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Note

Rapid typing of *Mannheimia haemolytica* major genotypes 1 and 2 using MALDI-TOF mass spectrometry



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ABSTRACT

Genotype 2 *M. haemolytica* predominantly associate over genotype 1 with the lungs of cattle with respiratory disease and ICES containing antimicrobial resistance genes. Distinct protein masses were detected by MALDI-TOF MS between genotype 1 and 2 strains. MALDI-TOF MS could rapidly differentiate genotype 2 strains in veterinary diagnostic laboratories.

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Bovine respiratory disease (BRD) is one of the most costly diseases to cattle production throughout the globe, and losses in the US are estimated at one billion dollars annually (Griffin et al., 2010). Although the disease is multifactorial, one of the major pathogens is *Mannheimia haemolytica* (*M. haemolytica*), which is the most commonly detected bacterial agent associated with the disease (Bell et al., 2014; Fulton et al., 2009; Panciera and Confer, 2010). *Mannheimia haemolytica* is a typical commensal resident of the bovine upper respiratory tract. However, under certain immunosuppressive conditions *M. haemolytica* proliferates in the lungs, and secretes a variety of virulence factors which ultimately cause pneumonia and potentially mortality (Singh et al., 2011). Distinct differences in capsular serotypes and pulsed field gel electrophoresis patterns have been shown between *M. haemolytica* strains located in the upper respiratory tract of cattle without signs of bovine respiratory disease versus strains from the lungs and nasopharynx of animals with bovine respiratory disease (Klima et al., 2014).

Recently, whole genome sequencing was employed on 1133 North American *M. haemolytica* isolates and a nucleotide polymorphism typing system was developed. Two major genotypes were discovered (1 and 2). Genotype 1 strains were mostly isolated from the nasopharynx of cattle without signs of BRD. In contrast, genotype 2 *M. haemolytica* predominantly associated with the lungs of cattle with BRD and integrative conjugative elements that contained antimicrobial resistance determinants (Clawson et al., 2016). A test that could rapidly and accurately

distinguish genotype 1 and 2 *M. haemolytica* could be beneficial to diagnostic laboratories and veterinary practitioners that currently identify *M. haemolytica* at the species level using culture. Within a single animal, *M. haemolytica* cultured from the nasopharynx may genetically differ from those cultured from the lung, making culture findings from nasal swabs and other antemortem clinical samples challenging to interpret (Capik et al., 2015). A test to rapidly distinguish genotypes would provide an enhanced ability to interpret *M. haemolytica* culture and subsequent antimicrobial susceptibility testing results from nasal swabs or other samples that contain *M. haemolytica* of either genotype.

Matrix-assisted laser desorption/ionization- time of flight mass spectrometry (MALDI-TOF MS) is an emerging technology in clinical and veterinary microbiology and has most frequently been used for bacterial identification, but has been applied to a wide variety of applications including fungi identification and detection of antimicrobial resistance (Clark et al., 2013; Seng et al., 2009). The MALDI-TOF MS method has recently been shown to have utility in discriminating or typing other members of the family Pasteurellaceae, including those in the genus *Haemophilus* and *Gallibacterium* (Alispahic et al., 2011; Nørskov-Lauritsen et al., 2012). This paper describes the use of MALDI-TOF MS to discover and evaluate a mass spectrum biomarker-based classifier(s) also known as a “model” (Khot and Fisher, 2013) that distinguishes *M. haemolytica* genotype 1 from genotype 2 isolates.

Thirty four isolates were used in this study that had been subjected to whole genome sequencing and nucleotide polymorphism typing, and that represented the major *M. haemolytica* genotypes 1 ($n = 23$) and 2 ($n = 11$) and subtypes 1b ($n = 6$), 1b recombinant ($n = 1$), 1c ($n = 3$), 1e ($n = 2$), and 1f ($n = 4$) and 1i ($n = 7$), 2b ($n = 6$), 2c ($n = 2$), 2d ($n = 1$) and 2e ($n = 2$) (Table 1).

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Table 1

Mannheimia haemolytica strains isolated from cattle that were utilized to gather MALDI-TOF mass spectra for analysis. The state and year in which the isolates were obtained, and their classification into major genotypes and minor subtypes are included.

Isolate Minilims number	State or province	Year of isolation	Genotype	Subtype
28284	Kentucky	2013	1	b
28454	Kentucky	2013	1	b
56333	Tennessee	2013	1	b
28229	Kentucky	2013	1	b
33924	Kansas ^a	2013	1	b
34142	Kansas ^a	2013	1	b
33749	Kansas ^a	2013	1	b recombinant with genotype2
23580	Missouri	2013	1	c
23533	Missouri	2013	1	c
23570	Missouri	2013	1	c
23217	Tennessee	2013	1	e
23473	Tennessee	2013	1	e
22604	Missouri	2013	1	f
28240	Kentucky	2013	1	f
39434	Kansas ^a	2013	1	f
33204	Kansas ^a	2013	1	f
23299	Tennessee	2013	1	i
23311	Tennessee	2013	1	i
23348	Tennessee	2013	1	i
28226	Kentucky	2013	1	i
23309	Tennessee	2013	1	i
33982	Kansas ^a	2013	1	i
34180	Kansas ^a	2013	1	i
1590	Nebraska	2010	2	b
1621	Nebraska	2011	2	b
1562	Idaho	2010	2	b
32563	Kansas ^a	2013	2	b
32635	Kansas ^a	2013	2	b
33170	Kansas ^a	2013	2	b
23456	Tennessee	2013	2	c
32864	Kansas ^a	2013	2	c
28488	Kentucky	2013	2	d
1629	Nebraska	2011	2	e
22549	Missouri	2013	2	e

^a Animal from which the strain was isolated was shipped to Kansas from sale barn location in either Kentucky, Missouri, or Tennessee.

For MALDI-TOF MS, isolates were received on chocolate agar following two passages from frozen stocks. Initial characterization utilized a previously described smear method (Khot et al., 2012). Briefly, a single colony of each isolate was smeared onto a stainless steel target in duplicate, covered in α -cyano-4-hydroxycinnamic acid in 50% acetonitrile–2.5% trifluoroacetic acid (HCCA Matrix Bruker Daltonics) and allowed to dry. Mass spectra from each isolate were acquired and compared to a database using a software package (Bruker MALDI Biotyper Compass). Data were collected between 2 K and 20 K m/z using automated detection and spectrum collection in linear mode (Microflex Bruker Daltonics). The instrument was calibrated using the autocalibration function prior to each run using bacterial test standards (Bruker Daltonics). Each spectrum collected was a sum of 500 laser shots collected in increments of 100 shots. All isolates were identified as *M. haemolytica* to the species level with scores of ≥ 2.3 (out of a maximum of 3).

To generate experimental and technical replicates, the isolates were serially passaged twice on 5% sheep's blood agar (Remel, Lenexa, KS) at 37 °C in a 5% CO₂ atmosphere. After 18 h incubation, isolated colonies were subjected to extraction using the manufacturer's recommended procedure using ethanol and formic acid (Khot et al., 2012). One μ L of each isolate was applied to a stainless steel target in quadruplicate wells (technical replicates) and allowed to dry and overlaid with 1 μ L HCCA matrix. Data was collected as above using three repeat measurements on the quadruplicate wells for a total of 12 replications per isolate. This process was repeated three times independently with fresh subcultures for a total of 36 mass spectra collected for each isolate.

Spectra from each isolate were then evaluated for consistency over the technical and biological replicates using FlexAnalysis 3.0 software (Bruker Daltonics). Spectra from each isolate were subjected to baseline subtraction and background smoothing features, and evaluated for any

individual spectra with peak mismatches or flatline spectra, which were subsequently removed from the dataset. Spectra were then uploaded into ClinProTools 3.0 (Bruker Daltonics) software for biomarker discovery. An initial "training set" of spectra was used to generate initial data for comparison which consisted of 4 technical replicates and a single biological replicate. Isolates utilized in this training set included 34142 genotype 1b, 23570 genotype 1c, 23217 genotype 1e, 22604 genotype 1f, 28226 1i, 32635 genotype 2b, 23456 genotype 2c, 28488 genotype 2d, and 22549 genotype 2e. These isolates were selected due to the availability of high quality closed genome data. Comparisons were made between each major genotype and subtype and a "Peak Statistic Table" was generated for each comparison. Peaks were found using this tool with the presence of a peak 9494 Da found in all genotype 2 strains and absent in all genotype 1, and a 9523 Da peak detected in all of genotype 1 strains and absent in all genotype 2 (Fig. 1). To develop an objective biomarker based model to enable consistent automated classification of genotypes, spectra were subjected to the "Quick Classifier" (QC) model in ClinProTools with parameters using p value "tta" as sort mode. The model overall used the presence or absence of a single biomarker peak with a mass of 9476.97–9509.73 with a weight of 8.47 as the sole classifier between genotypes. Other models, including the supervised neural network (SVNN) and genetic algorithm (GA) also identified and utilized the same biomarker peak alone (SVNN) or in combination with other peaks (GA) (data not shown). The QC model was selected due to simplicity, and was then saved and subjected to validation using representatives from each of the genotypes. After validation correctly classified all of the isolates into genotype 1 and genotype 2, the classification tool was used to classify all technical and biological replicates collected from each isolate, including those isolates and spectra in the training set. Overall, the model correctly classified all

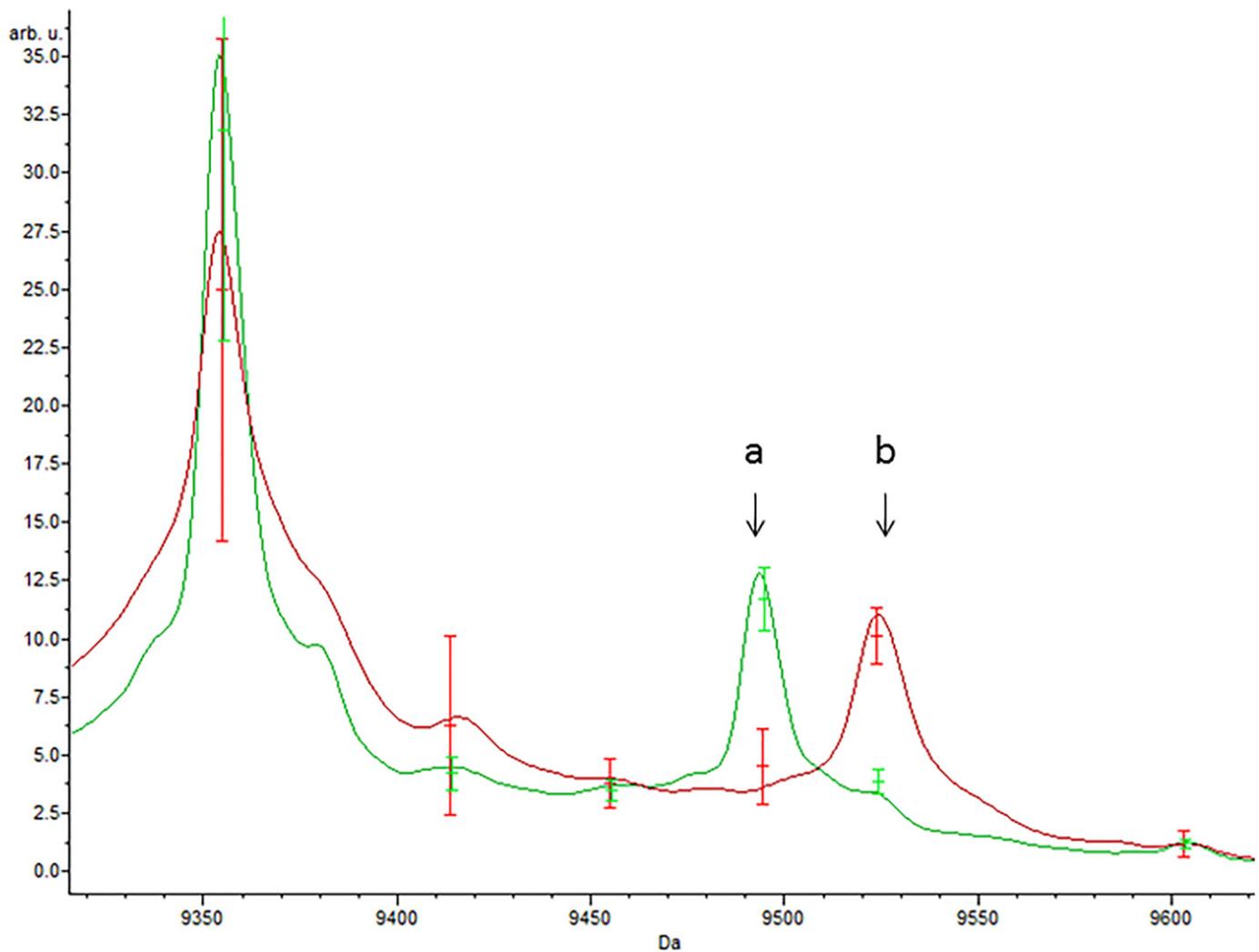


Fig. 1. Mass spectrum peaks differentiating *Mannheimia haemolytica* genotype 1 (red $n = 23$) from genotype 2 (green $n = 11$) as analyzed by MALDI-TOF MS. Bars indicate standard error. X-axis is mass of ions in Da and y-axis is the absolute intensity of the ions in arbitrary units (arb. u.) Peak distinguishing genotype 2 (a) is 9494 Da and peak distinguishing type genotype 1 (b) is 9523 Da. Mean peak mass represents 36 spectra collected from each isolate over 3 independent experiments.

of the isolates into group 1 and group 2 when the majority of each individual replicate spectrum was used for overall classification. The recombinant 1b2 genotype was classified as genotype 1. There were some individual spectra, the most of which was 8/36, for isolate 33170 Type 2b, where misclassification from type 2 into type 1 was noted. Other misclassifications included 23456 (1/36), 32635 (2/36), 32864 (1/36), 1562 (5/36), 1621 (1/36). This may have been due to slight variations due to error (≤ 300 ppm m/z tolerance used for calibration) or other variation in data collection. The model did have 100% agreement when using spectra generated from the direct smear method run in duplicate which is frequently used in diagnostic labs, to those run from ethanol and formic acid extraction, making this method rapidly implementable into clinical and diagnostic workflows. However, some variation may occur from run to run or between replicates, therefore technical replication should be included to ensure that misclassification does not occur.

In conclusion, this method using MALDI-TOF MS provides an effective, simple, and cost effective tool to rapidly type *M. haemolytica* into clinically and biologically relevant genotypes. Although the nature of the proteins being detected remains undetermined, future work to identify these proteins may provide insight into differences in virulence and pathogenicity of *M. haemolytica*. Further work may be required to ensure these findings apply to larger and more diverse collections,

however, the isolates utilized in this study represent the known genotypes and subtypes of *M. haemolytica*. This method should greatly enhance the ability of veterinary diagnostic labs to interpret *M. haemolytica* culture findings, especially from nasal swabs or the upper respiratory tract where both genotypes are likely to be present and interpretation can be challenging.

Financial conflicts of interest

Dr. Loy has served as a consultant for, and thus has disclosed a significant financial interest in Harrisvaccines. In accordance with its Conflict of Interest policy, the University of Nebraska-Lincoln's Conflict of Interest in Research Committee has determined that this must be disclosed.

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