NOTE *Bacteriology*

The use of MALDI-TOF mass spectrometry for rapid identification of *Mannheimia haemolytica*

Andrzej PUCHALSKI[1)]*, Renata URBAN-CHMIEL[1)], Marta DEC[1)], Diana STĘGIERSKA[1)] and Andrzej WERNICKI[1)]

[1)]*Sub-Department of Veterinary Prevention and Avian Diseases, Institute of Biological Bases of Animal Diseases, Faculty of Veterinary Medicine, University of Life Sciences, Akademicka 12, 20–033 Lublin, Poland*

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ABSTRACT. *Mannheimia haemolytica* is the most important bacterial pathogen isolated from cases of Bovine Respiratory Disease (BRD). Routine identification of these bacteria is usually performed using phenotypic methods. Our study showed that MALDI-TOF MS is a reliable alternative to these methods. All of the strains analyzed were identified as *M. haemolytica*. The identification results were compared to those obtained using conventional methods commonly used in microbiological diagnostics, based on detection and analysis of biochemical properties of microorganisms. The degree of agreement between the two methods for identifying *M. haemolytica* was 100%.
KEY WORDS: bovine respiratory disease, MALDI-TOF MS, *M. haemolytica*

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Bovine Respiratory Disease (BRD) is one of the most significant health problems in the rearing of calves and young feedlot cattle. Economic losses incurred by cattle farmers in the European Union as a result of this syndrome are estimated at about 600 million euros per year [4]. Their causes include reduced body weight gain, costs of prevention and treatment, and increased mortality rates in sick calves. In the complex etiology of the syndrome, both viruses and bacteria play an important role [9, 10]. Among these infectious agents, *Mannheimia haemolytica* merits particular attention, being responsible for the clinical forms of the syndrome with the most severe course and usually ending in a quick death. According to the classification developed by Angen *et al.* [3], the species *M. haemolytica* includes 12 capsular serotypes, of which the most frequently dominant isolates from disease cases are serotypes A1, A2 and A6, as well as atypical strains [14, 15, 17].

Given the multiple agents involved in the etiology of BRD and the importance of *M. haemolytica* in terms of the health of calves, rapid and accurate identification of these bacteria is essential for effective prevention and treatment of infected animals. Phenotypic tests commonly used in laboratory diagnostics to identify these bacteria are based on biochemical and serological analyses [2, 11]. Molecular biology methods based on analyses of genetic material are also used in diagnosis of *M. haemolytica*. Besides, DNA-DNA hybridization and 16S rRNA gene sequencing, which have been used for reclassification and identification of *Mannheimia* spp. [3], and pulsed-field gel electrophoresis (PFGE), which is considered the gold standard for genotyping of many species of bacteria [16]. Methods based on PCR reactions have also been developed: tRNA-intergenic spacer PCR [7], repetitive PCR [21], multiplex PCR [1] and real-time PCR [13].

A reliable alternative to phenotypic and genotypic techniques for identification of bacteria is MALDI-TOF MS (Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry). The method is based on analysis of bacterial proteins, mainly ribosomal, which are particularly abundant in the bacterial cells and are present in similar numbers of copies per cell. Each bacterial species contains a characteristic set of proteins called a molecular fingerprint [20]. During analysis carried out in a mass spectrometer, a spectrum of peaks is generated, corresponding to protein and peptide ions with varying mass-to-charge ratios. The protein spectrum obtained for the strain analyzed is compared with reference spectra, and then, the bacterium is automatically identified. Mass spectrometry is currently used successfully for rapid identification of a variety of bacterial species [6, 8, 18, 19].

The objective of this study was to evaluate the use of MALDI-TOF MS as a strategy of a single test enabling accurate and rapid identification of *M. haemolytica* strains isolated from cases of respiratory disease in calves.

The study used 63 strains of *M. haemolytica*, of which 54 were field isolates obtained in 2004–2012 from calves with clinical symptoms of BRD. The animals came from 40 breeding environments in different regions of Poland. The morphology of the bacterial colonies was evaluated on Columbia agar with 5% sheep blood (BTL, Lodz, Poland) following overnight incubation at 37°C in atmospheric air. Catalase-positive, Gram-negative rods growing in the form of small cream-colored colonies surrounded by a small hemolysis zone were chosen for further analysis. The isolates were stored at −80°C in brain heart infusion broth with 15%

*CORRESPONDENCE TO: PUCHALSKI, A., Sub-Department of Veterinary Prevention and Avian Diseases, Institute of Biological Bases of Animal Diseases, Faculty of Veterinary Medicine, University of Life Sciences, Akademicka 12, 20–033 Lublin, Poland.
e-mail: andrzej.puchalski@up.lublin.pl

glycerol until analysis.

To confirm the accuracy of identification, 9 reference strains of *M. haemolytica* were also analyzed: serotype A1 P 588, serotype A2 P 499, serotype A5 P 501, serotype A6 P 502, serotype A7 P 503, serotype A8 P 504, serotype A9 P 505 and *M. haemolytica* non-typeable A0164 obtained from the Federal Institute for Consumer Health Protection and Veterinary Medicine (BgVV, Jena, Germany), and *M. haemolytica* serotype A1 ATCC BAA-410 (LGC Standards, Dziekanow Lesny, Poland).

Preliminary identification of the isolates was performed by evaluating their phenotypic properties according to Angen *et al*. [2]. The biochemical analysis included determination of the ability of bacteria to ferment $_{L}$-arabinose, $_{D}$-xylose, mannitol, $_{D}$-sorbitol, maltose and trehalose and to produce urease, indole, aesculin, ODC (ornithine decarboxylase), NPG (β-glucosidase), ONPF (α-fucosidase), ONPG (β-galactosidase) and ONPX (β-xylosidase). The analysis was carried out using Diatabs® Diagnostic Tablets (Rosco Diagnostica, Emapol, Gdansk, Poland).

Serological evaluation of the isolated strains was conducted by indirect hemagglutination [12]. Antisera against reference serotypes 1, 2 and 5–9 were raised in rabbits according to the immunization schedule described by Biberstein [5].

The analysis was performed on 24 hr bacterial cultures on solid Columbia Agar (BTL), identified in the phenotypic tests as *M. haemolytica*, and on reference strains. Single colonies were transferred to a metal plate with 384 spots-MTP AnchorChip™ T F stainless steel MALDI target plate. Two spots were assigned to each strain. Next, 1 *µl* of 70% aqueous formic acid solution (Sigma-Aldrich, Poznan, Poland), and after it dried 1 *µl* of HCCA matrix solution (α-cyano-4-hydroxycinnamic acid, Sigma-Aldrich), at a concentration of 10 mg/m*l*, dissolved in 50% acetonitrile (Sigma-Aldrich), and 2.5% TFA (trifluoroacetic acid, Sigma-Aldrich), were layered onto the samples. The samples were analyzed in an UltrafleXtreme MALDI-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with a 1,000 Hz laser Nd-YAG (neodymium-doped yttrium aluminium garnet). Prior to the actual analysis, spectra were calibrated using a standard calibration mixture containing peptides and proteins isolated from an *E. coli* DH5 alpha strain (Bruker Daltonics). The spectrum of the bacteria was collected in a positive linear mode in a mass range from 2 to 20 kDa. Each sample was analyzed in 3 repetitions. The mass spectra were processed with the MALDI Biotyper 3.0 software package containing 3,995 reference spectra. The results were shown as the top 6 identification matches with confidence scores ranging from 0.000 to 3.000. According to the criteria proposed by the manufacturer, a log (score) below 1.700 does not allow for reliable identification; a log (score) between 1.700 and 1.999 allows identification to the genus level; a log (score) between 2.000 and 2.299 means highly probable identification at the genus level and probable identification at the species level; and a log (score) higher than 2.300 (2.300–3.000) indicates highly probable identification at the species level. Bruker's recommended criteria for identification were expanded in this study, with species identification accepted whenever the three best matches with the MALDI Biotyper database were identical. Data are representative of two independent experiments. For all repetitions of individual reference and field strains, we calculated the mean log (score) and the standard deviation. The identification results obtained by mass spectrometry were compared to the results obtained by the biochemical method. The number of strains with identical species identification was analyzed. Agreement of identification was expressed as the number of strains with identical identification obtained by mass spectrometry in comparison with the biochemical method. The degree of identification agreement between the two methods was expressed as a percentage, where the total number of strains analyzed was 100%.

Table 1. The mean log (score) of field strains obtained in MALDI-TOF MS

M. haemolytica	Log (score)		
	2.300–3.000	2.000–2.299	1.700–1.999
A1	27	16	0
A2	0	2	2
A6	3	2	0
non-typable	0	2	0
Total	30 (55.6%)	22 (40.7%)	2 (3.7%)

On the basis of the biochemical reactions, 54 field isolates were classified as *M. haemolytica*. They were characterized by the ability to ferment $_{D}$-xylose, mannitol, $_{D}$-sorbitol and maltose and by production of ONPF (α-fucosidase). On the basis of the results of the serological reaction, the strains were assigned to 4 serotypes. The most frequently occurring serotype (43 isolates) was A1, five isolates were classified as serotype A6, and four as serotype A2. Two isolates, characterized by negative reactions in the serological test, were classified as non-typeable strains.

For all reference strains used in our study, MALDI-TOF MS confirmed that they belonged to the species *M. haemolytica.* For seven reference strains, the log (score) ranged from 2.300 to 3.000, and for two, it ranged from 2.000 to 2.299. In the case of the field strains, as many as 96.3% attained a log (score) of over 2.000, which made it possible to identify them as *M. haemolytica*. In the case of two strains identified as *M. haemolytica* serotype A2 after phenotypic testing, the log (score) was 1.741 and 1.959 (Table 1). Despite the log (score) <2.000, we identified these strains as *M. haemolytica* as well, because the best three matches obtained for them indicated this species. Analysis of the spectra corresponding to the protein and peptide ions of individual *M. haemolytica* strains, ranging from 2 to 20 kDa, reveals that both the position of the main peaks and their mass-to-charge ratios exhibit a high degree of similarity between strains (Fig. 1).

We found high, 100% identification agreement between mass spectrometry and the biochemical method. However, in comparison with conventional phenotyping methods, MALDI-TOF MS is much faster, and labor and reagent costs are lower [8]. The suitability of MALDI-TOF MS for rapid

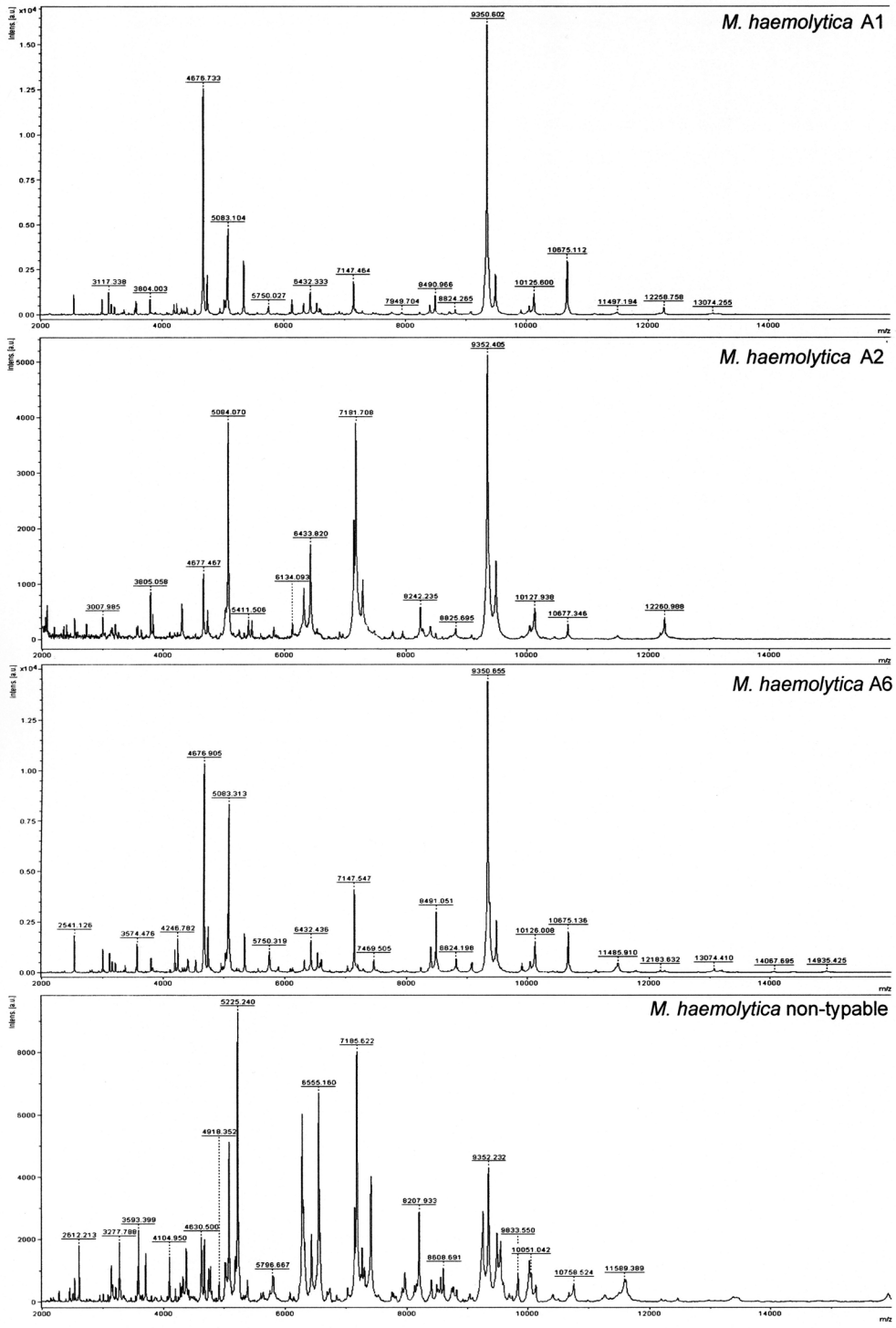

Fig. 1. Selected mass spectra of *M. haemolytica* strains.

identification of many species of bacteria isolated from animals, including *M. haemolytica*, has only been confirmed in a few publications [18, 19, 22]. Wragg *et al*. [22] compared the results of identification of 100 different reference strains, including nine strains of *M. haemolytica,* using MALDI-TOF MS, 16S rRNA gene sequencing and the Biolog GEN III microplate system. The performance of these three different systems for identifying bacteria to species was found to

be similar. Bizzini *et al*. [6] also showed that MALDI-TOF MS has the potential to reduce the need for molecular identification techniques, such as 16S rRNA gene sequencing, and might replace these time-consuming and expensive techniques for the majority of difficult-to-identify isolates in the clinical microbiology laboratory.

To sum up, MALDI-TOF MS based on identification of ribosomal proteins provides an inexpensive and rapid method for identifying *M. haemolytica*, which is essential for confirming infections. Longer, more labor-intensive and more expensive techniques can be reserved for the small group of isolates not identified with high probability by MALDI-TOF MS. Moreover, mass spectrometry offers the possibility of performing large-scale epidemiological testing pertaining to the contribution of *M. haemolytica* in clinical cases of bovine respiratory disease, thereby establishing a basis for effective prophylactic strategies.

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