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Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry identification of *Moraxella bovoculi* and *Moraxella bovis* isolates from cattle

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Abstract. Infectious bovine keratoconjunctivitis (IBK) is an economically significant disease caused by *Moraxella bovis*. *Moraxella bovoculi*, although not reported to cause IBK, has been isolated from the eyes of cattle diagnosed with IBK. Identification of *M. bovis* and *M. bovoculi* can be performed using biochemical or DNA-based approaches, both of which may be time consuming and inconsistent between laboratories. We conducted a comparative evaluation of *M. bovoculi* and *M. bovis* identification using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) with a database provided by Bruker Daltonics (termed the BDAL database), the BDAL database supplemented with spectra generated in our study (termed the UNLVDC database), and with PCR–restriction-fragment length polymorphism (PCR-RFLP) typing. *M. bovoculi* (n = 250) and *M. bovis* (n = 18) isolates from cattle with or without IBK were used. MALDI-TOF MS using the UNLVDC database correctly identified 250 of 250 (100%) of *M. bovoculi* and 17 of 18 (94%) of *M. bovis* isolates. With the BDAL database, MALDI-TOF MS correctly identified 249 of 250 (99%) of *M. bovoculi* and 7 of 18 (39%) of *M. bovis* isolates. In comparison, the PCR-RFLP test correctly identified 210 of 250 (84%) of *M. bovoculi* and 12 of 18 (66%) of *M. bovis* isolates. Thus, MALDI-TOF MS with the UNLVDC database was the most effective identification methodology for *M. bovis* and *M. bovoculi* isolates from cattle.

Key words: Infectious bovine keratoconjunctivitis; MALDI-TOF mass spectrometry; *Moraxella bovis*; *Moraxella bovoculi*

Infectious bovine keratoconjunctivitis (IBK), also known as "pinkeye," is an economically significant and highly prevalent disease of cattle.[23,24] Cattle with IBK frequently demonstrate clinical signs including increased lacrimation and photophobia that progress to conjunctivitis, keratitis, ulceration, and possible ocular rupture and blindness.[4] Two species of *Moraxella* are prominently associated with IBK.[1] *Moraxella bovis* is a primary causative agent of the disease.[19] In contrast, although *M. bovoculi* has not been reported to cause IBK, the organism has been isolated from eyes of cattle with IBK during outbreaks in the absence of detectable *M. bovis*.[3] As well, *M. bovoculi* was the most frequent isolate found in eye swabs submitted to a diagnostic laboratory during IBK outbreaks.[16]

At present, distinguishing between *M. bovis* and *M. bovoculi* in clinical laboratory specimens is time consuming and relies on complex biochemical testing, which does not always accurately identify members of either species.[2] Such difficulty may be the result of strain variation, particularly for *M. bovoculi*. Extensive genetic differences, including the presence or absence of virulence factors, have been found between *M. bovoculi* isolates obtained from the eye and nasopharynx of cattle with and without IBK.[7] A PCR–restriction-fragment length polymorphism (PCR-RFLP) assay has been used to identify *M. bovis* and *M. bovoculi*, but this assay can be costly and results are not consistent among isolates, especially *M. bovoculi* isolates obtained from the nasopharynx of cattle without signs of IBK.[2,7,16] A method to accurately identify *M. bovis* or *M. bovoculi* during an IBK outbreak could assist veterinarians in ultimately implementing proper prevention and treatment strategies.

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) is an emerging method of bacterial identification that is rapid, inexpensive, and provides advantages over many traditional identification methods.[5,20] MALDI-TOF MS has been implemented in many veterinary diagnostic laboratories, and has been used to identify significant veterinary pathogens at the species and subspecies levels.[17,18] We compared MALDI-TOF MS using

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both the manufacturer's provided database, termed the BDAL database, and the BDAL database supplemented by additional spectra generated in our study, termed the University of Nebraska–Lincoln Veterinary Diagnostic Center (UNLVDC) database, with the PCR-RFLP assay.

The *M. bovoculi* (n = 250) and *M. bovis* (n = 18) isolates used were from outbreaks of clinical IBK that were diverse in time and location, and from the eyes and nasopharynx in healthy adults and young cattle (Supplementary Table 1). Many of the isolates have been described previously.[7,16] All 268 isolates were subjected to whole genome paired-end sequencing (MiSeq instrument, Illumina, San Diego, CA) at the U.S. Meat Animal Research Center with either MiSeq reagent kit v.2 (2 × 250 bp, 500 cycles) or v.3 (2 × 300 bp, 600 cycles). Resulting sequences were mapped to a *M. bovoculi* reference genome, strain CP011374,[7] in Geneious v.10.[8] The mapped sequences of each isolate were visually checked for evidence of "mixed" isolate contamination, which was not detected, indicating that the sequences represented a single haploid isolate genome. We identified isolates at the species level through phylogenetic analyses of the same large ribosomal DNA (rDNA) locus that was employed in the PCR-RFLP assay[2] and the initial description of *M. bovoculi.*[3]

To conduct the phylogenetic analyses, target large rDNA loci were extracted from GenBank reference sequences,[3,6,8,9,12] and the sequence reads from each isolate were mapped to all of the reference sequences. Variants to the optimal reference sequence were identified, and rDNA locus consensus sequences were constructed using bcftools (https://samtools.github.io/bcftools/call-m.pdf) and aligned using Clustal Omega.[13,14,21] Sequence evolution was modeled, and a maximum likelihood phylogenetic analysis of the large rDNA locus was implemented.[10,15,22] Half of the *M. bovis* isolates had evidence of interspecies recombination in the internal transcribed spacer region of the rDNA locus (Supplementary Fig. 1A), therefore the same analysis was conducted using only the 16S portion of the locus aligned with ClustalW.[11] Less recombination was detected in the 16S locus (Supplementary Fig. 1B). This evidence of apparent interspecies recombination, or lateral gene transfer events within "conserved" rDNA genes, suggests that *M. bovis* and *M. bovoculi* may exchange genetic material at a rate that will require additional genomic sequencing and phylogenetic analyses to fully resolve to the species level. However, despite the presence of apparent recombinant alleles, the supplementary figure highlights strong phylogenetic separation and support for species-level allocation of the *M. bovis* and *M. bovoculi* isolates used to develop and validate the MALDI-TOF MS approach.

All 268 isolates were subjected to a PCR-RFLP assay with band sizes evaluated using capillary gel electrophoresis in comparison to reference markers (QIAxcel, Qiagen, Germantown, MD) as described previously.[2,16] For MALDI-TOF MS analyses, isolates were grown overnight on blood agar (5% sheep blood in trypticase soy agar; Remel, Lenexa, KS). Isolated colonies were prepared in duplicate according to the manufacturer's recommended procedures for the direct smear method using α-cyano-4-hydroxycinnamic acid matrix (Bruker Daltonics, Billerica, MA) and subjected to automatic detection in positive linear mode between 2 kDA and 20 kDA m/z, with a laser frequency of 60 Hz (Microflex LT MALDI-TOF MS, Bruker Daltonics) calibrated for reference masses of 3,637–16,952 Da using the manufacturer's supplied bacterial test standard. A maximum of 240 spectrum profiles were acquired per isolate. Identifications were determined using commercial software (Biotyper, Bruker Daltonics) and the latest manufacturer's database (BDAL v.6 containing 6,903 reference spectra) or a modified database that we developed (UNLVDC) that used the BDAL database with additional spectra from reference isolates of *M. bovis* and *M. bovoculi* that had been subjected to 16S rDNA sequencing and/or genomic sequencing and analyses for confirmation of their identities.[6] These additional spectra came from one *M. bovis* and one *M. bovoculi* isolate that were added because of low or no matching scores to existing entries in the BDAL library. Isolate spectra were added to BDAL v.6 to create the UNLVDC database by using the manufacturer's recommendations for custom database generation (Bruker Daltonics). MALDI-TOF MS with the BDAL database, MALDI-TOF MS with the UNLVDC database, and PCR-RFLP comparisons were all conducted using sequence information as the gold standard for species identification.

MALDI-TOF MS that used the UNLVDC database was able to correctly identify 100% (250 of 250) of *M. bovoculi* isolates and 94% (17 of 18) of *M. bovis* isolates. With the BDAL database, MALDI-TOF MS correctly identified 99% (249 of 250) of *M. bovoculi* isolates and 39% (7 of 18) of *M. bovis* isolates. In comparison, the PCR-RFLP test was able to correctly identify 84% (210 of 250) of *M. bovoculi* isolates and 66% (12 of 18) of *M. bovis* isolates. The top match scores for *M. bovoculi* were all ⩾2.2 and for *M. bovis* were all ⩾1.97, except for 2 isolates with scores of 1.72 and 1.74. Most identification disagreements resulted from isolates that were identified as *M. bovoculi* by sequencing and MALDI-TOF MS, but that did not demonstrate a restriction site with PCR-RFLP (the presence of which was consistent with *M. bovoculi*), and that had an amplicon size consistent with *M. bovis*. Interestingly, all of these isolates matched to database isolate *M. bovoculi* 23343, which also lacked a restriction site and was classified as *M. bovis* by PCR-RFLP. These strains may be similar to the genetically atypical, non-IBK strains of *M. bovoculi* described previously.[7] The *M. bovis* isolates that matched to database isolate *M. bovis* 2017003602-1 in the UNLVDC library were also those that demonstrated interspecies recombination at the ribosomal loci (Supplementary Fig. 1), indicating the potential for MALDI-TOF MS to detect these recombinants. Overall, MALDI-TOF MS combined with our customized reference database (UNLVDC) identified both *M. bovis* and *M. bovoculi* isolates with higher levels of agreement to sequencing

than either PCR-RFLP or MALDI-TOF with the BDAL database.

The ability of MALDI-TOF MS to accurately identify bacteria is dependent on the depth of the reference database. MALDI-TOF MS with the UNLVDC database accurately identified 100% of genetically diverse isolates of *M. bovoculi*, which may or may not all be represented with equal frequencies in the eyes of cattle with IBK.[6] In the future, it may be possible to develop a strain-specific or subtyping assay for *M. bovoculi* on the MALDI-TOF MS platform with the addition and analyses of more isolate spectra to the UNLVDC, or other reference databases, along with pertinent genotypic and phenotypic isolate information. Whole genome sequence information may be particularly important isolate information to have for identifying the extent of recombination that may have occurred between strain types, thus potentially impacting the development of a more specialized MALDI-TOF MS assay.

Notably, *M. bovis* was under-represented in our study. Even greater identifying resolution than that provided with the UNLVDC database may be obtained in the future with the addition of more spectra from genetically diverse *M. bovis* isolates. To that end, 3 of 17 *M. bovis* isolates in our study identified with the UNLVDC database had matching scores below 2.0, which is the manufacturer's recommended minimum score for reliable identification to the species level for many organisms. However, all *M. bovis* isolate scores using the current UNLVDC database were above the threshold recommended for identification to the genus level ($\geqslant$1.7), and 14 of 17 isolate scores exceeded the 2.0 threshold.

Database sharing is an important component of MALDI-TOF MS, as it may enable users to further enhance identification capabilities. Reference mass spectra (MSP) files or raw spectra files from isolates included in UNLVDC are available for MALDI-TOF MS users interested in identifying *M. bovis* or *M. bovoculi* isolates obtained from cattle. This may be especially useful to identify genetically recombinant *M. bovis* isolates, which were not identified using the BDAL database.

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Declaration of conflicting interests

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