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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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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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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.

Table 1 – Leptospira strains used as reference for MALDI-TOF MS measurements.

Specie	Serogruop	Serovar	Strain	Pathogenicity
Leptospira borgpetersenii	Ballum	Castellonis	Castellon 3	
Leptospira borgpetersenii	Celledoni	Whitcombi	Whitcombi	
Leptospira borgpetersenii	Javanica	Javanica	Veldrat Batavia 46	
Leptospira borgpetersenii	Mini	Mini	Sari	
Leptospira borgpetersenii	Sejroe	Hardjo	Hardjobovis	
Leptospira borgpetersenii	Tarassovi	Tarassovi	Perepelitsin	
Leptospira interrogans	Australis	Australis	Ballico	
Leptospira interrogans	Australis	Bratislava	Jez Bratislava	
Leptospira interrogans	Autumnalis	Autumnalis	Akiyami A	
Leptospira interrogans	Bataviae	Bataviae	Van Tienen	
Leptospira interrogans	Canicola	Canicola	Hond Utrecht IV	
Leptospira interrogans	Djasiman	Sentot	Sentot	
Leptospira interrogans	Hebdomadis	Hebdomadis	Hebdomadis	Pathogenic
Leptospira interrogans	Icterohaemorrhagiae	Copenhageni	M-20	
Leptospira interrogans	Icterohaemorrhagiae	Icterohaemorrhagiae	RGA	
Leptospira interrogans	Pomona	Kennewicki	Fromm	
Leptospira interrogans	Pomona	Pomona	Pomona	
Leptospira interrogans	Pyrogenes	Pyrogenes	Salinem	
Leptospira interrogans	Sejroe	Hardjo	Hardjoprajitno	
Leptospira interrogans	Sejroe	Wolffi	3705	
Leptospira kirshneri	Autumnalis	Butembo	Butembo	
Leptospira kirshneri	Cynopteri	Cynopteri	3522C	
Leptospira kirshneri	Grippotyphosa	Grippotyphosa	Moskova V	
Leptospira noguchi	Panama	Panama	CZ 214K	
Leptospira santarosai	Shermani	Shermani	1342 K	
Leptospira biflexa	Andamana	Andamana	CH 11	
Leptospira biflexa	Andamana	Bovedo	Bovedo	
Leptospira biflexa	Doberdo	Rufino	RPE	Non-pathogenic
Leptospira biflexa	Garcia	Garcia	Garcia	
Leptospira biflexa	Nazare	Nazare	Nazaré	
Leptospira biflexa	Seramanga	Patoc	Patoc-1	

Collection at the Bacterial Zoonoses Laboratory, Department of Veterinary Preventive and Animal Health of School of Veterinary Medicine and Animal Science, São Paulo University, Brazil.

All spectra were analyzed by standard pattern-matching algorithm using the MALDI Biotyper™ 3.1 software (Bruker Daltonics), and the raw spectra were compared with the reference spectra of the Bruker library (database version 3.3.1, 5627 reference spectra) with default settings. The ID criteria used was the recommended by the manufacturer: – a score ≥2.000 indicated species, a score between 1.700 and 1.999 indicated genus level and a score <1.700 was interpreted as no ID. For MainSpectra (MSP) and dendrogram construction, flat-liners and bad quality spectra were removed and additional measurements were carried out to obtain 20 spectra from each isolate/strain. Spectra were then loaded into Biotyper™ 3.1 software (Bruker Daltonics) for MSP creation and dendrogram clustering construction with the default settings (distance measure: correlation; linkage: average; score oriented). Each spot was measured in 1000-shot steps for a total of 4000 laser shots. Preparation of the BTS and calibration were performed following the manufacturer's instructions: calibration was successful when proteins of the mass spectra were in a range of ±200 parts per million (ppm).

In-house database and dendrogram construction

For each of the 31 strains, 30 individual spectra were used to create a MSP. Flat-liners and bad quality spectra were removed, and additional measurements were carried out to obtain 30 spectra from each isolate/strain. The MSP was obtained using MALDI-Biotyper software (Bruker Daltonics, Germany) and then loaded into the Bruker Daltonics database (version 3.1.2.0). Software settings for MSP creation were set to maximal mass error of each single spectrum: 2000; desired mass error for the MSP: 200; desired peak frequency minimum (%): 25; and maximal desired peak number of the MSP: 70. Dendrogram clustering was constructed with the default setting of 160 (distance measure: correlation; linkage: average; score oriented).

Determining the efficiency of the database search with Leptospira field isolates

Four mass spectra of each field isolate were obtained and challenged against our in-house *Leptospira* database. The results

were expressed in log score values, with values ≥2 indicating reliable species identification and values from 1.7 to 2.00 indicating reliable genus identification.

Differentiation of Leptospira interrogans and Leptospira kirschneri using ClinProTools™

ClinProTools™ (Bruker Daltonics) generates multiple mathematical algorithms to generate pattern recognition models for the classification and prediction of different classes (e.g., *L. interrogans* class 1, *L. kirschneri* class 2) from mass spectrometry-based profiling data. Various spectra of the different serovars (03 serovar to *L. kirschneri* and 12 serovar to *L. interrogans*) were used for each class, seeking to standardize the data for species distinction. Moreover, ClinProTools™ provides a list of peaks sorted according to the statistical significance to differentiate between both classes.[12] Thus, to recognize mass spectra patterns and biomarkers between *L. interrogans* and *L. kirschneri*, spectra peak analysis models with ClinProTools™ software v.3.0 (Bruker Daltonics) were created from an additional 210 mass spectra of 11 *L. interrogans* (15 high-quality mass spectra per isolate) and 3 *L. kirschneri* (15 high-quality mass spectra per isolate) isolates. Spectra were pretreated with a resolution of 800 ppm, a mass range of 2000–20,000 Da, a top hat baseline subtraction with 10% minimal baseline width, enabling null spectra exclusion, recalibration with 500 ppm maximal peak shift and 30% match celebrant peaks. ClinProTools™ models (Bruker Daltonics) were generated using three algorithms: Genetic Algorithm (GA), Supervised Neural Network (SNN), and Quick Classifier (QC). For each model, the recognition capability (RC) and cross validation (CV) percentage were generated to demonstrate the reliability and accuracy of the model. RC and CV percentages are indicators of the model's performance and serve as useful predictors of the model's ability to classify test isolates. We also carried out principal component analysis (PCA) included in ClinProTools software aiming to visualize homogeneity and heterogeneity of the protein spectra of *L. interrogans* and *L. kirschneri*. Principal component analysis (PCA) and the results were shown in 3D score plot.

Single-peak analysis

For each peak, the AUC for the discrimination of the groups was directly obtained from the ClinProTools™ v.3.0 software (Bruker Daltonics). For the five peaks with the highest AUC, the detection performances were verified using FlexAnalysis™ v.3.4 (Bruker Daltonics). After smoothing and baseline subtraction, the mass lists for each isolate were obtained using the centroid algorithm with a signal-to-noise (SN) threshold of 0.5 and a maximum of 500 peaks and exported to Microsoft Excel. The SN ratios of the peaks with a tolerance of 1000 ppm were exported to SPSS version 18.0. ROC (Receiver Operating Characteristic) curves were constructed, and their optimal cutoff values were determined with the maximum Youden index.[11]

Results

Reference spectra were created for all 31 leptospiral strains and applied as unassigned MSPs in the commercially available MALDI Biotyper™ database spectra library, which lacks leptospiral protein profiles (Fig. 1). The MSPs were clustered according to pathogenicity in the MALDI-TOF MS dendrogram, and the pathogenic species (red) are clearly differentiated from the nonpathogenic *Leptospira* species (green) (Fig. 2). Similarly, the pathogenic *L. borgpetersenii* and *L. interrogans* are located in separate clusters, but, as expected, poor discrimination was obtained for *L. interrogans* and *L. kirschneri*.

Representative mass spectra of *L. interrogans* and *L. borgpetersenii* obtained by direct analysis and by protein extraction protocol are shown in Fig. 1. In A and C, mass spectra were obtained without protein extraction and peaks with low intensity were observed. In contrast, B and D show higher quality mass spectra obtained after protein extraction protocol, with peaks with higher intensity.

The 22 field isolates belonging to *L. biflexa*, *L. interrogans* and *L. santarosai* had the correct species assigned by MALDI-TOF MS, and all isolates showed score values over 2.0 (Table 2), where it is possible to identify all isolates by the correct species ID following our *in-house* database. The PCA reproduces through different statistical tests the reduction of several variables of a set of data, where each point represents a spectrum and each color represents a grouping of similar data. Fig. 3A presents the PCA for *L. interrogans* species in red and *L. kirschneri* in green, there is a perceptible distinction between the two groups even with some closer points showing that the PCA analysis does not guarantee a clear separation between the species. B presents the PCA for the serovars that formed the class *L. kirschneri* in A, a clear separation between the serovars is observed. C presents the PCA for the serovars that form the class *L. interrogans*, which shows that the group representing *L. interrogans* serovar Bataviae can be completely separated, since the other clusters cannot be separated.

The three classification models from ClinProTools™ showed RC values ≥90% in the discrimination of *L. interrogans* and *L. kirschneri*. The best results were provided by the GA model, with RC and CV values of 100% and 97%, respectively. Details of these values are shown in Table 3. The strain distribution maps based on the GA model show that *L. interrogans* and *L. kirschneri* can be distinguished based on their peptide mass fingerprints, the best separating peaks of the current statistic sort order are displayed in Fig. 4.

The peaks with the highest AUC (>0.9) to discriminate between *L. interrogans* and *L. kirschneri* using ClinProTools™ were 3074 *m/z*, 3090 *m/z*, 3118 *m/z*, 6710 *m/z* and 8059 *m/z*. However, the performances of these peaks for the discrimination of the two groups using the FlexAnalysis™ software validation showed that only the peak at 8059 *m/z* had an AUC > 0.9, with sensitivity and specificity of 98.1% and 95.5%, respectively. The SN cut-off values of the peak 8057 *m/z* peak for the discrimination of the for *L. interrogans* (below the cut-off) and for *L. kirschneri* (above the cut-off) was 7.0. The ClinProTools™

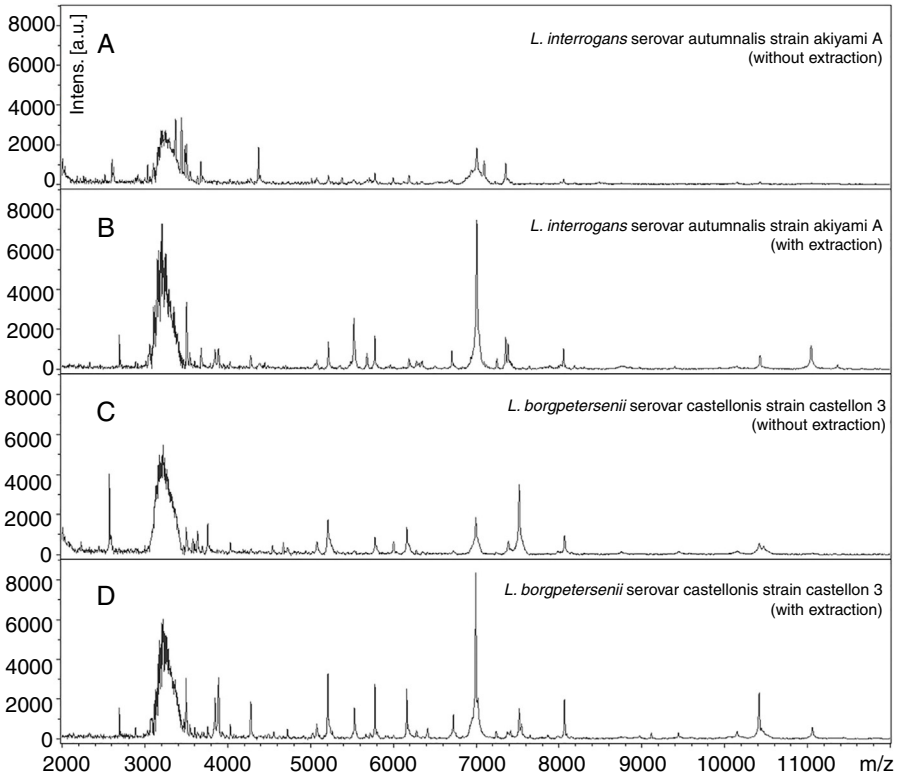

Fig. 1 – MALDI-TOF MS spectra obtained by analyzing the reference strains of *Leptospira interrogans* and *Leptospira borgpetersenii* with and without extraction as described in "Material and methods" section. These data show the importance of the protein extraction to obtain the better quality of spectra.

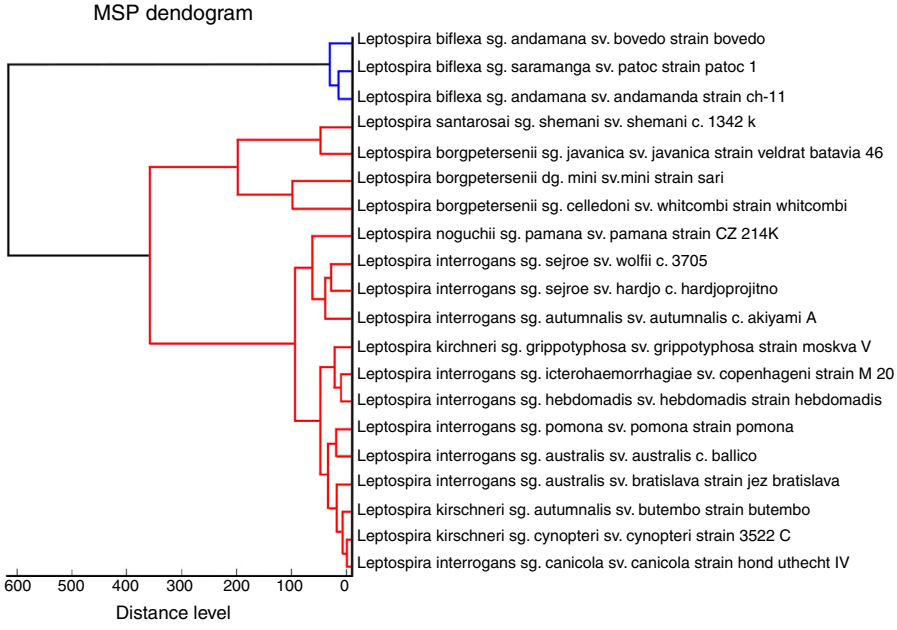

Fig. 2 – Comparison of the phylogenetic classification and MALDI-TOF dendrograms of the isolates of *Leptospira* spp. This figure contains only strains analyzed in this study.

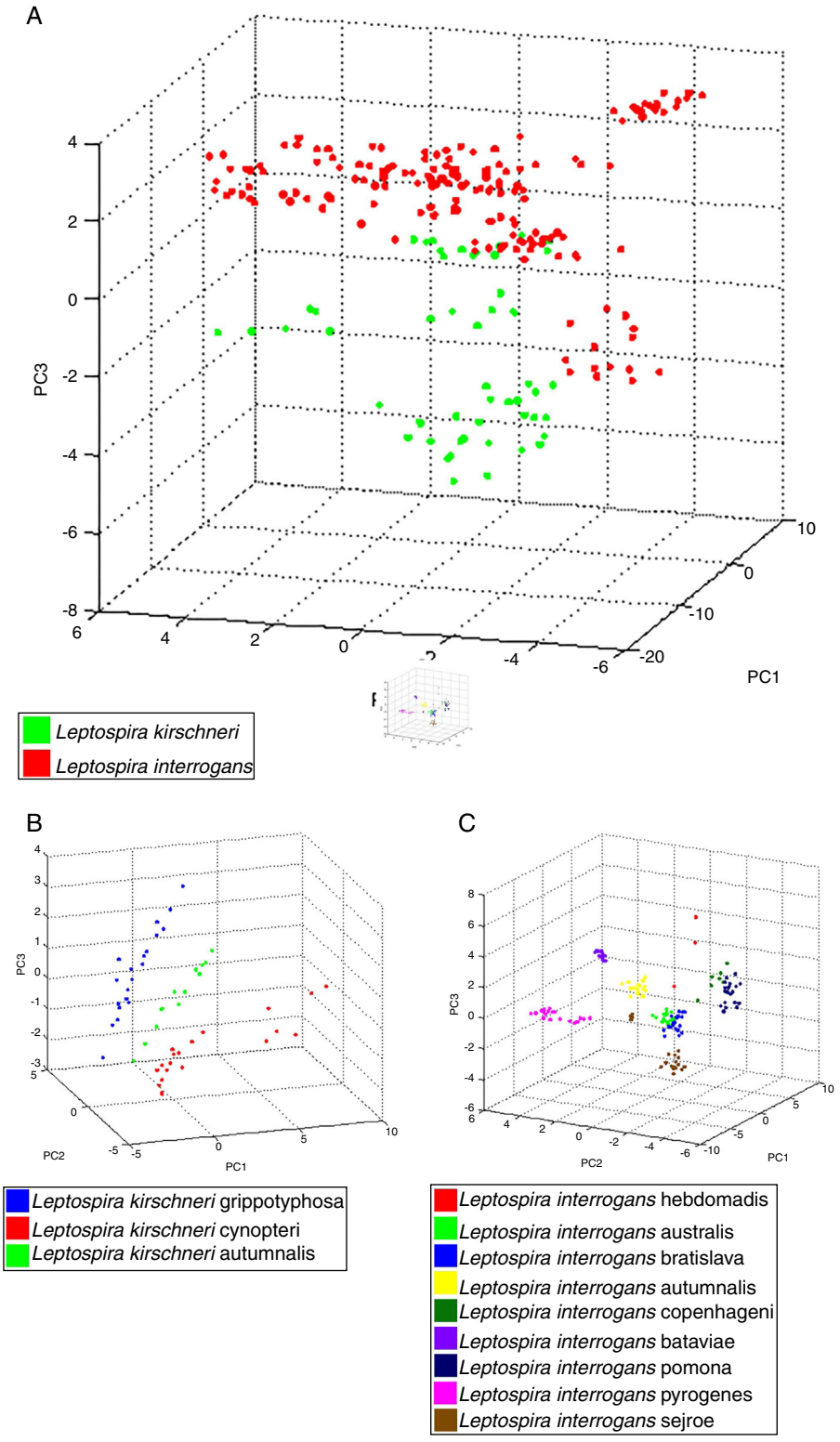

Fig. 3 – Principal component analysis (PCA) using tools ClinProTool™. In (A), PCA of strains analyzed, for data standardization by species, data from different serovars were used. In (B), we have PCA of different serovars of the *L. kirchneri* and in (C), we have PCA of different serovars of *L. interrogans*.

Table 2 – Identification results of 22 leptospiral field isolates by MALDI-TOF MS and 16S rRNA gene sequencing.

Strain identification	Genome species (16S rRNA Identification)	Serogroup	MALDI-TOF-MS Identification	
			Species	Score values
Ranarum	*L. biflexa*	Semaranga	*L. biflexa*	2.355
M85/06	*L. interrogans*		*L. interrogans*	2.565
M46/07	*L. interrogans*	Icterohaemorrhagiae	*L. interrogans*	2.070
M67/07	*L. interrogans*	Icterohaemorrhagiae	*L. interrogans*	2.535
M71/07	*L. interrogans*	Icterohaemorrhagiae	*L. kirschneri*	2.643
M5/90	*L. interrogans*	Icterohaemorrhagiae	*L. interrogans*	2.342
M64/06	*L. interrogans*	Icterohaemorrhagiae	*L. interrogans*	2.542
61H	*L. kirschneri*	Pomona	*L. kirschneri*	1.898
M110/06	*L. kirschneri*		*L. kirschneri*	1.866
16CAP	*L. meyeri*	Grippotyphosa	*L. meyeri*	2.828
19CAP	*L. meyeri*	Grippotyphosa	*L. meyeri*	3.000
LO9	*L. santarosai*		*L. santarosai*	2.574
M52/08-12	*L. santarosai*		*L. santarosai*	2.359
M52/08-19	*L. santarosai*		*L. santarosai*	1.833
U160	*L. santarosai*		*L. santarosai*	2.017
U164	*L. santarosai*	Tarassovi	*L. santarosai*	2.093
An776	*L. santarosai*	Bataviae	*L. santarosai*	2.366
10ACAP	*L. santarosai*	Grippotyphosa	*L. santarosai*	2.525
6BCAP	*L. santarosai*	Grippotyphosa	*L. santarosai*	2.457
21CAP	*L. santarosai*	Grippotyphosa	*L. santarosai*	2.614
M4/98	*L. santarosai*	Sejroe	*L. santarosai*	2.370
BOV 6	*L. santarosai*	Sejroe	*L. santarosai*	2.434

Collection at the Bacterial Zoonoses Laboratory, Department of Veterinary Preventive and Animal Health of School of Veterinary Medicine and Animal Science, São Paulo University, Brazil.

Table 3 – Complete results derived from the classification models.

Classification model	Cross validation (CV) (%)	Recognition capability (RC) (%)	Integration regions used for classification				
			Peak #1 (Da)	Peak #2 (Da)	Peak #3 (Da)	Peak #4 (Da)	Peak #5 (Da)
GA[a]	97.2	100.0	8057	4671	5472	8084	8305
SNN[b]	55.6	100.0	8057	8097	6710	8084	12,679
QC[c]	92.6	93.7	8057	–	–	–	–

Results obtained by analyzing of ClinProTools.
[a] Genetic Algorithm.
[b] Supervised Neural Network.
[c] Quick Classifier.

and single-peak analysis results for the differentiation of *L. interrogans* from *L. kirschneri* are summarized in Table 4 and exemplified in Fig. 5.

Discussion

During leptospirosis outbreaks, *Leptospira* species identification is an essential step for tracking and controlling the pathogen transmission. The determination of a serovar may be insufficient as different species may have the same serovar but may be distinct in their ability to cause mammalian infection.[13] DNA sequencing is an alternative method for the identification of *Leptospira* species, although it can be a costly, time-consuming and labor-intensive technique. MALDI-TOF MS has been successfully applied in the identification of *Leptospira* species, yielding comparable results to 16S rRNA sequencing, with fast, reproducible and less costly protocols.[6–8] However, the creation of an *in-house* MSP database with well-identified strains remains necessary until an updated commercial database with *Leptospira* MSPs becomes available. A score-oriented dendrogram produced by Biotyper™ software with 31 MSPs clustered the strains according to their pathogenicity clearly separated pathogenic and non-pathogenic *Leptospira* strains into different nodes. Our analysis reproduced the results that have been reported

Table 4 – Single-peak analysis for the discrimination of *L. kirschneri* and *L. interrogans*.

Peaks (*m/z*)	ClinProTools				FlexAnalysis				Sensitivity (%)	Specificity (%)
	AUC[a]	Dave[b]	PWKW[c]	PAD[d]	Ave[e]	Ave[f]	AUC[g]	Cutoff		
8059	0.99	3.68	0	0.00226	16.72	20.63	0.99	6.96	98	95
3090	0.92	2.90	<0.0001	0.000001	7.81	7.51	0.74	3.93	96	48
3074	0.91	4.14	<0.0000	<0.000001	7.13	8.04	0.83	5.41	100	59
3118	0.90	12.03	<0.0001	<0.000001	8.38	9.47	0.83	14.00	100	56
6710	0.87	2.85	0	<0.000001	14.26	16.36	0.87	3.22	85	86

Peaks with the best performances according to ClinProTools™ and FlexAnalysis™ softwares.

[a] AUC, area under the curve.

[b] Dave, difference between the maximal and the minimal average peak area/intensity of the groups.

[c] PWKW, *p*-value of Wilcoxon/Kruskal–Wallis test (range:0–1; 0 D good).

[d] PAD: *p*-value of Anderson–Darling test, <0.05 indicates data not normally distributed; gives information about the normal distribution (range: 0–1; 0 = not normally distributed).

[e] Ave, area/intensity average of a group from *Leptospira kirschneri*.

[f] Ave, area/intensity average of a group from *Leptospira interrogans*.

[g] AUCs and signal-to-noise cut off values were obtained from an ROC curve constructed using SPSS Version18.0 and FlexAnalysis.

by other centers that also constructed in house *Leptospira* MSP databases. Moreover, all field isolates had the correct species assigned, with scores above 2.0, which ensures the quality of our MSP database for *Leptospira* species ID. The distinction of *L. interrogans* and *L. kirschneri* using ClinProTools™ and single-peak analysis is also noteworthy. Although MALDI-TOF MS has already been successfully applied in *Leptospira* genus and species identification,[6–8] the misidentification of closely related species, such as *L. interrogans* and *L. kirschneri*, has also been reported and represents an important challenge in the implementation of MALDI-TOF MS for *Leptospira* identification. Here we observed that with proper analysis, *Leptospira* species can be distinguished based on their peptide mass fingerprints.

The ClinProTools™ software is a biomarker analyzer that has been widely applied in microbiology, providing a rapid and cost-saving method for epidemiological clustering, strain typing and subspecies identification.[14–16] Using both ClinProTools™ and single-peak analysis with FlexAnalysis™ has provided higher discriminatory power to detect biomarker peaks.[14–17] Our results corroborate previous findings that one isolate biomarker with 8000–8100 Da can effectively distinguish the closely related pathogenic species *L. interrogans* from *L. kirschneri*.[10] Indeed, we further described the SN cut-off value that has to be adopted to accurately differentiate these two taxa by a simple inspection of the mass spectrum. Recently, *L. kirschneri* serovar Mosdok was, for the first time, linked to human leptospirosis in the southern hemisphere; therefore, rapid species ID using MALDI-TOF MS may be the first step to implement control strategies.

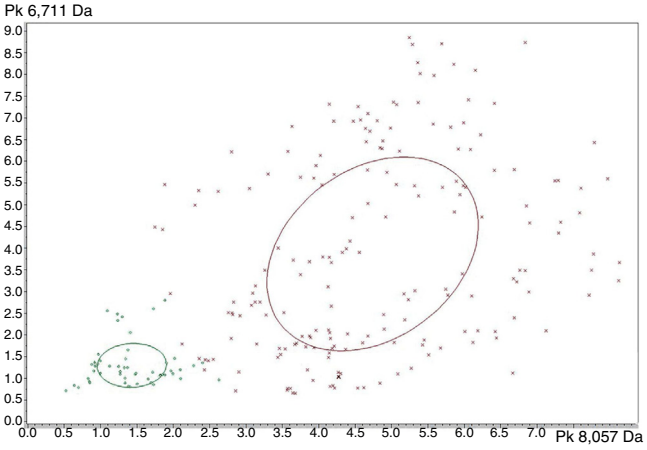

Fig. 4 – Strain distribution map corresponding to *Leptospira interrogans* (red) and *Leptospira kirschneri* (green). The *x*-axis shows the peak area/intensity values with respect to the most relevant peak (8057 Da) to distinguish *Leptospira interrogans* (red) from *Leptospira kirschneri* (green). The *y*-axis shows the peak area/intensity values with respect to the peak with (6711 Da) from *Leptospira interrogans* (red) and *Leptospira kirschneri* (green). The ellipses represent the spectra with greater distinction between the two groups, whereas prominent peaks in the *x*-axis and *y*-axis.

Conflicts of interest

The authors declare no conflicts of interest, even during the item proofs.

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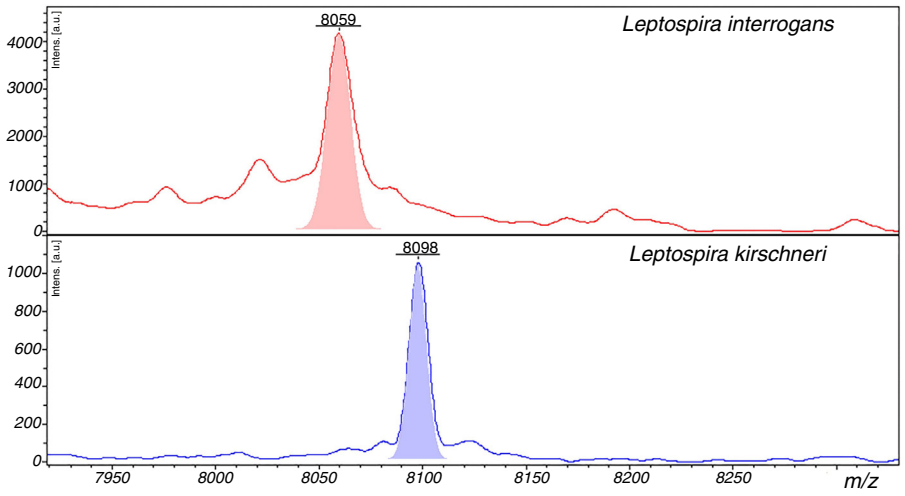

Fig. 5 – Representative spectra of *Leptospira interrogans* and *Leptospira kirschineri*. The representative peaks that allow differentiation of the strains in the spectra are showed, in red for *L. interrogans*, and in blue for *L. kirschneri*. The peak with *m/z* = 8059 in *L. interrogans* we detected as shown in Table 3 by ClinPro Tools analysis. The peak *m/z* = 8098 in *L. kirschineri* was previously identified by Rettinger et al.[10]

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