Advance Publication

The Journal of Veterinary Medical Science

Accepted Date: 1 Apr 2012
J-STAGE Advance Published Date: 13 Apr 2012

Note

Laboratory Animal Scinece

Rapid identification of *Mycoplasma pulmonis* isolated from laboratory mice and rats using matrix-assisted laser desorption ionization time-of-flight mass spectrometry

Running tite: Identification of *M.pulmonis* by MALDI-TOF MS

Kazuo Goto[1,2*], Mikachi Yamamoto[3], Miwa Asahara[2], Takashi Tamura[2], Mitsuru Matsumura[1,2], Nobuhito Hayashimoto[4], and Koichi Makimura[2,3].

Department of Clinical Laboratory Medicine, School of Medical Technology[1], Graduate school of Medical Technology[2], Laboratory of Space and Environmental Medicine, Graduate School of Medical Technology[3], Teikyo University, 2-11-1 Kaga, Itabashi-ku, Tokyo 173-8605, and Central Institute for Experimental Animals, ICLAS Monitoring Center, 1430 Nogawa, Miyamae-ku, Kawasaki, Kanagawa 216-0001[4], Japan.

*Corresponding author
Kazuo GOTO (e-mail: gotok@med.teikyo-u.ac.jp)
Department of Clinical Laboratory Medicine, School of Medical Technology, Teikyo University, 2-11-1 Kaga, Itabashi-ku, Tokyo 173-8605
tel:03-3964-1211 (ex 44552)
fax:03-5944-3354

Abstract

Mycoplasma species identification is based on biochemical, immunological, and molecular methods that require several days for accurate identification. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) is a novel method for identification of bacteria and has recently been introduced into the clinical microbiology laboratory as a rapid and accurate technique. This method allows a characteristic mass spectral fingerprint to be obtained from whole inactivated mycoplasmal cells. In this study, we evaluated the performance of the MALDI-TOF MS for the identification of *Mycoplasma* by comparison with standard sequence analysis of 16S rRNA. We developed the first database of MALDI-TOF MS profiles of *Mycoplasma* species, containing *Mycoplasma pulmonis*, *M. arthritidis*, and *M. neurolyticum*, which are the most common pathogens in mice and/or rats, and species-specific spectra were recorded. Using the database, 6 clinical isolates were identified. Six tracheal swabs from 4 mice and 2 rats were cultured on PPLO agar for 4 to 7 days, and the colonies were directly applied to analyze the protein profiles. Five strains were identified as *M. pulmonis*, and 1 strain from a mouse was identified as *M. neurolyticum* (spectral scores were >2.00); the results were consistent with the results of the 16S rRNA gene sequence analysis (homologies > 97.0%). These data indicate that MALDI-TOF MS can be used as a clearly rapid, accurate, and cost-effective method for the identification of *M. pulmonis* isolates, and this system may represent a serious alternative for clinical laboratories to identify *Mycoplasma* species.

Key words: MALDI-TOF MS, mouse, *Mycoplasma pulmonis*, rat

Mycoplasma infection causes severe problems in laboratory rodent colonies [1]. The Mycoplasma species isolated from mice and rats, *M. pulmonis [13], M. arthritidis* [6], *M. neurolyticum* [7], *M. muris* [12], and *M. collis* [5], have been associated with several disease manifestations. *M. pulmonis* is an especially common pathogen in mice and rats and the etiological agent of murine respiratory mycoplasmosis [11]. For diagnosis of *M. pulmonis* infection, serological testing, culture methods, and polymerase chain reaction (PCR) assays have been used [3]. The culture method is one of the most common and specific methods to detect bacteria; however, a laborious step is needed to identify the colonies [4], such as morphological studies, reversion studies, or the sterol requirement and metabolism inhibition test. Recently, sequence analysis of the 16S rRNA gene has also been used as a simple method for identification of the bacteria [16].

More recently, matrix-assisted laser desorption ionization time–of-flight mass spectrometry (MALDI-TOF MS) has been applied to analyze the protein composition and used for direct identification of pathogens in the culture agar and/or broth [8,14,15]. However, there has been limited work thus far on the use of MALDI-TOF MS for the identification of pathogens in laboratory animals. We demonstrate that MALDI-TOF MS can be used in the clinical laboratory for rapid and accurate identification of *M. pulmonis* from solid culture media.

M. pulmonis (strain m53) [9], *M. arthritidis* (PG6, ATCC19611), and *M. neurolyticum* (type A, ATCC19988) were used to construct a database for MALDI-TOF MS. Media for cultivation of *Mycoplasma* strains have been described previously [4]. Briefly, an agar medium containing 70 mL of PPLO broth (BD, MD, USA) supplemented with 10 mL of 25% (*w*/*v*) yeast extract, 20 mL of heat-inactivated horse serum (Invitrogen, CA, USA), 1 mL of 100,000 units of penicillin G, 1 mL of 2.5%

thallium acetate, and 1.3% Bacto agar (BD, MD, USA) was used. The agar medium was incubated at 37°C for 4 to 7 days in a humid chamber.

Four and 2 tracheal swabs were collected from the mice and rats, respectively. The animals were derived from different animal facilities. The swabs were inoculated on the PPLO agar and cultured for 4 days. Identification of isolates was performed by 16S rRNA sequence analysis.

To determine the 16S rRNA sequence of mycoplasma isolates, a region of the 16S rRNA sequence was amplified with conserved universal primers F (5′-AAT GGG TGA GTA ACA CGT A-3′) and R (5′-TGT ACA AAA CCC GAG AAC GT-3′), and the expected size of the PCR products was 1289 bp. The PCR products were directly sequenced using the Taq DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems, Inc., CA, USA). The sequences were compared with a DNA database using the DNA Data Bank of Japan homology search system (http://vip05.nig.ac.jp/index-e.html).

Identification of a colony on the PPLO agar plate was performed by depositing a thin smear of bacterial suspension on a MALDI steel plate. A saturated solution of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile and 2.5% trifluoroacetic acid was applied to the bacterial smear and dried. Measurements were performed with a Microflex mass spectrometer (Bruker Daltonics, Wissembourg, France) using the FlexControl software (version 3.0). Mass spectra were acquired in a linear positive extraction mode ranging from 2,000 to 20,000 Da. The spectrum was imported into the BioTyper software (version 2.0; Bruker, Karlsruhe, Germany). The degree of spectral concordance is expressed as a logarithmic identification score and interpreted according to the manufacturer's instructions: scores > 2.00 indicated species identification with a high level of confidence.

The spectra of *M. pulmonis*, *M. arthritidis*, and *M. neurolyticum* are shown in Fig. 1. Each peak represents the mass of an intact protein detected in the analysis, whereas the height of the peak depends on the amount of proteins measured. The spectrum peaks were different depending on each mycoplasma species.

The spectra of 6 isolates were compared with the results of the reference strains, *M. pulmonis*, *M. arthritidis*, and *M. neurolyticum*. A representative spectrum of an isolate identified as *M. pulmonis* (m53) is shown in Fig. 2. As shown in Table 1, high level identification (spectral scores > 2.00) was obtained for 5 isolates with *M. pulmonis* (m53). The sequence analysis of these 5 isolates showed high homology with *M. pulmonis* m53 (>97.0%). The 1 remaining isolate from the mice trachea was identified as *M. neurolyticum* (spectral scores > 2.00 with *M. neurolyticum* type A). The DNA sequence of the isolate was 100% homologous with *M. neurolyticum* type A.

MALDI-TOF MS, which is sensitive and efficacious, has been applied to analyze the protein composition of bacteria [8, 14, 15]. The method provides an easy-to-handle system to identify different bacterial pathogens and is useful in the determination of bacterial species. Databases for bacterial cluster analysis have now been established, although most of the databases are for human pathogens. In this study, we applied the system to laboratory animal pathogens, and we developed an initial reference database of *Mycoplasma* species. *M. pulmonis*, *M. arthritidis*, and *M. neurolyticum* are the most common pathogens in laboratory mice and rats. *M. pulmonis* in particular is epizootic in conventional animal facilities [2, 10]. To date, *M. pulmonis* has been identified by morphological and biochemical analyses. The 16S rRNA sequence analysis is one of the most powerful tools to identify bacterial species, including *Mycoplasma*; however, the analysis is costly and it is time-consuming to obtain the results. An *M. pulmonis*

smear was treated with a saturated solution of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile and 2.5% trifluoroacetic acid before measurements by a Microflex mass spectrometer. Because the organisms can be inactivated by treating them with the acid, the spread of pathogens within diagnostic laboratories can be restricted.

In this study, we developed the *Mycoplasma* species database for laboratory animals. Six clinical isolates could be identified by MALDI-TOF MS directly from a colony on a PPLO agar plate in 20 min without any ambiguity, and the results were completely consistent with sequence analysis. Once a MALDI-TOF MS database of a bacterial species is established, any isolates can be identified using the database. One of the 6 isolates was identified as *M. neurolyticum*. The mouse infected with *M. neurolyticum* showed no clinical signs, and only one colony was obtained on the PPLO agar. Using traditional phenotype-based diagnostic methods, the propagation step by PPLO broth was required to identify the bacteria from a colony. The serial passage of the *Mycoplasma* strains did not affect the MALDI-TOF MS results (data not shown). On the other hand, the results from the liquid medium (PPLO broth) were not as good as those obtained from the agar medium (data not shown). The materials in the broth, such as horse serum or yeast extract, might have interfered with the analysis. Identification of *Mycoplasma* from liquid growth media should be studied for using of this technology.

It is reported that *M. pulmonis* is closely related to *M. agassizzii*, which is isolated from desert tortoises by using 16S rRNA sequence analysis. We didn't compare the MALDI-TOF MS spectrum of *M. agassizzii* with that of *M. pulmonis*, because the purpose of this study was to identify murine mycoplasma species.

In conclusion, our results indicate that identification of *M. pulmonis* using

MSLDI-TOF MS was consistent with a molecular method of *Mycoplasma* identification. Furthermore, the identification of the bacteria by MALDI-TOF MS was clearly much more rapid than that by molecular methods, and MALDI-TOF is more cost-effective than the traditional methods, such as morphological studies, the metabolism inhibition test, and the immunological test. The current database generated by the results of the MALDI-TOF MS method for laboratory animals needs to be expanded. Once the database is more fully developed, we believe that MALDI typing will provide a suitable tool for diagnosing laboratory animal pathogens.

References.

1. Cassell, G. H., J. R. Lindsey, J. K. Davis, M. K. Davidson, M. B. Brown, and J. G. Mayo. 1981. Detection of natural *Mycoplasma pulmonis* infection in rats and mice by an enzyme linked immunosorbent assa (ELISA). *Lab. Anim. Sci.* **31**:676-682.
2. Easterbrook, J. D., J. B. Kaplan, G. E. Glass, J. Watson, and S. L. Klein. 2008. A survey of rodent-borne pathogens carried by wild-caught Norway rats: a potential threat to laboratory rodent colonies. *Lab. Anim.* **42**:92-98.
3. Goto, K., S. Kunita, E. Terada, and T. Itoh. 1994. Comparison of polymerase chain reaction and culture methods for detection of *Mycoplasma pulmonis* from nasal, tracheal and oral swab samples of rats. *Exp. Anim.* **43**:413-415.
4. Goto, K., T. Itoh, S. Ebukuro, N. Kagiyama, and R. Harasawa. 1993. Characterization of *Mycoplasma* strains isolated from house musk shrew (*Suncus murinus*) and their infectivity in mice and rats. *Exp. Anim.* **42**:363-369.
5. Hill, A. C. 1983. *Mycoplasma collis,* a new species isolated from rats and mice. Int. *J. Syst. Bacteriol.* **33**:847-851.
6. Hill, A. and G.J.R. Dagnall. 1975. Experimental polyarthritis in rats produced by *Mycoplasma arthritidis*. *J. Comp. Path.* **85**:45-52.
7. Hottle, G. A. and D. N. Wright. 1996. Growth and survival of *Mycoplasma neurolyticum* in liquid media. *J. Bacteriol.* **91**:1834-1839.
8. Kok, J., L. C. Thomas, T. Olma, S. C. A. Chen, and J. R. Iredell. 2011. Identification of Bacteria in blood culture broths using Matrix-assisted laser desorption ionization sepsityperTM and time of flight mass spectrometry. *PLoS ONE.* **6**:e23285-

9. Kunita, S., E. Terada, K. Goto, and N. Kagiyama. 1990. Sensitive detection of Mycoplasma pulmonis by using the polymerase chain reaction. *Exp. Anim.* **39**:103-107.

10. Liang, C., A. Shih, Y. Chang, C. Liu, Y. Lee, W. Hsieh, Y. Huang, W. Huang, X. Kuang, K. Lee, Y. Zhuo, S. Ho, S. Liao, Y. Chiu, C. Hsu, S. Liang, and C. Yu. 2009. J. Am. Assoc. *Lab. Anim. Sci.* **47**:381-386.

11. Lindsey, J. R. and H. Cassell. 1973. Experimental *Mycoplasma pulmonis* infection in pathogen-free mice. Models for studying mycoplasmosis of the respiratory tract. *Am. J. Pathol.* **72**:63-90.

12. McGarrity, G. J., Rose, D. L., Kwiatkowski, V., Dion, A. S., Phillips, D. M., and Tully, J. G. 1983. *Mycoplasma muris*, a new species from laboratory mice. *Int. J. Syst. Bacteriol.*, **33**:350-355.

13. Nelson, J. B. and M. J. Lyons. 1965. Phase-contrast and electron microscopy of murine strains of *Mycoplasma*. *J. Bacteriol.* **90**:1750-1763.

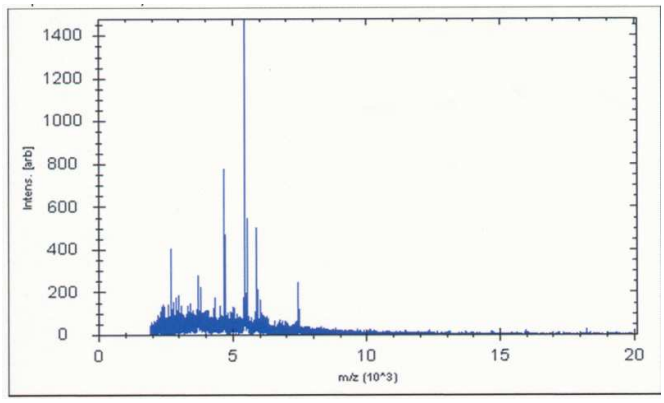
14. Saleeb, P. G., S. K. Drake, P. R. Murray, and A. M. Zelazny. 2011. Identification of Mycobacteria in solid-culture media by Matrix-assisted laser desorption ionization-time of flight mass spectrometry. *J. Clin. Microbiol.* **49**:1790-1794.

15. Szabados, F., J. Woloszyn, C. Rchter, M. Kaase, and S. Gatermann. 2010. Identification of molecularly defined Staphlococcus aureus strains using matrix assisted laser desorption/ionization time of flight mass spectrometry and the Biotyper 2.0 database. *J. Med. Microbiol.* **59**:787-790.

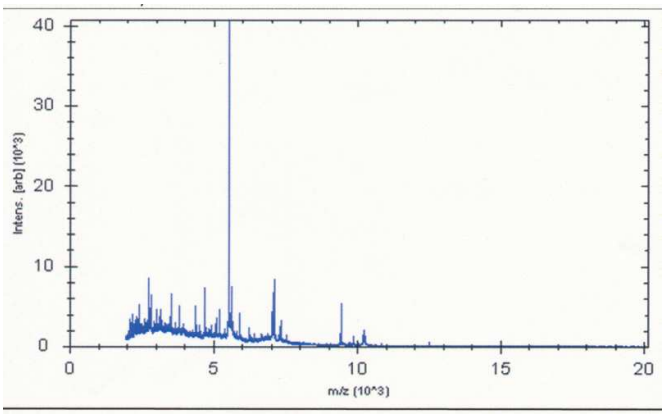
16. Woo, P. C., S. K. Lau, J. L. Teng, H. Tse, and K. Y. Yuen. 2008. Then and now: use of 16S rDNA gene sequencing for bacterial identification and discovery of novel bacteria in clinical microbiology laboratories. *Clin. Microbiol. Infect.* **14**:908-934.

Fig. 1. Overview of the mass spectra for *Mycoplasma pulmonis* (A), *M. arthritidis* (B),and *M. neurolyticum* (C)
Each peak represents the mass of an intact protein detected during the analysis, while the height of the peak depends on the amount of protein measured.

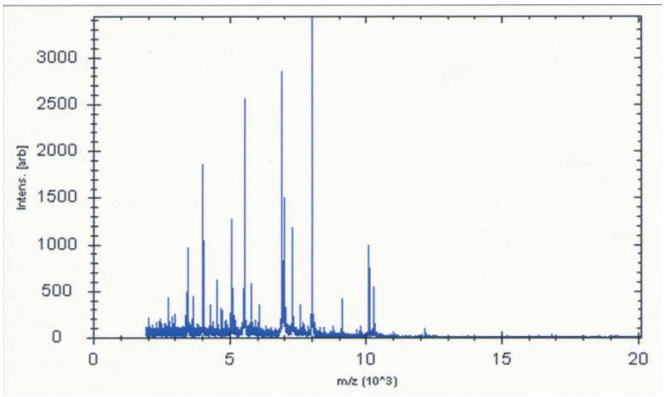
Fig. 2. Spectrum of an isolate (sample no. 1; up) with the database results for *Mycoplasma pulmonis* (m53) (down), with a score of 2.011 (A).



(A)



(B)



(C)

Fig. 1. Overview of the mass spectra for *Mycoplasma pulmonis* (m53) (A), *M. arthritidis* (PG6) (B),and *M. neurolyticum* (Type A) (C)

Each peak represents the mass of an intact protein detected during the analysis, while the height of the peak depends on the amount of protein measured.

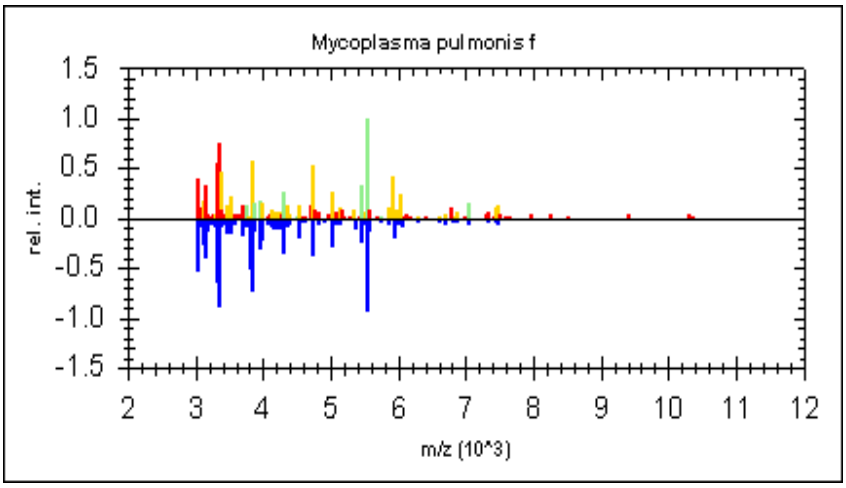


Fig. 2. Spectrum of an isolate (sample no. 1; up) with the database results for *Mycoplasma pulmonis* (m53) (down), with a score of 2.011

Table 1. MALDI-TOF MS identification of isolates compared to 16S rRNA sequence analysis

Isolate No.	Identification by 16S rRNA sequence (Homology %)		MALDI-TOF MS score against each species of Mycoplasma		
			M. pulmonis (m53)	*M. arthiritidis* (PG6)	*M. neurolyticum* (Type A)
1	*M. pulmonis*	(99.0%)	2.011	1.739	1.495
2	*M. pulmonis*	(99.0%)	2.228	1.693	1.497
3	*M. pulmonis*	(100.0%)	2.213	1.510	1.581
4	*M. pulmonis*	(97.0%)	2.256	1.685	1.575
5	*M. pulmonis*	(97.0%)	2.004	1.459	1.473
6	*M. neurolyticum*	(100.0%)	1.519	1.822	2.004