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# Comparison of multilocus sequence types found among North American isolates of *Mycoplasma bovis* from cattle, bison, and deer, 2007–2017

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**Abstract.** A prior multilocus sequence typing (MLST) study reported that *Mycoplasma bovis* isolates from North American bison possess sequence types (STs) different from those found among cattle. The 42 bison isolates evaluated were obtained in 2007 or later, whereas only 19 of 94 (~20%) of the available cattle isolates, with only 1 from North America, were from that same time. We compared STs of additional, contemporary, North American cattle isolates with those from bison, as well as isolates from 2 North American deer, all originating during the same timeframe, to more definitively assess potential strain-related host specificity and expand our understanding of the genetic diversity of *M. bovis*. From 307 isolates obtained between 2007 and 2017 (209 from cattle, 96 from bison, 2 from deer), we identified 49 STs, with 39 found exclusively in cattle and 5 exclusively in bison. Four STs were shared between bison and cattle isolates; one ST was found in cattle and in a deer. There was no clear association between ST and the health status of the animal of origin. An MLST-based phylogeny including 41 novel STs identified in our study reveals that STs found in bison fall within several divergent lineages that include STs found exclusively in cattle.

**Keywords:** bison; cattle; deer; *Mycoplasma bovis*; MLST.

*Mycoplasma bovis* is an increasingly important pathogen of cattle worldwide.[6] *M. bovis* is most frequently associated with respiratory disease, particularly pneumonia in calves, but additionally causes arthritis, conjunctivitis, otitis, mastitis, infertility, and abortion.[11,15] In the early 2000s, *M. bovis* also emerged as a pathogen in North American bison, in which it is a primary cause of pneumonia, polyarthritis, necrotic pharyngitis, pleuritis, dystocia, and abortion, often with significant mortality.[8,16]

A prior report[14] described the development of a multilocus sequence typing (MLST) method and related database for *M. bovis* that, to date, serves as the PubMLST reference typing scheme (https://pubmlst.org/mbovis/). Thirty-two sequence types (STs) were identified from the 94 cattle isolates and 42 bison isolates evaluated in that study, with 4 restricted solely to bison and the remaining 28 found exclusively in cattle. On that basis, it was hypothesized that the population of *M. bovis* causing disease in bison may be genetically distinct from strains associated with disease in cattle. A definitive conclusion could not be reached because of the host-related disparity in the chronologic and geographic origin of the isolates available for analysis at that time. All bison isolates were acquired in North America between 2007 and 2014; only 19 cattle isolates (20.2%) originated during that time, with only 1 from North America. Therefore, we compared the STs of additional, contemporary, North American cattle isolates with bison isolates, as well as 2 isolates recovered from North American deer, all isolated between 2007 and 2017, to more

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**Table 1.** Geographic and anatomic origin of *Mycoplasma bovis* isolates, sorted by host of origin.

| Host/Geographic origin | Anatomic origin | No. of isolates | Sequence type |
|---|---|---|---|
| Bison | | | |
| Canada | Fetal fluid or tissue | 3 | 2 (3)* |
| | Lung | 19 | 2 (19) |
| | Lymph node | 3 | 57 (3)* |
| | Nasal cavity | 12 | 135 (12)‡ |
| | Pharynx | 3 | 57 (3)* |
| | Placenta | 1 | 2 |
| | Tonsil | 1 | 2 |
| | Uterus | 1 | 2 |
| USA | Joint | 2 | 1 (2)‡ |
| | Larynx | 2 | 1 (2)‡ |
| | Lung | 19 | 1 (7), 2 (1), 4 (5), 5 (1), 46 (1), 133 (1), 136 (3)* |
| | Lymph node | 1 | 1 |
| | Mammary gland | 2 | 1 (2)‡ |
| | Nasal cavity | 23 | 1 (21)‡, 4 (2)‡ |
| | Pharynx | 2 | 2 (2)‡ |
| | Subcutaneous tissue | 1 | 1 |
| | Uterus | 1 | 1 |
| Bovine | | | |
| Canada | Ear | 1 | 10 |
| | Joint | 26 | 10 (12), 12 (1), 109 (3), 111 (1), 121 (4), 124 (1), 125 (2), 128 (1), 130 (1) |
| | Lung | 72 | 5 (1), 6 (2)*, 10 (15), 12 (5), 57 (1), 73 (1), 108 (2), 109 (2), 112 (1), 114 (1), 121 (12), 122 (1), 123 (7), 124 (1), 125 (6), 126 (1), 128 (2), 130 (11) |
| | Lung or joint† | 2 | 10 (1), 123 (1), |
| | Nasal cavity | 77 | 2 (31), 5 (11), 10 (22), 113 (1), 125 (11), 126 (1) |
| USA | Lung | 8 | 3 (5), 47 (2), 48 (1) |
| | Milk | 20 | 49 (1), 50 (1), 51 (1), 52 (1), 53 (1), 54 (1), 55 (1), 56 (1), 57 (1), 58 (1), 59 (1), 82 (1), 97 (1), 98 (1), 99 (1), 100 (2), 101, (1), 102 (1), 105 (1) |
| | Nasal cavity | 3 | 30 (1), 46 (1), 51 (1) |
| Mule deer | | | |
| USA | Lung | 1 | NT |
| White-tailed deer | | | |
| USA | Lung | 1 | 3 |

NT = nontypeable. Numbers in parentheses are number of isolates.
* All isolates are from a single animal.
† Records regarding anatomic source are unclear.
‡ All isolates represent a single herd.

definitively evaluate potential strain-related host specificity and to gain a more comprehensive understanding of the genetic diversity within the *M. bovis* populations recently circulating within North America.

We evaluated 307 isolates in our study, all obtained between 2007 and 2017. Isolates included 209 from cattle (31 from the United States, 178 from Canada), 96 from bison (53 from the United States, 43 from Canada), and 1 isolate each from a white-tailed deer and a mule deer (both from the United States; Table 1). On some occasions, multiple isolates were obtained from a single animal such that 122 cattle and 60 bison are represented in our study (Supplementary Table 1). Canadian cattle isolates were obtained from animals in feedlots in either Saskatchewan or Alberta; cattle isolates from the United States originated from 21 dairy farms in California or from feedlots or herds located in midwestern or southern states. Bison isolates from Canada were from herds in Alberta, Saskatchewan, or Manitoba; those from the United States were acquired from herds in Montana or one of several midwestern or southern states. Deer isolates were recovered from animals with pneumonia in either Minnesota (white-tailed deer) or Nevada (mule deer).

All isolates were assigned STs according to the PubMLST reference scheme.[14] STs for 42 bison isolates, 1 cattle isolate, and the white-tailed deer isolate have been reported previously[14] (indicated in bold font in Supplementary Table 1).

STs for an additional 21 cattle isolates were obtained from submissions to the *M. bovis* PubMLST database (database IDs 219–233, 292, 294–297, and 335). For 89 cattle isolates and 6 bison isolates, STs were deduced using MLST target sequences retrieved from privately held genome sequences, originally obtained for other purposes. Genome sequencing was performed (MiSeq; Illumina, San Diego, CA) using either 2 × 150 or 2 × 300 paired-end libraries, prepared with a Nextera XT DNA library preparation kit (Illumina), as detailed in the Reference Guide. Reads were trimmed using Trimmomatic[3] v.0.38, with settings slidingwindow 5:15, leading 5, trailing 5, and minlen 50, and assembled de novo using SPAdes[1] v.3.11.0 with default settings. The average depth of coverage across each assembly ranged from 26× to 437×, with a median of 72×. Remaining isolates were typed on the basis of PCR amplicon sequences obtained using primers and methodology reported previously.[14] Consensus sequences used for allele assignment were derived from a minimum of 2 high-quality reads with at least 1 from each strand. All isolates and MLST profiles included in our study have been submitted to the PubMLST *M. bovis* database (Supplementary Table 1).

We identified 49 STs among the isolates examined, including 41 not previously reported (Table 2). Of the latter, 15 STs consist of new combinations of existing alleles; 26 STs include 1 or more of 23 alleles not encountered previously (GenBank accessions for new alleles are MK540478–MK540500). Considering all STs, 39 were found exclusively in cattle, and 5 were found exclusively in bison (Table 2). ST2, ST5, ST46, and ST57 isolates originated from both bison and cattle; ST3 isolates were found in cattle as well as a white-tailed deer. No clear association between health status and any ST was apparent. Of the 6 STs most frequently identified (ST1, ST2, ST5, ST10, ST121, and ST125), each found in ≥9 individuals from feedlots or from a combination of herds and feedlots, all but ST121 represent both healthy animals and those with clinically apparent disease (Supplementary Table 1). ST121 isolates ($n = 16$) were cultured from 11 Canadian feedlot cattle with a diagnosis of pneumonia, arthritis, or both. It seems unlikely that all 11 animals originated from the same operation, but the possibility cannot be dismissed given that related records were unavailable. Accordingly, additional data are required to more fully evaluate the implication that infection with an ST121 isolate necessarily leads to disease.

Isolate KRB1, cultured from the pneumonic lung of a mule deer, was nontypeable because of the absence of the alcohol dehydrogenase-1 (*adh-1* gene) target sequence. No PCR amplicon could be obtained from KRB1 using the *adh-1* primers specified in the typing protocol.[14] Various combinations of alternative primers designed to amplify either the entire 1,050-bp open-reading frame or smaller segments thereof were similarly unsuccessful. Further evaluation of this isolate confirmed the absence of the *adh-1* gene. Five hundred ng of purified DNA from KRB1 and bison isolate NADC1, used as a positive control, was spotted onto a nitrocellulose membrane and hybridized to a digoxigenin-labeled probe comprised of 978 bp of the *adh-1* gene (bp 22–999), using standard methods as reported previously.[12] Probe synthesis and labeling, and chemiluminescent

**Table 2.** Distribution of *Mycoplasma bovis* sequence types (STs) by host and country of origin.

| | Cattle ($n = 209$) | | Bison ($n = 96$) | | Deer ($n = 2$) |
|---|---|---|---|---|---|
| ST | USA | Canada | USA | Canada | USA |
| 1 | | | 37 | | |
| 2 | | 31 | 3 | 25 | |
| 3 | 5 | | | | 1 |
| 4 | | | 7 | | |
| 5 | | 12 | 1 | | |
| 6 | | 2 | | | |
| 10 | | 51 | | | |
| 12 | | 6 | | | |
| 30* | 1 | | | | |
| 46* | 1 | | 1 | | |
| 47* | 2 | | | | |
| 48* | 1 | | | | |
| 49*0 | 1 | | | | |
| 50* | 1 | | | | |
| 51* | 2 | | | | |
| 52* | 1 | | | | |
| 53* | 1 | | | | |
| 54* | 1 | | | | |
| 55* | 1 | | | | |
| 56* | 1 | | | | |
| 57* | 1 | 1 | | 6 | |
| 58* | 1 | | | | |
| 59* | 1 | | | | |
| 73* | | 1 | | | |
| 82* | 1 | | | | |
| 97* | 1 | | | | |
| 98* | 1 | | | | |
| 99* | 1 | | | | |
| 100* | 2 | | | | |
| 101* | 1 | | | | |
| 102* | 1 | | | | |
| 105* | 1 | | | | |
| 108* | | 2 | | | |
| 109* | | 5 | | | |
| 111* | | 1 | | | |
| 112* | | 1 | | | |
| 113* | | 1 | | | |
| 114* | | 1 | | | |
| 121* | | 16 | | | |
| 122* | | 1 | | | |
| 123* | | 8 | | | |
| 124* | | 2 | | | |
| 125* | | 19 | | | |
| 126* | | 2 | | | |
| 128* | | 3 | | | |
| 130* | | 12 | | | |
| 133* | | | 1 | | |
| 135* | | | | 12 | |
| 136* | | | 3 | | |
| NT | | | | | 1 |

NT = nontypeable.
* ST not reported previously.

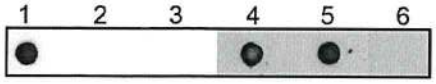


**Figure 1.** Dot blot of purified DNA (500 ng per dot in 5 μL of water) from NADC1 (columns 1 and 4) or KRB1 (columns 2 and 5) hybridized with a probe covering bp 22–999 of the *adh-1* gene (columns 1–3) or bp 42–811 of the *M. bovis* 16S rRNA gene (columns 4–6). Five μL of water was included as a negative control (columns 3 and 6).

detection, of the hybridized probe were carried out using commercial kits (Roche, Indianapolis, IN), as specified by the manufacturer. Although a strong hybridization signal was obtained with DNA from NADC1, no signal was apparent for KRB1 (Fig. 1, columns 1 and 2, respectively). DNA from both isolates hybridized strongly with a positive control probe encompassing a 770-bp fragment (bp 42–811) of the *M. bovis* 16S rRNA gene (Fig. 1, lanes 4 and 5). Other investigators similarly noted the absence of the *adh-1* gene in a 2008 isolate obtained from the milk of a Swiss cow with mastitis.[10] These data revealed that the current PubMLST scheme fails to fully characterize all isolates and prompted an ongoing, collaborative effort to modify the reference typing method to correct this deficiency (Register KB, et al., manuscript in preparation).

The *M. bovis* bison isolates evaluated herein, comprising only 9 STs, exhibit relatively limited diversity compared to those we evaluated from North American cattle. Cattle isolates originated from roughly twice as many individual animals as bison isolates (122 vs. 60, respectively) but include nearly 5 times as many STs (a total of 44 STs). It should be noted that the number of cattle herds represented by these isolates is likely to far exceed the number represented by bison isolates. Approximately 88% of cattle isolates (185 of 209) were obtained from feedlots to which animals had been transported from many different operations, whereas only 1 bison isolate was acquired from a feedlot. Bison isolates otherwise originated from only 21 different herds or ranches (Supplementary Table 1). Nonetheless, it seems probable that the proportion of North American bison herds represented in our study exceeds the proportion of North American cattle herds represented. Using currently available estimates for bison (3,539 private herds, 21 public herds, and at least 17 on tribal lands; National Bison Assoc, Public buffalo herds, 2019, https://bisoncentral.com/public-herds/; All About Bison, Tribal buffalo, 2019, https://allaboutbison.com/natives/tribal-buffalo/; Statistics Canada, 2016 census of agriculture, https://www150.statcan.gc.ca/t1/tbl1/en/tv.action?pid=3210042701),[17] isolates were obtained from ~0.6% of all North American herds. Records documenting the number of operations from which feedlot cattle were sourced are not available. On the assumption that all originated from different operations, the cattle isolates included in our study represent a maximum of 121 individual sources (Supplementary Table 1) or ~0.01% of all operations in North America (estimated at 959,538 as of 2019 for Canada and 2016 for the United States; Statistics Canada, Cattle and calves statistics, number of farms reporting and average number of cattle and calves per farm, Table 32-10-0144-01. 2019, https://www150.statcan.gc.ca/t1/tbl1/en/tv.action?pid=3210014401; USDA, Overview of the United States cattle industry, 2016, https://downloads.usda.library.cornell.edu/usda-esmis/files/8s45q879d/9z903258h/qz20sw09p/USCattSup-06-24-2016.pdf). Using results from arbitrarily primed PCR as a measure of genetic heterogeneity, others have demonstrated a potential link between management practices and the degree of *M. bovis* genetic diversity, with greater variability detectable among cattle isolates from a feedlot and a multiple-source herd compared to a closed herd.[5] On that basis, one might predict less diversity among bison isolates than among those from cattle, given that the frequency and degree to which animals from different herds are mixed is substantially lower for bison than for cattle.

A previous study using the MLST method also used here detected only a single ST in each of 8 bison from which more than 1 isolate of *M. bovis* was typed.[14] That study did not include more than a single isolate from any bovine animal but, using amplified fragment length polymorphism[7] or pulsed-field gel electrophoresis,[2] others have shown that multiple genotypes can be found in individual cattle sampled over a period of days or weeks. Whether different genotypes were simultaneously present in any of those animals is unclear. In our study, multiple STs were identified in 6 of the 41 cattle from which more than a single isolate was obtained (cattle 57, 58, 61, 64, 67, 88; Supplementary Table 1), all arising from samples collected on a single occasion. Two STs each were found in 2 of these cattle (67 and 88); 3 STs each were cultured from the remaining 4 cattle (57, 58, 61, 64). In 3 instances, 3 STs were cultured from a single site (a joint for cattle 57 and 64, and the lung for calf 58). All samples yielding multiple STs were collected from animals with pneumonia, arthritis, or both. Only 1 ST per animal was found in the nasal cavity of the 17 healthy cattle from which multiple isolates were obtained. Although we encountered 1 instance in which the nasal cavity was colonized by 2 STs (calf 88), that animal was pneumonic at the time of sample collection. In contrast, only a single ST was found in each of the 17 bison from which >1 isolate was recovered, 11 of which appeared healthy at the time they were sampled. These data raise the intriguing notion that the degree of genetic diversity within *M. bovis* populations residing in animals with disseminated disease may be greater than in apparently healthy animals carrying the bacterium in the nasal cavity. Should ongoing characterization of additional isolates from healthy and diseased cattle and bison provide further support, a central question is whether the expanded repertoire of genotypes plays any role in the transition from healthy carriage to systemic disease.

Consistent with prior observations,[14] ST1 and ST4 isolates were found exclusively in bison (Table 2). Interestingly, we detected those STs only among isolates from the United States (Table 2), where they comprise 83.0% of all

bison isolates so far typed. ST1 and ST4 isolates were recovered from a variety of anatomic locations, including the upper and lower respiratory tract and the reproductive tract, and from both healthy bison as well as those displaying clinical signs consistent with mycoplasmosis (Supplementary Table 1; *M. bovis* PubMLST database). Also restricted to the United States was ST3, which we found only in cattle and a white-tailed deer from a relatively limited geographic region (Iowa, Kansas, or Minnesota). Although ST2 was previously found solely in bison,[14] no Canadian cattle isolates had been typed by the method used in our study at the time of that investigation. We identified 31 ST2 cattle isolates, all from Canada, together with 25 Canadian bison isolates and 3 from bison in the United States (Table 1). Thus, it appears ST2 clones of *M. bovis* may be much more common in Canada than in the United States.

Our data reveal that some STs are shared between cattle and bison, which has not been reported previously, to our knowledge. The overall frequency with which *M. bovis* genotypes are shared between these hosts appears to be much higher in Canada than in the United States; 72.1% of the Canadian bison isolates evaluated have STs also found among cattle isolates compared to only 9.4% of bison isolates from the United States. A similar comparison limited to bison isolates obtained during roughly the first half of the time period represented in our study, 2007–2012, indicates this disparity has existed since at least that time. Only 9.5% of such isolates from the United States ($n = 42$) have STs also found among cattle compared to 100% of the Canadian isolates originating during the same time ($n = 18$). Ongoing assessment of the geographic and host-specific distribution and frequency of *M. bovis* genotypes circulating among these populations may be of benefit in efforts to develop efficacious vaccines, particularly those designed for use in bison (Veterinarian discusses pathology, prevention of *Mycoplasma bovis* diseases in bison. High Plains J, Aug. 19, 2016, https://www.hpj.com/livestock/veterinarian-discusses-pathology-prevention-of-mycoplasma-bovis-diseases-in-bison/article_e6123948-113b-59fd-b8d2-2d6caa765a67.html).

Considering all *M. bovis* STs reported previously[4,14] or submitted to the *M. bovis* PubMLST database, together with the novel STs reported herein, 157 STs have been defined. Probable genetic relationships among their allelic profiles were inferred using PHYLOViZ v.2.0,[13] implementing the goeBURST algorithm[9] with eBURST distance and clonal complexes formed at the SLV level (Fig. 2). STs found in bison, both those shared with cattle and those so far found exclusively in bison, fall within several divergent lineages that include additional STs found in North American cattle, suggesting that STs infecting bison are no more closely related to one another than to others within the same lineage. Our data neither support nor disprove a previously proposed hypothesis that newly evolved genotypes with an expanded host range and/or heightened virulence underlie the recent emergence of *M. bovis*–related disease in bison.[14] Comparative genomic studies currently underway in our laboratory and others are expected to shed additional light on the population structure and epidemiology of the bacterium and will perhaps reveal host-specific genetic features suitable for further study.

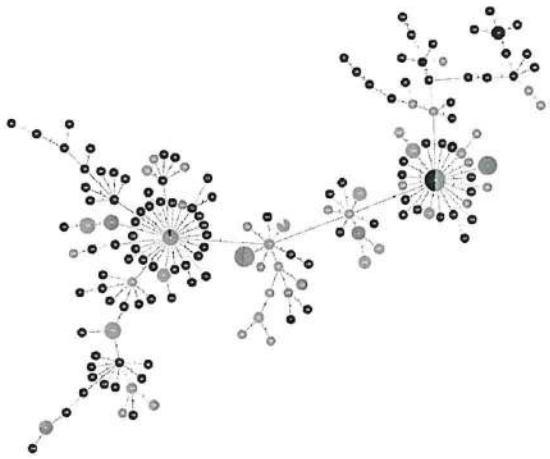

**Figure 2.** Minimum spanning tree depicting evolutionary relationships among the 157 sequence types (STs) thus far defined for *Mycoplasma bovis*. STs are indicated by the numeral in each circle. Blue circles (STs found in North American cattle between 2007 and 2017), red circles (STs found in North American bison between 2007 and 2017), and yellow circles (the ST found in North American deer between 2007 and 2017), or portions thereof, are sized in proportion to the number of isolates included in our study with the indicated ST (log scale). Black circles represent STs found in all other isolates so far reported or found in the *M. bovis* PubMLST database and are not proportional to the number of isolates known to have the indicated ST. Numerals on connecting branches indicate the number of loci at which the corresponding STs differ from one another. Connecting branches are depicted using a grayscale in which lighter branches represent profiles differing at more loci than profiles linked by darker branches.

## Authors' note

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## Declaration of conflicting interests

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## Supplemental material

Supplementary material for this article is available online.

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