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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