BRAZILIAN JOURNAL OF MICROBIOLOGY XXX (2018) XXX–XXX

BRAZILIAN JOURNAL OF MICROBIOLOGY

http://www.bjmicrobiol.com.br/

Bacterial, Fungal and Virus Molecular Biology

Identification of pathogenic and nonpathogenic *Leptospira* species of Brazilian isolates by Matrix Assisted Laser Desorption/Ionization and Time Flight mass spectrometry

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ARTICLE INFO

Article history:
Received 20 November 2016
Accepted 21 March 2018
Available online xxx

Associate Editor: Roxane Piazza

Keywords:
Leptospira
Brazil
Identification
Mass spectrometry
MALDI-TOF

ABSTRACT

Matrix Assisted Laser Desorption/Ionization and Time of Flight mass spectrometry (MALDI-TOF MS) is a powerful tool for the identification of bacteria through the detection and analysis of their proteins or fragments derived from ribosomes. Slight sequence variations in conserved ribosomal proteins distinguish microorganisms at the subspecies and strain levels. Characterization of *Leptospira* spp. by 16S RNA sequencing is costly and time-consuming, and recent studies have shown that closely related species (e.g., *Leptospira interrogans* and *Leptospira kirschneri*) may not be discriminated using this technology. Herein, we report an in-house *Leptospira* reference spectra database using *Leptospira* reference strains that were validated with a collection of well-identified Brazilian isolates kept in the Bacterial Zoonosis Laboratory at the Veterinary Preventive Medicine and Animal Health Department at Sao Paulo University. In addition, *L. interrogans* and *L. kirschneri* were differentiated using an in-depth mass spectrometry analysis with ClinProTools™ software. In conclusion, our in-house reference spectra database has the necessary accuracy to differentiate pathogenic and non-pathogenic species and to distinguish *L. interrogans* and *L. kirschneri*.

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https://doi.org/10.1016/j.bjm.2018.03.005

Introduction

Leptospirosis is a mammalian zoonosis caused by *Leptospira* strains belonging to the order Spirochaetales. Mammals, including humans, are affected by different clinical manifestations, depending on the virulence, motility, and ability of the leptospiral pathogen to survive in the host. Susceptibility to infection is dependent on age, genetic factors and skin integrity during the infection. *Leptospira* biology and leptospirosis physiopathology were comprehensively presented and discussed in a recent publication.[1] The antigenic diversity among serovars differentiates pathogenic (*Leptospira interrogans*) and non-pathogenic or saprophytic (*Leptospira biflexa*) species.[2] At least 22 species have been classified by molecular techniques.[2–4] The microscopic agglutination test (MAT) is the most commonly used diagnostic method in the clinic; however, limitations have been previously reported and discussed.[3,5] The characterization of *Leptospira* spp. using molecular techniques such as 16S RNA sequencing is costly and time-consuming,[6] especially taking into account the large number of microorganisms identified in the clinical practice. This method depends on one or several target genes, however the data for all the peptides with a mass range of 2–20 kDa could be collected using MALDI-TOF MS as demonstrated by Xiao et al.[7] on molecular fingerprinting of pathogenic and non-pathogenic *Leptospira*. MALDI-TOF MS is a well-established technique for the rapid characterization of bacteria, and its use is continuously increasing.[8] This technology can differentiate microorganisms' species by the analysis and comparison of proteins or protein fragments derived from ribosomes. It is important to note that slight sequence variations in conserved ribosomal proteins are sufficient to distinguish microorganisms at the subspecies and strain levels.[8] MALDI-TOF MS has been proposed to be a powerful tool for the identification of *Leptospira* at the species level.[6–8,10] However, the misidentification of *L. interrogans* as *L. kirschneri* by MALDI-TOF MS has been described, and potential biomarkers to differentiate these species have been investigated.[10] In the present paper, we focused on (i) the characterization of pathogenic and non-pathogenic *Leptospira* species of a *Leptospira* Brazilian collection using MALDI-TOF MS after creating an in-house database and (ii) the differentiation of *L. interrogans* from *L. kirschneri* by in-depth mass spectrometry analysis.

Material and methods

Leptospira strains and isolates

Thirty-one reference leptospiral strains and 22 field isolates belonging to pathogenic (*Leptospira interrogans*, *Leptospira borgpetersenii*, *Leptospira kirschneri*, *Leptospira noguchii* and *Leptospira santarosai*) and non-pathogenic (*Leptospira biflexa*) species were analyzed. The *Leptospira* isolates were recovered from bovine, dog, human, *Rattus norvegicus*, and *Rattus rattus* urine samples taken from Sao Paulo, Rio de Janeiro and Londrina (Table 1). The strains and isolates were maintained in the Laboratory of Bacterial Zoonosis – School of Veterinary Medicine and Animal Science/University of Sao Paulo (USP) and stored in Fletcher semi-solid medium (Fletcher Medium Base, Difco™, NJ, USA) at 30 °C. The species of the field isolates were previously identified by 16S rRNA sequencing (data not shown).

Sample preparation for MALDI-TOF analysis

The strains and isolates were grown and diluted (1:25) for seven days at 30 °C in Ellinghausen-McCullough-Johnson-Harris medium (EMJH Difco™, NJ, USA), and bacterial cells were counted using a Petroff Hausser counting chamber (HS Hausser Scientific, Horsham, PA) by dark field microscopy. *Leptospira* cultures were centrifuged at 11,000 × *g* for 10 min at room temperature, and the pellet was washed twice with 3 mL of phosphate buffered saline (PBS) and suspended in sterile deionized water to a final bacterial concentration of 1×10^8 organisms per mL. Ethanol/formic acid protein extraction was performed by addition of 300 μL of the culture into 900 μL of ethanol (99.8%, PA) followed vortexing and 10-min of incubation. This inactivation procedure was followed by a 10-min centrifugation at 11,000 × *g* at room temperature, the supernatant was removed, and the pellet was air dried until the ethanol was completely evaporated. This process was repeated and the material was then dissolved in 30 μL of 70% formic acid (Sigma–Aldrich) followed by the addition of 30 μL of acetonitrile (Fluka Analytical Sigma–Aldrich, Munich, Germany). Centrifugation was performed at 11,000 × *g* for 2 min at room temperature. Two microliters of the clear supernatant were spotted on a 384 target polished steel plate (Bruker Daltonik GmbH, Bremen, Germany) and allowed to dry. Following this, the dried spot was overlaid with 2 μL of matrix solution, a saturated solution of α-Cyano-4-hydroxycinnamic acid (HCCA, 99% Bruker Daltonik GmbH, Bremen or Sigma–Aldrich, Munich, Germany) (10 mg/mL) in acetonitrile (Fluka Analytical Sigma–Aldrich) and 0.1% trifluoracetic acid (1:2) (TFA-Reagent PlusW 99%, Sigma–Aldrich). Finally, samples were allowed to dry at room temperature. *Escherichia coli* DH5α was used as a positive control, and a non-inoculated matrix solution was used as a negative control. During data acquisition, it was observed that some isolates underwent osmotic lysis in deionized water, which was corrected by replacing sterile deionized water by saline solution (0.85% NaCl) buffered with Sorensen's solution (69 mM Na_2HPO_4/8 mM NaH_2PO_4, pH 7.6).[9] This solution has lower osmolarity than PBS, but kept cells intact without interfering with the ionization of the bacterial proteins as well as the mass fingerprint of our previously data that were generated in saline solution. Additional mass spectra were then obtained with fresh culture passages to ensure the minimum number of spectra for the generation of single Main Spectrum Profiles (MSP).

Instrument settings for MALDI-TOF MS analysis

A Microflex LT™ (Bruker Daltonics, Bremen, Germany) instrument was used with the software Flex Control™ version 3.4 (Bruker Daltonics). For mass calibration and instrument parameter optimization bacterial test standard (BTS, Bruker Daltonics) was used. The acquisitions were done in linear positive mode within a mass range from 2000 to 20,000 *m/z* with the manufacturer's suggested settings in automated collecting spectra mode.

Please cite this article in press as: Karcher D, et al. Identification of pathogenic and nonpathogenic *Leptospira* species of Brazilian isolates by Matrix Assisted Laser Desorption/Ionization and Time Flight mass spectrometry. *Braz J Microbiol*. (2018), https://doi.org/10.1016/j.bjm.2018.03.005