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Multilocus sequence typing of *Mycoplasma bovis* reveals host-specific genotypes in cattle versus bison☆

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ABSTRACT

Mycoplasma bovis is a primary agent of mastitis, pneumonia and arthritis in cattle and the bacterium most frequently isolated from the polymicrobial syndrome known as bovine respiratory disease complex. Recently, *M. bovis* has emerged as a significant health problem in bison, causing necrotic pharyngitis, pneumonia, dystocia and abortion. Whether isolates from cattle and bison comprise genetically distinct populations is unknown. This study describes the development of a highly discriminatory multilocus sequencing typing (MLST) method for *M. bovis* and its use to investigate the population structure of the bacterium. Genome sequences from six *M. bovis* isolates were used for selection of gene targets. Seven of 44 housekeeping genes initially evaluated were selected as targets on the basis of sequence variability and distribution within the genome. For each gene target sequence, four to seven alleles could be distinguished that collectively define 32 sequence types (STs) from a collection of 94 cattle isolates and 42 bison isolates. A phylogeny based on concatenated target gene sequences of each isolate revealed that bison isolates are genetically distinct from strains that infect cattle, suggesting recent disease outbreaks in bison may be due to the emergence of unique genetic variants. No correlation was found between ST and disease presentation or geographic origin. MLST data reported here were used to populate a newly created and publicly available, curated database to which researchers can contribute. The MLST scheme and database provide novel tools for exploring the population structure of *M. bovis* and tracking the evolution and spread of strains.

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1. Introduction

Mycoplasma bovis is an important pathogen of cattle worldwide, causing respiratory disease, otitis media, arthritis and mastitis (Maunsell et al., 2011). Recently, *M. bovis* outbreaks with extremely high morbidity and mortality have also been reported in North American bison (Dyer et al., 2008, 2013; Janardhan et al., 2010; Register et al., 2013). Disease presentations so far documented in bison include pneumonia, arthritis, necrotic pharyngitis, dystocia and abortion. Whether outbreaks in bison are due to the emergence of novel genetic variants of *M. bovis* is currently unknown.

Various typing methods based on comparison of the mobility of DNA restriction fragments or PCR amplicons in agarose gels, including pulsed-field gel electrophoresis

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(PFGE; McAuliffe et al., 2004; Punyapornwithaya et al., 2010; Arcangioli et al., 2012; Pinho et al., 2012), insertion sequence (IS) typing (Miles et al., 2005; Thomas et al., 2005; Aebi et al., 2012), random amplified polymorphic DNA (RAPD) analysis (McAuliffe et al., 2004; Pinho et al., 2012), amplified fragment length polymorphism (AFLP) fingerprinting (Kusiluka et al., 2000; McAuliffe et al., 2004; Soehnlen et al., 2011; Castillo-Alcala et al., 2012; Hendrick et al., 2013) and arbitrarily primed polymerase chain reaction (AP-PCR; Butler et al., 2001) have been used to evaluate cattle isolates of *M. bovis*. Although informative, these approaches are generally challenging to standardize, suffer from a degree of subjectivity in the analysis of DNA fragment patterns and, in some cases, require special equipment. More recently, a multiple-locus variable-number tandem-repeat analysis (MLVA) typing method has been developed that appears to be highly discriminatory (Pinho et al., 2012; Amram et al., 2013; Spergser et al., 2013; Sulyok et al., 2014). Because MLVA is based on the relative mobility of PCR amplicons, comparisons within and between laboratories may not be straightforward, particularly when amplicon patterns are inspected manually. Some users have employed a capillary electrophoresis gene analyzer and fluorescently labeled amplicons to simplify and better standardize data interpretation. Clear-cut comparisons between laboratories are also hampered by the absence of a uniform nomenclature for classification of genotypes.

Multilocus sequence typing (MLST) is a robust, scalable and highly standardized method that easily and unambiguously differentiates among strains based on comparison of partial DNA sequences from housekeeping genes (Ibarz Pavón and Maiden, 2009). Six to ten loci are typically required to achieve a level of resolution sufficient for population studies. An MLST scheme that includes four loci has been reported for *M. bovis* but has so far been evaluated with a total of only 39 isolates, most from a limited geographic region (Manso-Silván et al., 2012; Sulyok et al., 2014). Here we report the development of a highly discriminatory MLST method for *M. bovis* that demonstrates cattle and bison isolates comprise unique sets of sequence types (STs). The MLST data were used to populate a newly created and publicly available database intended to serve as a tool for epidemiologic studies and further investigating the population structure of *M. bovis*.

2. Materials and methods

2.1. Bacterial strains and genomic DNA

The geographic, anatomic and host of origin of the *M. bovis* isolates used in this study are summarized in Table 1. Cattle isolates include two well-characterized strains available from the American Type Culture Collection (Jasper and the type strain, PG45), 21 identified from clinical cases submitted to the Iowa State University Veterinary Diagnostic Laboratory (R.F. Rosenbusch, unpublished), one cultured at the National Animal Disease Center from the nasal cavity of a cow with respiratory disease (Robert Briggs, personal communication) and 69 previously studied and/or identified by others (Rasberry and Rosenbusch, 1995; Lu and Rosenbusch, 2004; Rosenbusch et al., 2005; Ben Shabat et al., 2010; Pinho et al., 2012; Qi et al., 2012; Zou et al., 2013). Bison isolates have been previously described (Janardhan et al., 2010; Dyer et al., 2013; Register et al., 2013) or were cultured and identified at the Montana Veterinary Diagnostic Laboratory (A.W. Layton, personal communication), VIDO-Intervac, University of Saskatchewan (Jose Perez-Casal, personal communication), Veterinary Diagnostic Services, Winnipeg, MB, Canada (Shelagh Copeland, personal communication) or the National Animal Disease Center (K.B. Register, unpublished). All isolates were derived from axenic cultures arising from single colonies and confirmed to be *M. bovis* using a species-specific PCR (Clothier et al., 2010).

Table 1
M. bovis isolates used in this study.

Host	Anatomic origin	Geographic origin	No. of isolates
Bison ($n = 42$)	Lung	United States	13
		Canada	13
	Larynx/pharynx	United States	4
	Lymph node	United States	1
	Subcutaneous tissue	United States	1
	Joint	United States	2
	Mammary gland	United States	2
	Uterus	United States	1
		Canada	1
	Placenta	Canada	1
	Fetus	Canada	3
Cattle ($n = 94$)	Lung	United States	30
		China	3
		Israel	2
		Lithuania	3
		Australia	1
		Hungary	2
	Milk	United States	18
		Israel	1
	Nasopharynx	United States	5
	Joint	United States	4
		Israel	3
	Ear	United States	6
		Israel	1
	Trachea	Israel	1
	Vulva	Israel	1
	Unknown	United States	11
		United Kingdom	2

M. bovis was cultivated for 18–24 h 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). Genomic DNA was purified from broth cultures using a commercially available kit (Qiagen) and quantified by UV spectrophotometry.

2.2. MLST design, PCR amplicon sequencing and data analysis

Candidate MLST loci were selected using the publically available genome sequences for isolates PG45 (ATCC 25523^T, GenBank ID CP002188.1), HB0801 (GenBank ID CP002058) and Hubei-1 (CP002513.1) and privately held genome sequences from three additional isolates obtained in the United States. The Vector NTI Advance 11 software package (Life Technologies) was used for DNA sequence