infection

The seven adult bison used in this study, four cows and three steers, were sourced from an Iowa herd with no history of respiratory or other disease problems, transported to the National Animal Disease Center (NADC) as calves and raised to adulthood. During this study they were housed in separate pens in a Biosafety Level 3-Agriculture (BSL-3-Ag) containment facility with the capability to safely and humanely handle bison. A calf born to one of the cows prior to the move into the BSL-3-Ag containment facility was co-housed with its mother for the duration of the investigation. The adult bison ranged from three to four years of age at the time the work reported here was carried out.

Cattle used in this study include three calves, two yearlings and one adult, all seronegative for *M. bovis*. The cattle were moved into the BSL-3-Ag containment facility one week prior to the initiation of the study, to a room separate from the bison, where they were housed in individual pens.

On the day of experimental infection (day 0), a rumen temperature probe programmed to detect and transmit core body temperature in real time (Advanced Telemetry Systems, Isanti, MN, USA) was orally dosed to the adult bison and all cattle. Rumen temperatures were continuously recorded for the duration of the study using a remote system, as previously described (Falkenberg et al., 2014). Following the collection of blood and nasal swabs (one swab from each nostril) from all animals, Nasal MAD™ Intranasal Mucosal Atomization Devices were attached to 5 ml syringes prepared with inoculum and the contents of a single syringe was delivered as a fine mist into the nasal cavity of each adult bison and all cattle, with 2.5 ml delivered to each nostril. The bison calf was not experimentally infected but was co-housed in direct contact with the experimentally infected mother. Animals were monitored twice daily for clinical signs of disease. Additional blood samples and nasal swabs were obtained from all animals on day 11 post-infection (PI) and immediately prior to euthanasia. Tissue samples from the lung, tracheobronchial lymph node, liver and spleen were collected at necropsy, both from areas with grossly visible lesions (if present) as well as those without. An ear notch used for bovine viral diarrhea virus (BVDV) antigen testing was also collected from all animals on the day of necropsy, to assess their status with regard to persistent infection acquired *in utero*. Logistical challenges necessitated a two-week period over which animals were euthanized and necropsied. Bison were euthanized on day 28 PI or day 34 PI, while cattle were euthanized on day 28 PI or day 41 PI, with the exception of one bovine that was euthanized on day 7 PI because of an unrelated injury. The study was conducted in accordance with a protocol approved by the Institutional Animal Care and Use Committee of the NADC.

2.4. Serology

Blood was obtained from all bison and cattle by jugular venipuncture prior to the beginning of the study (between 2 and 10 months prior, depending on the animal), immediately prior to experimental infection (day 0), at 11 days PI and at necropsy (28 days to 41 days PI, depending on the animal). Cattle sera were tested for anti-*M. bovis* IgG

using the Bovicheck *M. bovis* ELISA kit (Biovet, Canada). We have previously reported that *M. bovis* ELISAs developed for use with cattle perform poorly or sub-optimally with bison sera (Register et al., 2013a). In the current study bison sera were tested for *M. bovis*-specific IgG with a custom ELISA that utilizes a Tween 20 extract prepared from bison isolates of *M. bovis* as the capture antigen (Register et al., 2013a). The final result for each serum sample is the average of two independent ELISA evaluations performed on two different occasions. To facilitate comparisons between cattle and bison sera, positive results from the custom ELISA were graded according to the system used for the Bovicheck *M. bovis* ELISA. Specifically, an S/P ratio was calculated by dividing the average absorbance of each sample by the average absorbance of the positive control and assigning a degree of positivity based on the following scale: 1 + = 0.31 - 0.52; 2 + = 0.53 - 0.68; 3 + = 0.69 - 0.89; 4 + = > 0.89.

Sera from blood collected on day 0 from all bison were further tested for antibodies reactive with BVDV, bovine herpes virus 1 (BHV-1), bovine respiratory syncytial virus (BRSV), parainfluenza-3 virus (PI-3) and bovine adenovirus 3 (BAdV3), using a commercially available ELISA (BIO K 028/5 Multiscreen AbELISA Bovine respiratory, Bio-X Diagnostics S.A., Belgium). Cattle sera were not tested similarly, since a vaccine previously administered to those animals prevents us from using the results to infer naturally-occurring exposure.

2.5. Processing of nasal swabs and tissues and detection of infectious agents

The two nasal swabs collected from an individual animal were placed into a single tube containing 150 µl of PPLO broth. Tubes were kept on ice and transported to the lab within a few hours of collection. Following vigorous agitation of the transport tubes one swab was used for *Mycoplasma* culture and the other for isolation of *Pasteurella multocida*, *Mannheimia haemolytica* and *Histophilus somni*. Tissue samples were weighed, minced into small pieces with sterile scissors and homogenized in C tubes using a gentleMACS Octo Dissociator (Miltenyi Biotec) in a volume of PPLO broth sufficient to achieve a dilution of 1:10 (weight/volume).

For *Mycoplasma* culture, selective PPLO broth (PPLO broth supplemented with 0.05% thallium acetate and 500 IU/ml penicillin G) inoculated with a nasal swab or with 300 µl of tissue homogenate was incubated at 37 °C in an atmosphere of 5% CO_2 for at least 24 h, or until growth was visually apparent, up to 72 h in the event of no detectable growth. At the end of the incubation period, 2 µl of every culture was used as the template in a PCR for detection of *M. bovis* (Clothier et al., 2010) as well as a PCR that amplifies ~780 bp of the 16S rRNA gene from a variety of *Mycoplasma* species known to infect cattle (Miles et al., 2004). An aliquot of each culture was also serially diluted and plated on selective PPLO agar. Plates were incubated until *Mycoplasma*-like colonies were evident or up to five days, in the case of negative cultures. Colonies picked from PPLO agar for purposes of MLST were used to inoculate fresh PPLO broth, which was incubated and subsequently confirmed to contain *M. bovis* as described above.

The remaining swab from each animal and 100 µl of each tissue homogenate was used to inoculate plates made with Columbia blood agar base containing 5% defibrinated bovine blood and 0.01% thiamine monophosphate, supplemented with the selective agents amikacin (2 ug/ml), vancomycin (4 ug/ml) and amphotericin B (4 ug/ml) to facilitate isolation of *P. multocida*, *M. haemolytica* and *H. somni*. Plates were incubated at 37 °C for up to 48 h and examined daily for growth of colonies with a morphology typical of *P. multocida*, *M. haemolytica* or *H. somni*. Suspect colonies were picked to a nonselective blood agar plate and incubated for 24 to 48 hs, then delivered to the USDA/APHIS/VS/National Veterinary Services Laboratories for identification on the basis of partial 16S gene sequencing.

Ear notches were individually immersed in 500 µl of phosphate-buffered saline (PBS) for 30 min and frozen at -20 °C. After thawing, the PBS extract was tested for BVDV antigen, in duplicate, using a commercially available kit (IDEXX Laboratories).

2.6. MLST

The MLST methodology used to type *M. bovis* isolates has been reported previously (Register et al., 2015). A commercially available kit (Qiagen) was used to purify genomic DNA from axenic broth cultures arising from single, well-isolated colonies picked from PPLO agar plates. DNA was quantified by UV spectrophotometry and ~2 ng was used for each PCR. Amplicons were treated with ExoSAP-IT (USB Corporation) and sequenced in both directions at the Iowa State University DNA Facility on a 3730xl Genetic Analyzer using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). Consensus sequences were deduced from a minimum of two high quality reads, with at least one from each strand.

2.7. Gross pathology and histopathology

All tissues collected at necropsy were examined for grossly visible lesions. Samples used for histologic evaluation were immediately placed in neutral-buffered 10% formalin, processed by routine paraffin embedding techniques, cut in 4 µm sections and stained with hematoxylin and eosin.

2.8. Statistical analysis

A total of 27,898 temperature readings was used to calculate the group average

daily temperature for bison and for cattle from day 0 PI through day 33 PI. Daily readings used for group comparisons were aggregated to obtain group averages for six, 4-hour periods within each 24-hour period of monitoring: 12:00 am-3:59 am, 4:00 am-7:59 am, 8:00 am-11:59 am, 12:00 pm-3:59 pm, 4:00 pm-7:59 pm and 8:00pm-11:59 pm. Due to the time of day at which monitoring first began and at which it ended, days 0 PI and 33 PI include data for only three or two of the 4-hour periods, respectively. Student's one-tailed *t*-test was used to evaluate the statistical significance of differences in group daily average temperatures. Data compilation and analysis were performed using Excel (Microsoft Office).

3. Results

3.1. Clinical signs

Fig. 1 depicts the group average core body temperature of bison and cattle through day 33 PI for animals that could be monitored for the majority of the study. The bison group includes only five animals due to technical problems that caused significant interruptions in the continuous monitoring of the two remaining. Data points for this group from days 29 PI through 33 PI include only two bison, since three were euthanized on day 28 PI. Missing from the cattle group are data for one calf that had to be euthanized due to a broken leg on day 7 PI. Data points for cattle from days 29 PI through 33 PI include only three animals, since two were euthanized on day 28 PI.

Beginning on day 3 PI, and for several days thereafter, the group average core body temperature for bison was elevated primarily due to three of the five bison that were monitored. No elevation in temperature was apparent in the cattle, either individually or as a group. Group average daily temperatures for bison are significantly higher than those for cattle on days 3–17 PI (Fig. 1). Considering individuals, the fluctuation between the lowest and highest average daily temperature recorded for each bovine over the entire study ranged from 0.3 to 1.0 °C, variability consistent with normal, diurnal temperature patterns in cattle (Falkenberg et al., 2014), with an average of 0.6 °C. In contrast, daily variation in the temperature of individual bison ranged from 0.9 to 3.5 °C, with an average of 1.9 °C. While there are no previous reports

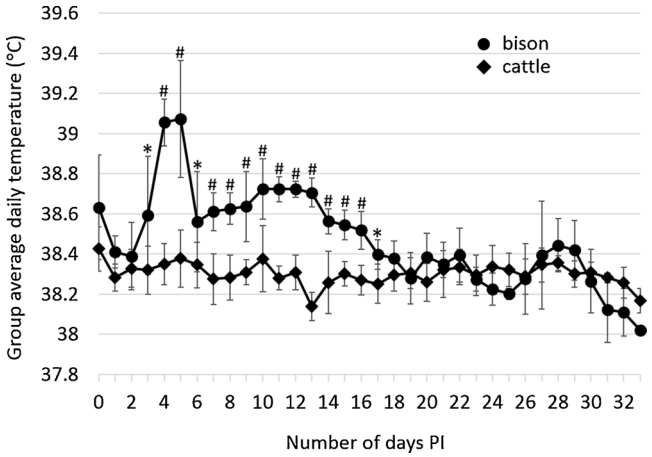

Fig. 1. Group average daily core body temperature recorded for bison or cattle, ± SD. The bison group includes only five animals due to technical problems that caused significant interruptions in the continuous monitoring of the remaining two. The cattle group also includes only five animals, since one calf had to be euthanized on day 7 PI due to a broken leg. Data points for days 29–33 PI include only two bison, since three were euthanized on day 28 PI, and only three cattle, since two were euthanized on day 28 PI. Group average daily temperatures for bison are significantly higher than those for cattle on days 3–17 PI (* $p \leq 0.03$; # $p \leq 0.002$).

describing daily temperature patterns in bison, daily fluctuations observed in this study after day 17 PI are similar to those of cattle. These observations suggest the elevated temperature in bison from days 3–17 PI is the result of challenge with *M. bovis*.

No other clinical signs were apparent in any animal at any time during the study.

3.2. Serology

M. bovis ELISA results for individual animals are shown in Table 2. No IgG reactive with *M. bovis* was detected in the serum of any animal prior to experimental infection. By 11 days PI, 6/7 bison inoculated with *M. bovis* were weakly to moderately positive, as were all surviving cattle. Bovine calf #4 had to be euthanized due to a broken leg on day 7 PI and was seronegative at that time. All experimentally infected bison and surviving cattle were seropositive on necropsy day and all except bovines #3 and #5 displayed an increase in the level of anti-*M. bovis* IgG as compared to the level found on day 11 PI. The bison calf that remained in contact with its experimentally infected mother was seronegative on day 11 PI but strongly positive by necropsy day.

Considering the group average S/P ratios, bison and cattle had similar levels of anti-*M. bovis* serum IgG on day 11 PI (S/P ratio = 0.48 and 0.51, respectively). A valid comparison between the average group levels of serum antibody in bison and cattle on necropsy day cannot be made because the animals in each group were euthanized at different times over a roughly 2-week period.

All bison were seronegative for IBR and BVDV. Bison #361 tested weakly positive for BRSV and BAdV3 but all others were negative for those viruses. The bison calf and #99 had no detectable antibodies to PI3; of the remaining bison, four (#96, #97, #369 and #377) tested weakly positive while #351 and #361 tested moderately positive.

3.3. Detection of infectious agents in nasal swabs and tissues

Results from culturing the nasal cavity and tissue samples for *M. bovis* are summarized in Table 2. Considering nasal swabs, viable *M. bovis* was recovered from 6/7 adult bison, the bison calf and 4/6 bovines on day 0 prior to experimental infection, despite the seronegative status of all animals. The nasal cavity of bison #351 was culture-positive only on day 0, although *M. bovis* was recovered from lesions grossly apparent in the lung of this animal at the time of necropsy. Nasal swabs from all other animals were positive on at least one of the two post-inoculation sampling days; the bison calf, 4/7 adult bison and 3/5 bovines surviving for the duration of the study were positive on both subsequent sampling days.

M. bovis was cultured from the lungs of the five adult bison in which macroscopic lesions were apparent on necropsy day (see Section 3.5) but was not found in the lung of the bison calf nor in the lung of any bovine. The tracheobronchial lymph nodes of two bison and two bovines were culture-positive on necropsy day. *M. bovis* was not found in

Table 2
M. bovis ELISA and culture results.

	day 0		day 11		necropsy day[a]					
		culture		culture		culture				
Animal	ELISA[b]	Nasal cavity/ST[c]	ELISA[b]	Nasal cavity	ELISA[b]	Nasal cavity/ST[c]	Lung/ST[c]	Lymph node/ST[c]	Liver	Spleen
bison #96	neg	neg	1+	pos	2+	pos/1,2	neg	pos/2	neg	neg
bison #97	neg	pos/1	1+	pos	4+	neg	neg	neg	neg	neg
bison #351	neg	pos/1	1+	neg	3+	neg	pos/1	neg	neg	neg
bison #99	neg	pos/1	neg	neg	2+	pos/2	pos/1	neg	neg	neg
bison #361	neg	pos/1	2+	pos	3+	pos/1	pos/1	pos/1	neg	neg
bison #369	neg	pos/1	2+	pos	4+	pos/1,2	pos/1	neg	neg	neg
bison #377	neg	pos/1	1+	pos	3+	pos/2	pos/1	neg	neg	neg
bison calf	neg	pos/1	neg	pos	4+	pos/2	neg	neg	neg	neg
bovine #4	neg	pos/1,2	NA[d]	NA[d]	neg	pos/1	neg	neg	neg	neg
bovine #3	neg	pos/1,2	2+	neg	2+	pos/1,2	neg	pos/2	neg	neg
bovine #5	neg	neg	1+	pos	1+	pos/1,2	neg	pos/2	neg	neg
bovine #40	neg	pos/1	1+	pos	4+	pos/2	neg	neg	neg	neg
bovine #1144	neg	neg	3+	pos[e]	4+	pos[e]	neg	neg	neg	neg
bovine #3132	neg	pos/1	1+	pos	4+	neg	neg	neg	neg	neg

[a] day 7 PI for bovine #4; day 28 PI for bison #96, #97 and #351 and bovines #3 and #5; day 34 PI for bison #99, #361, #369, #377 and the bison calf; day 41 PI for bovines #40, #1144 and #3132.

[b] ELISA-positive samples were assigned a degree of positivity based on an S/P ratio, calculated by dividing the average absorbance of each sample by the average absorbance of the positive control, using the following scale: 1+ = 0.31 - 0.52; 2+ = 0.53 - 0.68; 3+ = 0.69 - 0.89; 4+ = > 0.89.

[c] MLST sequence type (ST) found in independent clones of *M. bovis*.

[d] euthanized due to a broken leg on day 7 PI.

[e] first-passage broth culture was PCR positive for *M. bovis* but no *Mycoplasma*-like colonies were recovered.

the liver or spleen of any animal.

P. multocida was identified in nasal swab cultures from three bovines: #5 (day 11 PI), #40 (day 11 PI and necropsy day) and #1144 (day 0, day 11 PI and necropsy day). No other bacterial pathogens were identified from any animal at any time point. Testing of ear notches indicated none were persistently infected with BVDV.

3.4. MLST

For each of the seven bison and 4 bovines from which nasal swabs were culture-positive for *M. bovis* on day 0 (Table 2), three independent clones per animal were genotyped using MLST to assess whether the population of *M. bovis* present in the nasal cavity prior to experimental inoculation could be distinguished from the isolates subsequently administered. All day 0 *M. bovis* isolates from bison were found to be ST1, the same ST as two of the four isolates in the inoculum (Table 1). ST1 was also found exclusively in two bovines, while a mixture of ST1 and ST2 was identified from the two remaining calves (Table 2).

Three independent *M. bovis* clones derived from each of the nasal swabs and tissue samples positive for *M. bovis* on necropsy days were similarly evaluated (Table 2). Bison isolates recovered from lung tissue are exclusively ST1 while both ST1 and ST2, in nearly equal proportions, were identified both in nasal swabs and lymph node samples. Considering nasal swabs and tissue samples from individual bison, multiple STs were found in nasal swabs only from #96 and #369, although both ST1 and ST2 were recovered from 4/7 bison positive for *M. bovis* on necropsy day. Despite the detection of only ST1 in the nasal cavity of the bison calf on day 0, all isolates recovered on necropsy day were typed as ST2. Regarding bovine nasal swabs collected on necropsy day, a mixture of ST1 and ST2 was present in the nasal cavity of two bovines, while either ST1 or ST2 alone was found in the two additional, culture-positive animals (Table 2). Although only ST1 was detected in the nasal cavity of #40 on day 0, ST2 alone was found in the nasal cavity of that animal on necropsy day. ST2 alone was also recovered from the only bovine tissues collected at necropsy that were culture-positive for *M. bovis*, tracheobronchial lymph nodes from #3 and #5.

3.5. Gross pathology and histopathology

Five of seven adult bison experimentally infected with *M. bovis* had grossly visible lesions including mild, multifocal pockets of emphysema, locally extensive pleural and intra-lobular edema and locally extensive areas of hyperemia (Fig. 2). Two bison had small (approximately 2 cm in diameter), focal, encapsulated abscesses within the lung parenchyma. In one bison multifocal, subacute, mild microabscesses were present in the bronchial lymph node, although no grossly apparent lesions were noted in the lung.

Histologic examination of lung sections revealed moderate to severe lesions in 4/7 adult bison (Fig. 3, panels A–D). Lesions observed in these animals include: moderate to severe, subacute, suppurative bronchopneumonia; interstitial thickening with fibrinoproteinaceous material and moderate pyogranulomatous infiltrates; expansion of alveoli by moderate to severe pyogranulomatous infiltrates, seroproteinaceous material, and red blood cells; multifocal moderate to severe, chronic, granulomas within lung parenchyma and intralobular septa; epithelial metaplasia; hyperplasia of perivascular- and bronchial-associated lymphatic tissue, and occasional thrombi within arterioles. Four adult bison additionally demonstrated multifocal, subacute, moderate splenitis (Fig. 3, panel E) characterized by suppurative infiltrates surrounding white pulp with occasional hypoplasia of white pulp. Three of these four bison further had reactive lymph nodes characterized by increased numbers of tingible body macrophages and follicular hyperplasia, two of which also contained small pockets of neutrophils. Lesions were lacking in hepatic tissue but mild, focal congestion and mild, multifocal, periportal, lymphocytic infiltrates were occasionally noted.

No grossly visible lesions or abnormalities were noted at necropsy in any of the cattle experimentally infected with *M. bovis*. Microscopic examination of lung sections from the five bovines for which they are available (tissues from #3132 were inadvertently discarded) revealed normal tissue morphology and no evidence of infiltrative lesions, exudates or other pulmonary pathology. Of the two bovine lymph nodes

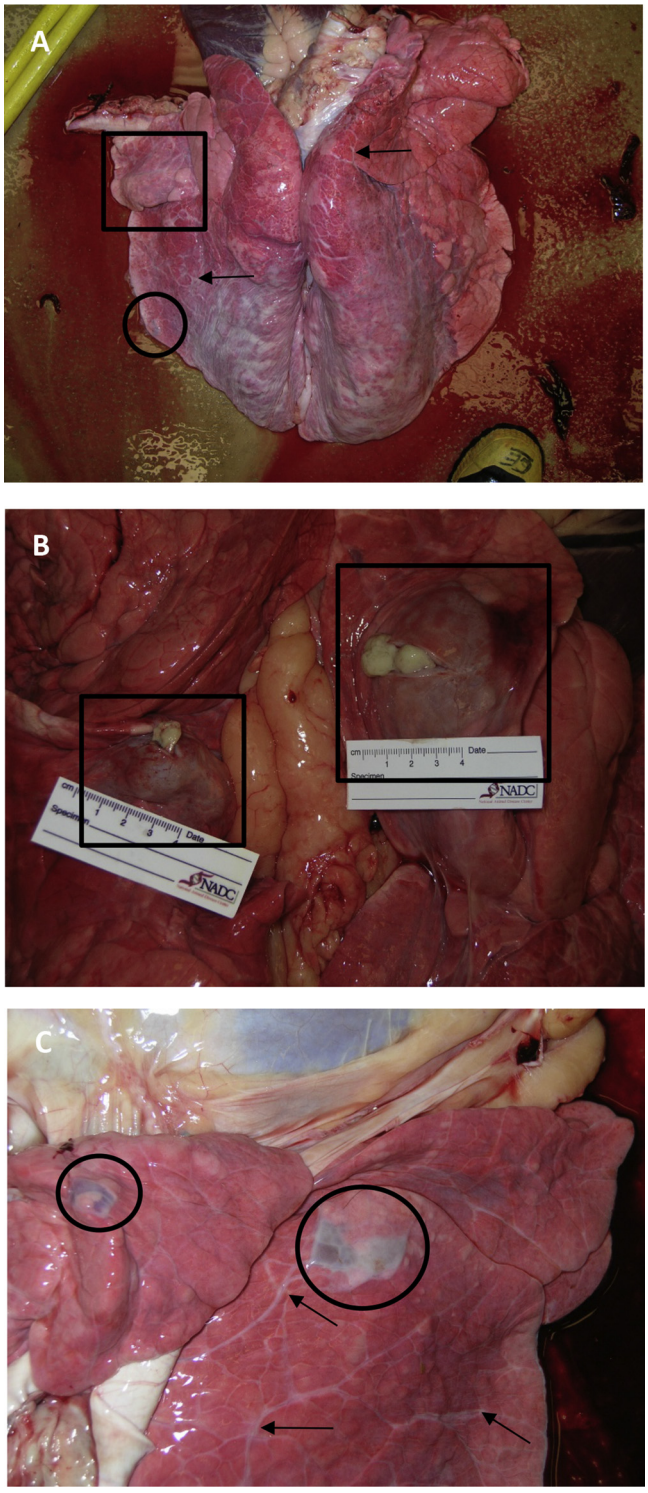

Fig. 2. Grossly visible lung lesions in bison experimentally infected with *M. bovis* include mild, multifocal pockets of emphysema (panels A and C, denoted by circles), locally extensive pleural and intra-lobular edema and locally extensive areas of hyperemia (panels A and C, denoted by arrows) and focal, encapsulated abscesses within the lung parenchyma approximately 2 cm or greater in diameter (panels A and B, denoted by squares).

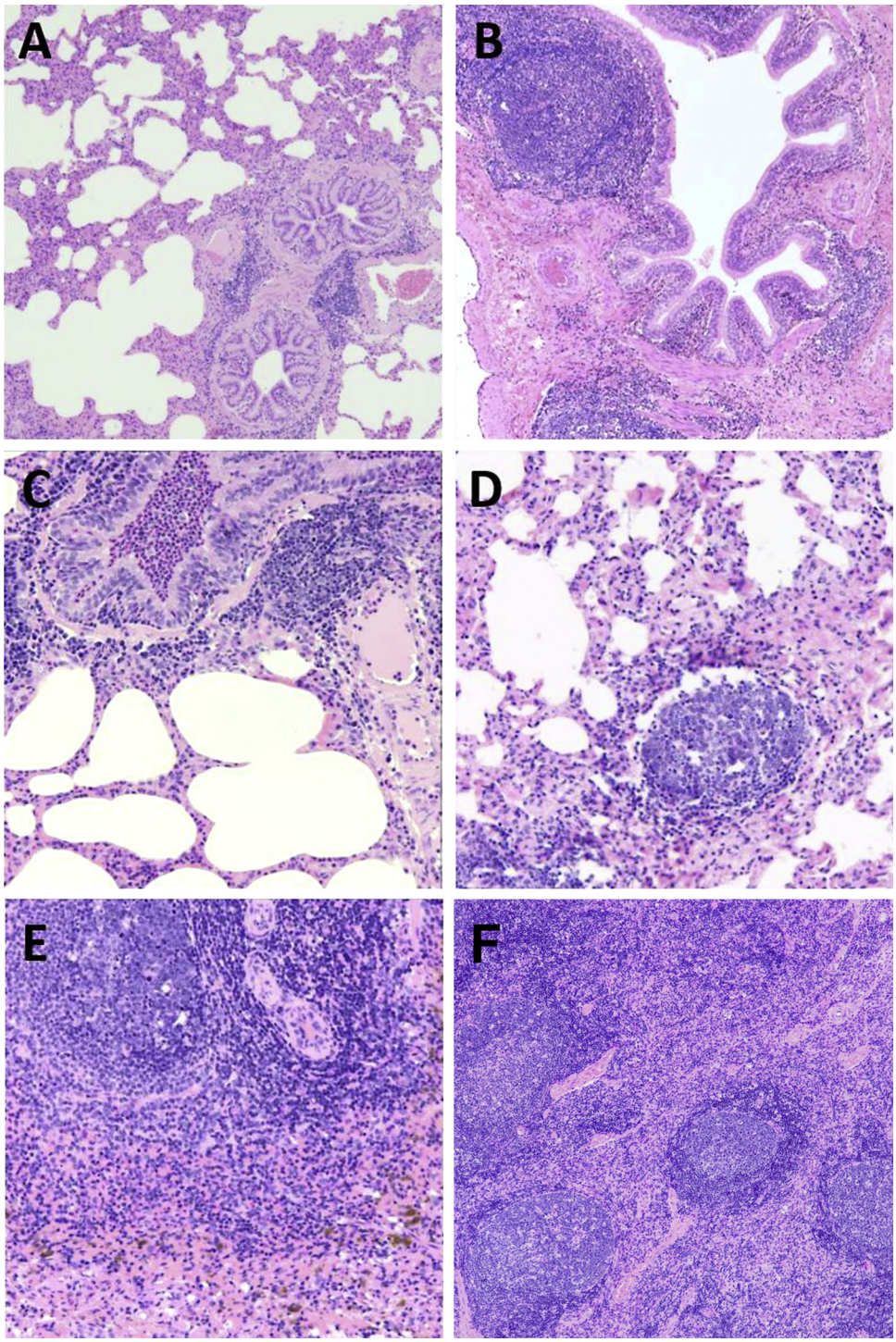

Fig. 3. Histologic lesions observed in the lung (A–D) or spleen (E) of bison and lymph node of a bovine (F) experimentally infected with *M. bovis*. (A) Interstitial thickening and hyperplasia of bronchial-associated lymphatic tissue; (B) Peribronchial, lymphohistiocytic infiltrates; (C) Purulent bronchopneumonia with mild, purulent, interstitial pneumonia and hyperplasia of bronchial-associated lymphatic tissue; (D) Focal granuloma in lung parenchyma; (E) Multifocal, moderate splenitis; (F) Reactive lymph node exhibiting follicular hyperplasia, sinus histiocytosis and numerous tingible body macrophages within the germinal centers. Magnification = 200 x (panels A, B and F) or 400 x (panels C–E).

that were culture-positive for *M. bovis*, tissue sections from #5 displayed mild signs of reactivity characterized by follicular hyperplasia, sinus histiocytosis and numerous tingible body macrophages within the germinal center, while sections from #3 had no apparent abnormalities. Additionally, mild to moderate multifocal suppurative lymphadenitis was noted in the lymph node of #40. Four bovines, including #3 and #5, demonstrated mild, multifocal, suppurative splenitis of the perimarginal zone while the liver of #3 was characterized by mild, diffuse, perivascular lymphocytic hepatitis.

4. Discussion

Since the early 2000's, when *M. bovis* first emerged as a disease problem in bison, much circumstantial evidence has accumulated suggesting that the bacterium is a primary pathogen in that host. However, until now, no definitive test of this hypothesis has been carried out. Here we report that *M. bovis* obtained from the lesions of naturally-occurring mycoplasmosis in bison was re-isolated from lesions that developed following intranasal exposure of healthy bison to pure cultures of the original isolates, thereby satisfying the postulates held as a standard with respect to disease causation (Koch, 1890). Furthermore, neither culture nor serology results provide any evidence to suggest that

other bacterial or viral pathogens commonly found in bison may have contributed to the clinical signs and lesions observed. Although the nasal cavities of nearly all bison were colonized by *M. bovis* prior to experimental inoculation, no clinical signs of disease were apparent until pyrexia was noted on roughly day 3 PI and the character of lesions observed at necropsy is consistent with development during the timeframe of this study. *M. bovis* is a frequent inhabitant of the nasal cavity in healthy cattle but only rarely causes respiratory disease in the absence of other bacterial or viral pathogens. Such carriers often fail to seroconvert unless the bacterium becomes invasive. Whether healthy bison might also harbor *M. bovis* in the upper respiratory tract was not known prior to our study. Our findings demonstrate that nasal colonization in the absence of disease and without seroconversion also occurs in bison. Why *M. bovis* triggered disease in the bison studied here only following experimental infection remains unknown. It may be that the number of bacteria in the inoculum delivered to the upper respiratory tract was sufficient to overwhelm innate immune defenses, which otherwise successfully contain the population acquired naturally. Alternatively, different strains, even those sharing the same MLST-based ST, may differ in their capacity to cause disease. We are further evaluating isolates obtained on day 0 PI for comparison with those recovered from disease outbreaks in an effort to address this question.

While it would have been desirable to test genetically distinct bison isolates individually, in different groups of bison, several considerations led to a decision to include a mixture of genotypes in the inoculum used for this study. A total of only seven adult bison was available, fortuitously provided to us, and it is unlikely we will have the capability to carry out additional studies in bison given the cost of procurement and housing and the logistics of working with these animals. Considering the uniqueness of this opportunity and the paucity of information related to virulence attributes of *M. bovis* we concluded that challenge with a mixture of isolates comprising the two STs most frequently identified from disease outbreaks in bison, ~84% of those so far typed, (Register et al., 2015; https://pubmlst.org/mbovis/) was a reasonable approach. In fact, several studies of naturally occurring disease outbreaks in cattle have reported the presence of multiple genotypes in different animals from a single herd and even in different anatomic sites of a single animal (Biddle et al., 2005; Butler et al., 2001; Castillo-Alcala et al., 2012; Kusiluka et al., 2000; Soehnlen et al., 2011; Sulyok et al., 2014). Regarding bison, MLST analysis of 70 Canadian isolates obtained from case herds of a case-control study (Bras et al., 2017) revealed multiple STs in 5 of the 11 bison herds from which more than a single animal was sampled; in one instance two different STs were isolated from different anatomic sites of the same bison (unpublished data). Thus, naturally occurring outbreaks in which multiple genotypes of *M. bovis* simultaneously infect a herd or a single animal may not be uncommon.

Since little is known about the genetic basis of virulence for *M. bovis*, bacterial factors contributing to a commensal versus a pathogenic lifestyle have not been identified. Nor is it clear why *M. bovis* has only relatively recently become a health threat to bison when it has been recognized as a cattle pathogen for over half a century (Hale et al., 1962). One theory proposed to explain the recent appearance of *M. bovis* as a primary pathogen in bison is that novel genotypes with an expanded host range and/or heightened virulence have recently emerged. MLST data reveal that genotypes isolated from bison are, indeed, distinct from those of cattle isolates (Register et al., 2015). The *M. bovis* pubMLST database (https://pubmlst.org/mbovis/) currently includes submissions for 302 cattle isolates and 35 bison isolates, from which 127 STs have been identified. Of those, only four STs are associated with isolates from bison, none of which have previously been found in cattle. Because MLST is based on nucleotide sequences from housekeeping genes those data, per se, provide little insight as to why bison-associated genotypes might differ in virulence or host range. We hope to further explore these questions through a comparative genomics study currently underway. The absence of clinical signs and lesions in cattle used for the study reported here suggests that strains of *M. bovis* acting as primary pathogens in bison are unlikely to do so in cattle, especially considering that the challenge load per body weight administered to cattle was higher than the load shown to cause disease in bison. Whether clones of *M. bovis* associated with disease in bison might possess enhanced virulence in cattle as compared to cattle-specific strains when additional pathogens are involved, as in bovine respiratory disease complex, remains to be determined, although co-infection with *P. multocida* in three bovines used for this study failed to trigger disease in those animals.

We were unable to completely discriminate between *M. bovis* isolates recovered from the nasal cavity of bison prior to experimental inoculation and those recovered at necropsy. While all pre-inoculation isolates were typed as ST1, we found both ST1 and ST2 in the nasal cavity following experimental infection. The inoculum seems the most likely origin of at least the ST2 population, especially in the three bison from which only that genotype was isolated on necropsy day, but the possibility remains that ST2 clones might have been found on day 0 had we analyzed more than three clones per nasal swab culture. It is difficult to say whether the exclusive isolation of ST1 from the lungs of bison in this study is meaningful. There is no information to suggest that ST1 isolates are, in general, more invasive than ST2 isolates. Moreover, considering isolates so far typed by MLST, the number of ST2 clones recovered from pneumonic bison lungs is double the number of ST1 clones (Register et al., 2015; https://pubmlst.org/mbovis/). The distribution of ST1 and ST2 in the nasal cavity in the days immediately following experimental infection is perhaps a more likely explanation. Because the pre-existing population was largely ST1, subsequent exposure to an equal mixture of ST1 and ST2 would be expected to shift the population towards dominance of ST1, at least temporarily, with the degree dependent on the pre-existing load of *M. bovis* in the nasal cavity.

The detection of ST2 in the nasal cavity of the bison calf on the day of necropsy, when only ST1 was detected on day 0, is consistent with natural transmission of *M. bovis* from the experimentally infected mother (#369), with which it was in close contact for the duration of the study. A high level of anti-*M. bovis* serum IgG was present in this calf on necropsy day (S/P = 0.97), comparable to that found in the mother (S/P = 0.98), the most strongly seropositive bison in the study. While a few dark red foci were noted in the right cranial and middle lung lobes of the calf, *M. bovis* was not recovered from those lesions. Seroconversion in cattle has been associated with an increased risk of respiratory disease (Martin et al., 1990). Whether seroconversion in bison might signal impending disease awaits further investigation. Given that *M. bovis* is well-adapted to the colonization of mucosal surfaces, where it can persist without causing apparent disease, the lack of clinical signs and discernable lesions in the bison calf is perhaps not surprising.

MLST analysis was also unable to discriminate between the pre-existing population of *M. bovis* in the nasal cavity of cattle on day 0 and the bison isolates to which they were subsequently exposed. Consequently, it is unclear whether isolates included in the inoculum were able to partially or completely displace the population of *M. bovis* already resident in the nasal cavity. The two bovines that were culture-negative on day 0 (#5 and #1144) were positive on each of the subsequent sampling dates, suggesting that bison-associated strains are capable of colonizing the bovine upper respiratory tract. However, prior to being relocated to single pens in the BSL-3-Ag containment facility, calves #5 and #1144 were housed in direct contact with one or more of the other bovines used in this study. Therefore, we cannot exclude the possibility that they were infected by penmates prior to the time this study commenced but at a level too low to be detected by our sampling method. Nonetheless, serology data suggest exposure of calves to bison isolates resulted in a level of colonization sufficient to trigger an antibody response. All cattle were seronegative prior to experimental exposure while all those surviving until day 11 PI

seroconverted by that time, consistent with the time to seroconversion reported in other challenge studies (Grissett et al., 2015). Antibody levels remained steady on day 28 PI (bovines #3 and #5) but increased by day 41 PI in all bovines tested at that time (#40, #1144 and #3132).

Until now, ST1 and ST2 isolates of *M. bovis* have been associated exclusively with mycoplasmosis in bison (Register et al., 2015; https://pubmlst.org/mbovis/). Their isolation on day 0 from the cattle used in this study indicates they are not solely restricted to bison and calls attention to our limited understanding of the population structure and genetic characteristics of strains residing as commensals in healthy animals as compared to those associated with disease. It has been suggested that strain-specific differences in virulence may exist (Nicholas, 2011), but relatively few isolates from healthy animals have been evaluated. Thomas et al. (2003a,b) reported that a single isolate from a healthy cow was significantly impaired in its ability to adhere to cells *in vitro* and that this phenotype may discriminate between pathogenic and nonpathogenic strains. Using DNA-based methods to evaluate a total of 200 isolates from cattle with clinically apparent disease and 19 isolates from healthy cattle, other investigators were unable to distinguish between the two groups (Castillo-Alcala et al., 2012; Rosales et al., 2015). Of the 302 cattle isolates so far typed by the MLST method used here, only 13 were obtained from healthy animals (Register et al., 2015; https://pubmlst.org/mbovis/). Considering the 11 different STs found among that group, only one (ST5) has also been found in isolates recovered from cases of mycoplasmosis. It may be that ST1 and ST2 have not been previously recognized in cattle because of the emphasis thus far on evaluating disease-associated isolates. An alternative explanation for isolation of ST1 and ST2 on day 0 from the bovines used here is unintended transmission of *M. bovis* from the bison to the cattle prior to the beginning of the study. While this seems unlikely, given the physical separation of the two groups and the conditions under which they were housed and managed, both before and during the study, this possibility cannot be dismissed with absolute certainty. MLST analysis of a larger and more diverse group of isolates from healthy cattle, including those from herds at the NADC from which the bovines used here were sourced, and more extensive genetic characterization of bison and bovine day 0 isolates may shed further light on this question.

Though *M. bovis* is most frequently encountered as a pulmonary pathogen it can also cause extrapulmonary pathology. Lesions observed in this study were found both in the lung and in other organs. The perivascular lymphocytic infiltrates present in the liver of bovine #3 and, to a lesser extent, in a few of the bison mirror observations reported by others (Maunsell and Donovan, 2009). These lesions are likely due to hematogenous spread of *M. bovis*, which may also account for the splenitis present in four of the bovines and four of the bison. Follicular hyperplasia of the lymph node, seen in bovine #5, is well documented in *M. bovis*-infected calves (Maunsell and Donovan, 2009). Surprisingly, there is only a single report describing suppurative lymphadenitis in bovines attributable to a *Mycoplasma*, in this instance, due to *M. mycoides, subsp. mycoides SC* (Scanziani et al., 1997). Nevertheless, our findings in bovine #40 and in two of the bison suggest that *M. bovis* is also capable of causing this pathology.

5. Conclusion

Data reported here are the first to unequivocally demonstrate that exposure of healthy bison to *M. bovis*, in the absence of other known microbial pathogens, results in the development of lesions typical of mycoplasmosis. The genotypes of *M. bovis* evaluated here appear unable to similarly act as primary pathogens in healthy cattle, but further study in a model of polymicrobial bovine respiratory disease is warranted. The finding that healthy, seronegative bison can act as inapparent carriers of the bacterium will likely complicate efforts to monitor its spread and to control related disease.

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Conflict of interest

The authors declare no conflicts of interest.

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