MALDI-TOF Mass Spectrometry for the research of specific biomarkers of *Leishmania* infection by analyzing strains isolated from biological samples

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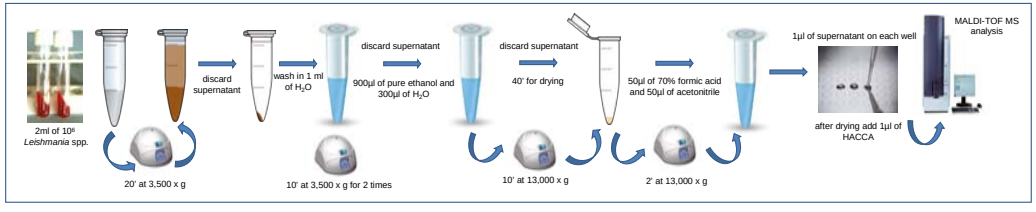
INTRODUCTION AND PURPOSE

MALDI-TOF Mass Spectrometry (MS) is widely recognized as a powerful tool for the identification of bacteria and fungi at the species level, but few data are available for protozoa. In parasitology, the application of MALDI-TOF MS has been limited to obtaining general parasitic proteome studies (i.e. *Echinococcus multilocularis*), to the characterisation of specific biomarkers for discriminating between environmental and human *Cryptosporidium* and *Giardia* species for water management and to subtype protozoa (i.e. *Blastocystis* spp.). However, MALDI-TOF MS was recently used to propose an approach able to identify *Leishmania* spp. at the species level from *in vitro* culture. The aim of this study was the investigation of the potential role of MALDI-TOF MS in the rapid identification of specific protein biomarkers of *Leishmania* spp. by analyzing strains isolated in our laboratory from clinical samples.

METHODS

In this study, we analysed two *Leishmania* strains isolated in our laboratory from skin biopsies of two patients with suspected cutaneous Old World Leishmaniasis: strain 1174 was previously characterized as *L. major* zymodeme MON25, and strain 1585 was previously identified as *Leishmania* spp. by a 18S-rDNA Real-time PCR assay able to differentiate, by melting temperature analysis, among clinically relevant *Leishmania* groups. Showing this strain the same melting peak as the strain 1174, it could be classified in the *Leishmania* group including *L. major*. In this study aliquots of *L. major* strain 1174 and *Leishmania* spp. strain 1585 cultivated in Evan's modified Tobies's medium were submitted to formic acid/acetonitrile protein extraction for MALDI-TOF MS analysis. Then, the strain 1585 was analyzed by MALDI-TOF MS to verify the presence/absence of the same protein peaks observed for strain 1174.



The MicroFlex LT mass spectrometer (Bruker Daltonics, Germany supplied by Becton Dickinson, Italy) was used for the analysis of the obtained protein extracts. A minimum of 20 replicates for each strain was analyzed in order to ensure the reproducibility of the results. The acquisition of the spectra was performed by the MBT_Standard method in manual mode. All the spectra were analyzed by FlexAnalysis software (Version 3.0 Bruker Daltonics, Germany) and subsequently imported into ClinProTools software version 2.2 (Bruker Daltonics, Germany) in order to verify the presence of specific *Leishmania* peaks to be used as molecular biomarkers. In order to compare the 2 strains, an equal number of spectra per strain was analyzed: statistical testing of the datasets was performed on the basis of Principal Component Analysis (PCA) and the results were displayed in a three-dimensional score plot, automatically generated by the software. The presence/absence of discriminating peaks was evaluated by the comparison of each average spectrum automatically created from the replicates of each strain and those of Evan's modified Tobies's medium.

RESULTS

The 2 strains yielded a protein profile (Fig. 1a) which was found to be reproducible over 4 independent experiments and no differences were observed when strains were grown in different lots of media. In Fig. 1b the plot of the spectra in a three-dimensional space is reported showing the replicates of the spectra of *L. major* strain 1174 and *Leishmania* spp. strain 1585 and the replicates of spectra of Evan's modified Tobies's medium as three separate clusters. The profiles obtained for each of the 2 *Leishmania* spp. strains analyzed in this study showed the presence of 3 specific peaks (9,692, 11,184 and, 13,700 Da) (Fig. 1c) that were not present in the medium used for their cultivation.

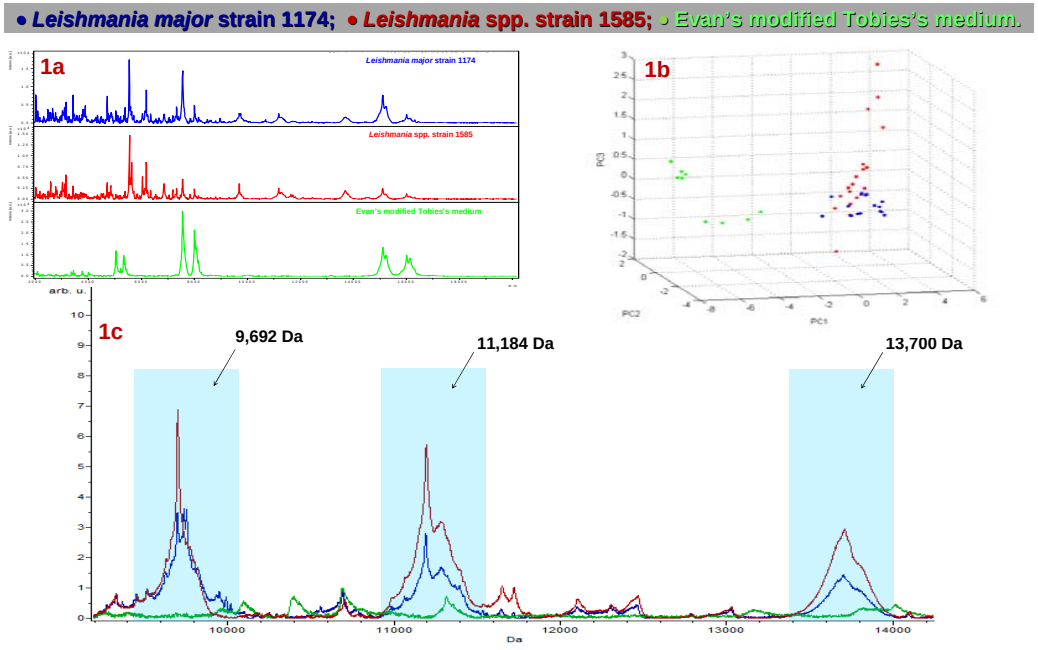


Fig. 1a. Spectra obtained by MALDI-TOF MS analysis; **Fig. 1b.** Principal Component Analysis (PCA); **Fig. 1c.** m/z window bins with the details of 3 discriminating peaks.

CONCLUSION

In our area, in a 22-year period (1992-2013), 15 cases of leishmaniasis (7 cases of visceral and 8 cases of cutaneous leishmaniasis) were diagnosed, corresponding to an infection rate of 11.2%. Despite the low rate of infection in our area, the severity of a disease as visceral lesihmaniasis and its documented spread in continental regions of Italy and Europe underline that suitable tools are mandatory for diagnosis. The detection of the same 3 peaks in both of the *Leishmania* strains analyzed (absent in the culture medium) may be useful for the identification of *Leishmania* species isolated from biological samples by MALDI-TOF MS. Our future goal will be to extend the analysis to other *Leishmania* spp. strains isolated in our laboratory in order to confirm the presence/absence of species-specific biomarkers for a possible rapid identification of *Leishmania* strains directly from biological samples.

References: 1) Martiny D, Bart A, Vandenberg O, Verhaar N, Wentink-Bonnema E, Moens C, van Gool T. Subtype determination of *Blastocystis* isolates by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS) (2013) Eur J Clin Microbiol Infect Dis DOI 10.1007/s10096-013-1980-z. 2) Schulz A, Mellenthin K, Schönian G, Fleischer B, Drosten C. (2003) Detection, differentiation, and quantitation of pathogenic *Leishmania* organisms by a fluorescence resonance energy transfer-based Real-time PCR assay. J. Clin. Microbiol. 41, 1529-1535; 3) Cassagne C, Pratlong F, Jeddi F, Benikhlef R, Aoun K, Normand AC, Faraut F, Bastien P, Piarroux R. (2013) Identification of *Leishmania* at the species level with matrix-assisted laser desorption ionization time-of-flight mass spectrometry. Clin Microbiol Infect. 10.1111/1469-0691.12387; 4) Calderaro A, Piccolo G, Montecchini S, Buttrini M, Gorrini C, Rossi S, Arcangeletti MC, De Conto F, Medici MC, Chezzi C (2013) MALDI-TOF MS analysis of human and animal *Brachyspira* species and benefits of database extension. J Proteom 78:273-280. 5) Calderaro A, Gorrini C, Piccolo G, Montecchini S, Buttrini M, Rossi S, Piergianni M, Arcangeletti MC, De Conto F, Chezzi C, Medici MC (2014) Identification of *Borrelia* Species after Creation of an In-House MALDI-TOF MS Database. PLoS One 12;9(2); 6) Calderaro A, Montecchini S, Rossi S, Gorrini C, Dell'Anna ML, Piccolo G, Medici MC, Arcangeletti MC, Chezzi C, De Conto F. (2014) A 22-year survey of leishmaniasis cases in a tertiary-care hospital in an endemic setting. Int J Environ Res Public Health. 11(3):2834-45.