Microbiological Research 166 (2011) 77—86

Available online at www.sciencedirect.com

ScienceDirect

Microbiological Research

www.elsevier.de/micres

Rapid discrimination of *Legionella* by matrix-assisted laser desorption ionization time-of-flight mass spectrometry

Yoshihito Fujinami*, Hitomi S. Kikkawa, Yohei Kurosaki, Koichi Sakurada, Mineo Yoshino, Jiro Yasuda

National Research Institute of Police Science, 6-3-1, Kashiwanoha, Kashiwa 277-0882, Japan

Received 18 November 2009; received in revised form 16 February 2010; accepted 20 February 2010

KEYWORDS
Legionella pneumophila;
Pulsed-field gel electrophoresis;
Matrix-assisted laser desorption ionization time-of-flight mass spectrometry

Summary

Molecular typing is an important tool in the surveillance and investigation of human *Legionella* infection outbreaks. In this study, two molecular typing methods, pulsed-field gel electrophoresis (PFGE) and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS), were used to discriminate 23 *Legionella pneumophila* strains. The usefulness of MALDI-TOF-MS was demonstrated. The MALDI-TOF-MS fingerprinting with filtered small acid-soluble molecules gave different molecular profiles among strains, and the clustal analysis with MALDI-TOF-MS showed a high discrimination of strains the same as that with PFGE. In addition, MALDI-TOF-MS data could be generated within a few hours after the initial culture, although PFGE analyses took several days to complete. Thus, MALDI-TOF-MS offers a simple and rapid discrimination technique that could aid in the tracking of fast-spreading outbreaks of *Legionella*.

Introduction

Legionella pneumophila, the causative agent of Legionnaires' disease and the type species of the genus *Legionella*, was first recognized in 1976, following an epidemic of airborne acute pneumonia among veterans of the American Legion in Philadelphia, Pennsylvania. The findings led to the discovery of a new species and genus (Brenner et al., 1979). Since then, this genus has become

*Corresponding author. Tel.: +81 471 35 8001; fax: +81 471 33 9159.
E-mail addresses: fujinami@nrips.go.jp (Y. Fujinami), kikkawa@nrips.go.jp (H.S. Kikkawa), kurosaki@nrips.go.jp (Y. Kurosaki), sakurada@nrips.go.jp (K. Sakurada), yoshino@nrips.go.jp (M. Yoshino), yasuda@nrips.go.jp (J. Yasuda).

0944-5013/$ - see front matter © 2010 Elsevier GmbH. All rights reserved.
doi:10.1016/j.micres.2010.02.005

very large, with 56 species currently recognized (http://www.dsmz.de/microorganisms/bacterial_nomenclature_info.php?genus=LEGIONELLA). In 2002, a large outbreak of Legionellosis occurred in a bathhouse with spa facilities in the Miyazaki Prefecture of Japan. Two hundred and ninety-five patients (including suspected cases) who had pneumonia and/or symptoms of fever, cough, and so forth were reported; 37% of them were hospitalized and seven people died (Okada et al., 2005).

Currently, pulsed-field gel electrophoresis (PFGE) is the most commonly employed molecular typing technique in epidemic bacterial infections (Liu et al., 1999; Matsumoto et al., 2005; Terajima et al., 2002; Watanabe et al., 2002). In addition, PFGE is used to clarify the route and source of not only large-scale but also localized infections with *Legionella* disease (Amemura-Maekawa et al., 2005). Thus, PFGE is very sensitive to subtle variations in the genome of an organism. However, PFGE has some limitations, including the length of time required to obtain results and the lack of reproducibility of electrophoresis images (Mitsuda et al., 1998).

In recent years, matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) has become a valuable tool for the identification of numerous microbes due to improvements in the device and associated software (Maier et al., 2006). The accuracy and speed of data acquisition make this method a potentially important tool for the study of biological public health hazards, quality control in food processing, blood screening, and disease diagnosis (Camara and Hays, 2007; Mazzeo et al., 2006; Parisi et al., 2008). Mass spectra obtained from unidentified bacterial samples can be correctly identified through comparison with spectra from reference libraries of known organisms (Maier et al., 2006). However, there are no reports that this method has been used to discriminate *Legionella* strains. In addition, few studies comparing results from MALDI-TOF-MS and PFGE for other bacteria exist in the literature (Arnold et al., 2005; Barbuddhe et al., 2008). Therefore, MALDI-TOF-MS is expected to be a simple, rapid, and safe method to analyze extracts of *Legionella* species and strains. In this study, we examined the usefulness of MALDI-TOF-MS compared with that of PFGE in identifying 23 *Legionella* strains.

Materials and methods

Reference strain

Ten reference strains of *L. pneumophila* and eight strains of other *Legionella* species (*L. bozemanii*, *L. micdadei*, *L. dumoffii*, *L. gormanii*, *L. longbeachae*, *L. jordanis*, *L. anisa*, and *L. feeleii*) were obtained from the American Type Culture Collection (ATCC). These strains are shown in Table 1.

Collection of water samples from hot springs

Thirteen waters samples were obtained from two hot springs, Tone and Numata, in Japan (Table 1). Two spas (F spot and M spot) were 20 km apart. Isolation of *Legionella* from natural water samples was performed by culture according to the Detection Manual of Pathogenic Microbes (http://www.nih.go.jp/niid/reference/pathogen-manual-60.pdf) (Okada et al., 2005). One-liter samples of water were concentrated by filtration on a 0.22 μm pore-diameter polycarbonate membrane (Nalgene). After filtration, bacteria collected on the membranes were resuspended in 10 ml of water, and 0.1 ml of the suspension was spread on a 90 mm Petri dish containing Buffered Charcoal Yeast Extract (BCYE) agar supplemented with vancomycin, polymyxin B, cycloheximide, and glycine (GVPC medium) (Merck). The inoculated plates were then incubated for 7 days at 37 °C. Smooth colonies showing a grayish-white color were counted as suspicious legionellae to be confirmed. Up to six to seven colonies of suspected *Legionella* were subcultured onto BCYE agar, BCYE agar without L-cysteine, and blood agar for verification. The isolated colonies growing only on BCYE agar were determined to be *Legionella* colonies. Pure cultures of *Legionella* were enumerated by spreading onto BCYE medium (without antibiotics). Furthermore, it was confirmed that an isolate did not grow up on blood agar plates. All strains were exactly harvested at 4 days after inoculating for PFGE and MALDI-TOF-MS analysis.

Confirmation by specific polymerase chain reaction (PCR) method

The isolated colony was resuspended in 1 ml of autoclaved ultrapure water. In the case of bacterial cells from a fresh culture, they were suspended at only about 10^8 cells/ml in autoclaved ultrapure water. To release the DNA directly from bacterial cells, the suspension was frozen at −80 °C, heated in boiling water for 5 min, and supplemented with 1/10 volume of protease K (1 mg/ml) and lysis buffer (100 mM Tris–HCl [pH 8.3], 50 mM KCl, 15 mM $MgCl_2$, 0.1% Tween 20). After 1 h of incubation at 55 °C, the solution was frozen at −80 °C and then heated in boiling water for 5 min. Extracted DNA was tested by PCR using the *L. pneumophila*