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Identification of *Mycobacterium* Species by MALDI-TOF Mass Spectrometry

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Abstract

Matrix-assisted laser desorption ionization – time-of-flight (MALDI-TOF) mass spectrometry enables to identify microorganisms by comparison of the protein content with reference spectra in the database. The aim of this study was to evaluate the efficacy of phenotypic identification of mycobacteria by MALDI-TOF mass spectrometry in laboratory practice. Seventy five isolates of mycobacteria were identified by molecular and phenotypic method, and the results were compared by MALDI-TOF. For MALDI-TOF, material was processed according to the Bruker Daltonics protocol and Mycobacterial Library database version 2.0, with 313 reference mycobacteria spectra. All except one of the 72 isolates agreed with regard to the species and genus by both methods. Forty three isolates were identified as the *M. tuberculosis* complex by MALDI-TOF. Thirty one isolates of nontuberculous mycobacteria were consistently identified by both methods to the species level. We conclude that MALDI-TOF mass spectrometry is an accurate method of bacterial identification. Simplicity, speed, and economic availability of the method makes it suitable for mycobacteria identification in a routine laboratory.

Keywords

Bacterial isolates • Mass spectrometry • *Mycobacterium tuberculosis* • Phenotype bacterial identification • Protein identification

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1 Introduction

Mycobacterial infections are a public health problem causing significant morbidity and mortality (Zumla et al. 2013; Jarzembowski and Young 2008). Tuberculosis (TB) is contagious, airborne, and a top infectious killer worldwide, although it is a curable and preventable disease. A WHO report indicates that 9.6 million people (5.4 million men, 3.2 million women, and 1.0 million children) fell ill with *M. tuberculosis* and 1.5 million people died from tuberculosis in 2014. Rapid and accurate diagnosis of mycobacterial infections is important for initiating early treatment and for preventing drug resistance (Balada-Llasat et al. 2013).

It is essential to differentiate mycobacterial isolates to the species level to detect if they are true pathogens or environmental contaminants. Tuberculous mycobacteria belonging to the *Mycobacterium tuberculosis* complex are of notable clinical importance. Likewise, a large group of nontuberculous mycobacteria are of clinical interest, such slowly growing *M. avium-M. intracellulare* complex, *M. kansasii, M. marinum, M. xenopi, M. simiae,* and *M. ulcerans*, and rapidly growing *M. abscessus, M. chelonae*, and *M. fortuitum* (Balada-Llasat et al. 2013). Conventional methods for the identification of mycobacterial species are simple to perform, but they require extensive incubation period, as long as 12 weeks. Several new tests have become available for detection of *Mycobacterium* species. Molecular methods are rapid, but expensive and often exclusive to reference laboratories. Matrix-assisted laser desorption ionization – time-of-flight (MALDI-TOF) technology, used in mass spectrometry for the analysis of biomolecules, presents an alternative method for the identification and differentiation of mycobacteria. The method is increasingly on the rise as early and rapid identification of mycobacteria is essential for disease control (Dixon et al. 2015; Quinlan et al. 2015; Biswas and Rolain 2013; Tonolla et al. 2010). The increase in the use of MALDI-TOF is reflected in the outstanding rise in the number of publications on the subject, from one in 1995 to 395 in 2015.

The present study demonstrates the efficacy of MALDI-TOF mass spectrometry for the phenotypic identification of mycobacteria isolates from clinical material as compared with conventional methods. We also discuss the use of MALDI-TOF mass spectrometry for the diagnosis of fastidious bacteria, and the advantages and limitations of MALDI-TOF compared with other currently used identification methods in clinical laboratory.

2 Methods

The study was approved by the Ethics Committee of Jessenius Faculty of Medicine in Martin, Slovakia.

2.1 MALDI-TOF Mass Spectrometry

MALDI-TOF analysis identifies bacteria on the basis of their protein profile. The identification is based on comparison of the mass spectra of bacterial proteins with the known protein reference spectra in the database. Samples of microbial material are mixed with a matrix on a conductive metal plate, which results in the crystallization of a sample within the matrix. Then, the metal plate is introduced in the mass spectrometer where it is bombarded with brief pulses of nitrogen laser. The matrix absorbs laser energy, which leads to desorption of bioanalytes that are subsequently vaporized and ionized in the gas phase. The ionized molecules are accelerated in the electrostatic field and are ejected through a metal flight tube that is subjected to a vacuum until they reach a detector, with smaller ions traveling faster than larger ions. Thus, analytes are separated according to their TOF, which creates a mass spectrum that is composed by mass to charge ratio (m/z) peaks with varying intensities. The spectrum is a microbial fingerprint that is compared with a database for the identification at the species or genus level (Croxatto et al. 2012). MALDI-TOF MS is a precise, fast, and relatively

Table 1 Identification of mycobacteria by conventional/molecular and MALDI-TOF MS methods

Isolates (n)	Conventional/molecular	MALDI-TOF MS
43	*Mycobacterium tuberculosis*	*Mycobacterium tuberculosis* complex
4	*Mycobacterium avium*	*Mycobacterium avium*
2	*Mycobacterium fortuitum*	*Mycobacterium fortuitum*
1	*Mycobacterium chelonae*	*Mycobacterium chelonae*
3	*Mycobacterium kansasii*	*Mycobacterium kansasii*
1	*Mycobacterium* spp.	*Mycobacterium novocastrense*
7	*Mycobacterium xenopi*	*Mycobacterium xenopi*
8	*Mycobacterium gordonae*	*Mycobacterium gordonae*
6	*Mycobacterium intracellulare*	*Mycobacterium chimaera-intracellulare* complex

inexpensive method and a number of studies have confirmed its suitability in laboratory practice (Quinlan et al. 2015; Biswas and Rolain 2013; Balada-Llasat et al. 2013; Clark et al. 2013).

2.2 Study Design

Seventy five isolates of mycobacteria were identified by molecular and phenotypic identification methods, and they were compared with the identification by MALDI-TOF MS. Most of the isolates (72 isolates) came from clinical material (clinical strains previously isolated from patient specimens). There were only three isolates from an external control laboratory.

2.3 Identification of Mycobacteria by Conventional Methods

Mycobacteria were cultured on solid Ogawa's medium and liquid Sula's medium. They were identified by conventional methods: microscopy of Ziehl-Neelsen stained preparations, growth rate, and ability to growth at different temperatures, shape and pigmentation of the colonies, growth on the thiophene-2-carboxylic acid hydrazide (TCH) medium, nitrate-niacin test, and sensitivity to antibiotics.

2.4 Identification of Mycobacteria by MALDI-TOF Mass Spectrometry

For MALDI-TOF MS identification, material was processed according to the optimized extraction protocol for mycobacteria of Bruker Daltonics GmbH (Bremen, Germany). The protocol used zirconia-silica beads and the database of Mycobacteria Library (v2.0 Bruker Daltonics) with 313 reference spectra of mycobacteria. The obtained spectra from clinical samples were analyzed against spectra in the database using the Biotyper software. The reliability of identification obtained by MALDI-TOF MS was assessed from the best match based on the m/z ratio and from the logarithmic score ranging from 0 to 3. The scores $\geq$2.000 were interpreted for genus- and species-level identification, scores between 1.700 and 1.999 for genus-level identification, and the scores $<$1.700 were assumed unreliable for identification.

3 Results

All but one out of the 75 isolates were determined in conformity to the genus, species, or complex with both conventional and MALDI-TOF MS methods. The results are shown in Table 1.

The results obtained with MALDI-TOF MS corresponded to those obtained with conventional methods in case of 71 out of the 72 isolates from clinical material. Forty three isolates of *M. tuberculosis* were identified by MALDI-

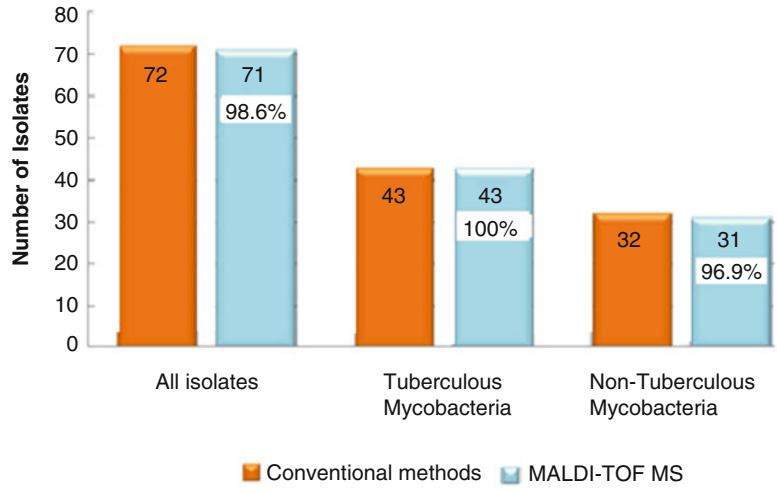

Fig. 1 Identification of tuberculous and non-tuberculous mycobacteria by conventional and MALDI-TOF MS methods

TOF MS as the *M. tuberculosis* complex, with 100% success. Thirty one out of the 32 isolates of non-tuberculous mycobacteria were consistently identified with both methods to the species or complex level (Fig. 1). The closely related species, identified consistently within a complex, were considered identical identification. One isolate, identified as *M. novocastrense* by MALDI-TOF MS, was designated as *Mycobacterium* species with hybridization techniques. All non-tuberculous mycobacterium strains were confirmed by PCR in the reference laboratory of the National Institute for Tuberculosis, Lung Diseases and Thoracic Surgery in Vysne Hagy, Slovakia.

4 Discussion

Mycobacteria cause significant morbidity in humans. Rapid and accurate identification of mycobacteria is important for the improvement of patient outcomes. Several diagnostic techniques, such as biochemical, sequencing, and probe methods, are used for mycobacterial identification (Balada-Llasat et al. 2013). Unambiguous diagnosis of active tuberculosis is a time-consuming process, requiring as long as 12 weeks for positive identification of the organism. This long time frame presents challenges for case identification. Early identification of mycobacteria is essential for the disease control (Biswas and Rolain 2013). MALDI-TOF MS is a powerful method for the detection and identification of proteins by molecular weight determination of individual, specific fragments. The recent developments of MALDI-TOF MS are rapidly changing the routine diagnostics scene. The method is accurate and easy to use, allowing for a quick determination of molecular protein weight, with minimal sample requirements (Benagli et al. 2011).

In the present study, MALDI-TOF MS correctly identified 98.6% of mycobacterial isolates from clinical material to the genus, species, or complex level. Tuberculous mycobacteria were identified as the *M. tuberculosis* complex with 100% success. For non-tuberculous mycobacteria, the results of MALDI-TOF MS corresponded to the conventional methods with 96.9% success. Only was one isolate imprecisely identified. It was designated as *M. novocastrense* by MALDI-TOF MS and as *Mycobacterium* species with hybridization techniques. *M. novocastrense* is rapidly growing photochromogenic mycobacterium, with yellow pigmented colonies when incubated in the light, which was confirmed by phenotypic identification. The identification of *M. novocastrense* was confirmed by PCR.

MALDI-TOF MS has many advantages. The method is quick, reliable, and cost-effective compared to conventional and molecular techniques. There are available extraction protocols for mycobacteria. The identification of mycobacteria is possible within 1–2 h. Databases of protein spectra, used as reference for comparison, are continually expanded by adding new spectra of mycobacteria since the identification of microorganisms is limited by the database (Benagli et al. 2011). The MALDI-TOF MS method has some other limitations concerning its use for the identification of mycobacteria due mainly to the high pathogenicity of some of these microorganism and the structure of their cell wall, requiring inactivation and special protein extraction protocols (Alcaide et al. 2016).

The genus Mycobacterium includes, as major groups, the pathogens of the *M. tuberculosis* complex and the non-tuberculous mycobacteria. The members of non-tuberculous mycobacteria are cited increasingly as the cause for opportunistic infections among immune-compromised patients. This trend and the rise of antibiotic resistance in this genus necessitate improved differentiation of mycobacteria. Although MALDI-TOF MS accurately classifies isolates as members of *M. tuberculosis* complex, it is unable to separate them into individual species. While MALDI-TOF MS can differentiate between *M. chelonae* and the *M. abscessus* group, and between *M. avium* and *M. intracellulare*, it cannot differentiate *M. intracellulare* from *M. chimera, and M. massiliense* from *M. abscessus* (Saleeb et al. 2011). The differentiation of *M. massiliense* from *M. abscessus* complex is of clinical interest because *M. massiliense* is one of the subspecies of *M. abscessus* complex and exhibits higher rates of response to antibiotic treatment for lung infection than do the other members of that complex (Kehrmann et al. 2016). The currently available databases of reference spectra of known proteins need extension, especially for less common organisms. New MALDI Biotyper System v3.0, created in 2015, contains 542 additional references of mainly clinical isolates. This new database includes a total of 855 reference spectra from 149 species (Pranada et al. 2015). A new Mycobacteria Library v4.0 covers 159 of the currently known 169 *Mycobacterium* species. Eight hundred and eighty strains, of which more than 450 are clinical isolates, cover the natural variability of *Mycobacterium* species. This latest version secures high sensitivity of mycobacteria identifications.

In the laboratory diagnosis of tuberculosis, smear microscopy is usually confirmed by culture. That is the gold standard which, however, requires approximately 45 days of incubation time. Automated and semi-automated liquid culture systems have reduced the culture time and increased sensitivity. Yet conventional methods used for the identification of tuberculosis still have low sensitivity (Şamlı and İlki 2016). The identification of tuberculous and non-tuberculous mycobacteria, based on conventional phenotypic tests, is time-consuming, labor-intensive, expensive, and often provides erroneous or inconclusive results (Griffith et al. 2007). Molecular methodologies, although rapid, are expensive, need different genetic markers, and are often exclusive to reference laboratories (Quinlan et al. 2015). For the identification of bacteria, which are difficult to culture, MALDI-TOF MS is a powerful, rapid, precise, and cost-effective method, compared to conventional phenotypic or molecular techniques (Biswas and Rolain 2013).

The cost of MALDI-TOF MS identification is significantly less compared to other methods, including genomic sequencing or biochemical techniques. MALDI-TOF MS generates less waste than other methods that are based on molecular and biochemical tests that use many disposable materials (Balada-Llasat et al. 2013). The identification of mycobacteria from isolates with the use of MALDI-TOF MS takes approximately 1–2 h, compared to a few weeks of other phenotypic identification, which has brought into laboratory practice a revolutionary shift in the speed and accuracy of identification.

5 Conclusions

MALDI-TOF MS is a diagnostic method which clarifies and accelerates the diagnosis, especially in slow growing bacteria, which are difficult to culture, such as *Mycobacterium* species. This method is a new laboratory option for the diagnosis of clinical infections and a valid alternative to conventional methods. In this study we confirmed that MALDI-TOF MS represents a rapid, reliable, and cost-effective identification technique and the method of choice for the identification of clinically important *Mycobacterium* species in a routine laboratory. Rapid and accurate diagnosis of mycobacterial infections is essential for the commencing of early treatment and the prevention of disease spread from person to person.

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Conflicts of Interest The authors declare no conflicts of interest in relation to this article.

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