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FULL PAPER

Evaluation of MALDI-TOF MS for identification of nontuberculous mycobacteria isolated from clinical specimens in mycobacteria growth indicator tube medium.

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SUMMARY

Nowadays, there is a rising worldwide incidence of diseases caused by nontuberculous mycobacteria (NTM) species, especially in immunocompromised patients and those with underlying chronic pulmonary diseases. Recently, matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) became a method of choice for the identification of NTM species. The aim of this study was to evaluate MALDI-TOF MS for the identification of NTM isolates compared to the PCR-restriction enzyme analysis (PRA)-*hsp*65 method. In this study, a total of 152 NTM strains isolated from various clinical specimens were retrospectively analysed. MALDI-TOF MS successfully identified 148 (97.4%) of the 152 NTM isolates but failed to identify four (2.6%) of them. Bruker mycobacteria library gave spectral scores higher than 2.0 for 45 (29.6%) of NTM isolates, between 1.6 and 2.0 for 98 (64.5%) of NTM isolates, and lower than 1.6 for nine (5.9%) NTM isolates. The discordant results between MALDI-TOF MS and PRA-*hsp*65 analysis were confirmed by sequence analysis. In conclusion, MALDI-TOF MS is a technique capable of performing accurate, rapid, cost-effective, and easy identification of NTM isolates.

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INTRODUCTION

Nontuberculous mycobacteria (NTM) are ubiquitous environmental microorganisms that consist of more than 160 species, some of which may cause various diseases in humans including pulmonary disease, skin infections after inoculation, cervical lymphadenitis in children, and disseminated disease in severely immunocompromised individuals (van Ingen, 2015). A worldwide increase has attracted attention due to the frequency of NTM laboratory isolation rates and their prevalence for related infections in the last three decades. Differentiation between contamination and infection remains challenging. Rapid, accurate diagnosis and differentiation to the species and subspecies level are important issues due to the differences in antibiotic susceptibilities. Treatment regimens for NTM may differ depending on the species and inappropriate treatment may lead to antibiotic resistance or unnecessary exposure to drug toxicities. The American Thoracic Society and Infectious Disease Society of America recommended the identification of clinically significant NTM isolates to the species level (Falkinham III, 2016; Griffith *et al*., 2007; Wassilew *et al*., 2016).

Key words:
Nontuberculous mycobacteria, MALDI-TOF MS, Identification, PRA-*hsp*65.

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For the species-level identification of NTM, biochemical tests are considered slow and unable to identify less common species. On the other hand, rapid and accurate molecular methods have currently surpassed biochemical tests for the identification of NTM and high-performance liquid chromatography is considered the method of choice but remains limited to the reference laboratories. Repetitive polymerase chain reaction (rep-PCR), random amplified polymorphic DNA (RAPD) PCR, pulsed-field gel electrophoresis (PFGE) of large restriction fragments, restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), partial gene sequencing, and multiplex PCR (using *hsp*65, 16S rDNA, *rpo*B, etc.) are among these molecular methods. Commercially available PCR-based hybridization assays including the Genotype CM/AS, and InnoLiPA Mycobacteria allow the differentiatiation of 21 and 14 NTM species, respectively (Falkinham III, 2016; Wassilew *et al*., 2016).

Telenti *et al*. (1993) developed a method for differentiating among NTM species based on evaluation of the gene coding for the 65-kDa heat shock protein by PCR and restriction enzyme analysis. This method was based on the amplification of 440 bp fragment of the *hsp*65 by PCR, followed by digestion of the product with the restriction enzymes *Bst*EII and *Hae*III. PRA-*hsp*65 is a simple and rapid method without the need for specialized equipment and has been used widely (Simner *et al*., 2015).

Recently, matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) has been effectively used for the identification of bacteria and yeasts,

and has also been applied for the rapid and accurate identification of mycobacteria (Ceyssens *et al*., 2017; Ge *et al*., 2016; Marekovic et al., 2015; Mediavilla-Gradolph *et al*., 2015; Tudo *et al*., 2015; van Belkum *et al*., 2017). MALDI-TOF MS analysis of NTM species level is based on unique spectral fingerprints produced by extracted proteins, and involves steps including inactivation, extraction, and analysis. Following the inactivation and extraction steps, an aliquot is spotted onto a steel plate and overlaid with a chemical matrix. The sample plate is loaded into the instrument and mycobacterial proteins are ionized using a laser and separated based on the mass-to-charge ratio of the ions. The proteomic fingerprints of the isolates are then compared with those in a reference database (Angeletti, 2017; van Belkum *et al*., 2017).
The purpose of this study was to evaluate MALDI-TOF MS and compare to the PRA-*hsp*65 method for the identification of NTM species isolated from clinical specimens.

MATERIALS AND METHODS

Clinical NTM strains

A total of 152 NTM strains (from 152 specimens) isolated from various clinical specimens (respiratory: sputum, bronchoalveolar lavage, gastric lavage; non-respiratory: pus, peritoneal fluid, biopsy, urine) in the Microbiology Department between 2000 and 2016 were retrospectively analysed. The clinical specimens were decontaminated using N-acetyl-L-cysteine-sodium hydroxide solution, then neutralized with a phosphate buffer, and concentrated via centrifugation (CLSI document M48-A, 2008). The processed specimens were used for acid-fast bacillus microscopy via the Ehrlich-Ziehl-Neelsen (EZN) method, as well as culture in Bactec 460 TB/Bactec MGIT 960 liquid medium (BD, Sparks, MD, USA) and Löwenstein-Jensen (L-J) solid medium (BD, Sparks, MD, USA). All of the cultures were incubated at 37°C for 6-8 weeks. From the positive liquid medium vials or L-J cultures, EZN stained smears were prepared and BACTEC NAP Test/MGIT TBc Identification Test (BD, Sparks, MD, USA) were performed for differentiation of NTM species and *M.tuberculosis* complex members. Subcultures in L-J medium were used to analyze phenotypic characteristics including growth rate (fast or slow) and pigment production (photochromogenic, scotochromogenic or nonchromogenic) (CLSI document M48-A, 2008; Siddiqi, 1995; Siddiqi and Rusch-Gerdes, 2006). All of the NTM isolates were stored at -80 degrees until they were tested. They were subcultured in MGIT medium for the analysis.

MALDI-TOF MS analysis

Protein extraction from Bactec MGIT tubes (BD, Sparks, MD, USA) was performed according to the manufacturer's MycoEX protocol. The biomass was collected by aspirating 1.2 ml of liquid medium from the bottom of the MGIT tubes and centrifuged at 13,000 rpm for two minutes. The supernatants were discarded and 300 µl high-performance liquid chromatography grade water was added into the tube. The cells were inactivated for 30 min at 95°C in a thermoblock. Then 900 µl of ethanol was added, mixed by using a vortex, and was centrifuged at 13,000 rpm for two minutes. The supernatants were removed and the pellets were dried at room temperature before addition of zirconia/silica beads and 20 µl of pure acetonitrile. After one minute of vortexing, 70% formic acid equal to the volume of acetonitrile was added and was mixed again using a vortex. After centrifugation for two minutes at 13,000 rpm, one µl of the supernatant was placed on a MALDI target plate and allowed to dry. Then the spot was overlaid with 1 µl of hydroxycinnamic acid matrix (MycoEX Method v3.0, 2014).
The identification of NTM isolates and data analysis were performed by the Bruker Microflex LT MALDI-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany), using the MALDI Biotyper 3.1 software. The obtained protein profiles were analyzed and compared to Mycobacteria Library 3.0 database (Bruker Daltonics, Bremen, Germany). Confidence scores of ≥2.0 were considered as the identification at the species level, scores of 1.6-2.0 were considered as the identification at the genus level, and scores of <1.6 were considered unreliable identification (Mediavilla-Gradolph *et al*., 2015).

PRA-hsp65 method

For DNA extraction, a loopful of NTM isolate grown on L-J medium was suspended in 500 µl of ultrapure water, and inactivated for 10 min at 100°C. After being sonicated for 15 min, it was frozen at -20°C at least, for 18 hours (Chimara *et al*., 2008).
PCR and restriction enzyme analysis of the *hsp*65 gene were performed as described previously by Telenti et al. (Telenti *et al*., 1993). For PCR amplification, five µl of lysate was added to the PCR mixture (final volume was 50 µl) containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl2, 10% glycerol, 200 µM deoxynucleoside triphosphate, 0.5 µM of each primer, and 1.25 U of Taq polymerase. The reaction was subjected to 45 cycles of amplification (1 min at 94°C, 1 min at 60°C, 1 min at 72°C), which was followed by 10 min of extension at 72°C. Primers Tbll (5'-ACCAACGATGGTGTGTCCAT) and Tb12 (5'-CTTGTCGAACCGCATACCCT) amplified a 441-bp fragment of the gene *hsp*65.
Restriction digestion of PCR products was carried out with 5 U each of *Bst*EII and *Hae*III. After adding 0.5 µl of enzyme to the mixture of 2.5 µl of restriction buffer and 11.5 µl of water. The mixture was incubated at 60°C for *Bst*EII digestion and at 37°C for *Hae*III digestion. Restriction products were separated by electrophoresis in a 3% agarose gel with 50 and 100 bp ladders as molecular size standard (Telenti *et al*., 1993). The patterns observed were analysed in the PRASITE query (http://app.chuv.ch/prasite).

Sequence analysis

The genomic DNAs of NTM isolates were extracted by the Mickle method. The 441 bp fragment of the *hsp*65 gene was amplified and showed by agarose gel electrophoresis. Partial PCR products were characterized by DNA sequencing using the forward primers on an automatised ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). For species identification of the resulting DNA sequences were analyzed using the basic local alignment search tool (http://www.ncbi.nih.gov/BLAST) (Ozcolpan *et al*., 2015).

RESULTS

A total of 152 NTM isolates were classified according to the Runyon classification. Among all isolates, 109 (71.7%),

Table 1 - *Summary of PRA-hsp65 results of the 152 NTM isolates.*

Species	Bst*EII fragments (bp)*	Hae*III fragments (bp)*	*Number of isolates (n, %) (n=152)*
M.abscessus type II	235 / 210 / 0	200 / 70 / 60 / 50	34 (22.4)
M.abscessus type I	235 / 210 / 0	145 / 70 / 60 / 55	32 (21.0)
M.fortuitum type I	235 / 120 / 85	145 / 120 / 60 / 55	24 (15.7)
M. avium subsp. *avium* type I	235 / 210 / 0	130 / 105 / 0 / 0	16 (10.5)
M.lentiflavum type I	440 / 0 / 0	145 / 130 / 0 / 0	8 (5.3)
M.simiae type I	235 / 210 / 0	185 / 130 / 0 / 0	5 (3.3)
M.szulgai type I	440 / 0 / 0	130 / 105 / 70 / 0	4 (2.6)
M.chelonae type I	320 / 130 / 0	200 / 60 / 55 / 50	4 (2.6)
M.kansasii type I	235 / 210 / 0	130 / 105 / 80 / 0	4 (2.6)
M.fortuitum type II	235 / 120 / 85	140 / 120 / 60 / 55	4 (2.6)
M.peregrinum type II	235 / 210 / 0	140 / 125 / 100 / 50	4 (2.6)
M.chimaera type I	235 / 120 / 100	145 / 130 / 60 / 0	2 (1.3)
M.gordonae type III	235 / 120 / 100	130 / 115 / 0 / 0	2 (1.3)
M.gordonae type IV	320 / 115 / 0	130 / 115 / 60 / 0	1 (0.7)
M.porcinum type I	235 / 210 / 0	140 / 125 / 100 / 50	1 (0.7)
M. xenopi type I	235 / 120 / 85	160 / 105 / 60 / 0	1 (0.7)
M.celatum type I	235 / 210 / 0	130 / 80 / 60 / 0	1 (0.7)
M. mucogenicum type I	320 / 130 / 0	140 / 65 / 60 / 0	1 (0.7)
M.peregrinum type III	235 / 130 / 85	145 / 140 / 100 / 60	1 (0.7)
Unidentified			3 (1.9)

20 (13.2%), 14 (9.2%) and nine (5.9%) of them were classified as rapidly growing, nonchromogenic, scotochromogenic, and photochromogenic, respectively.

Using the PRA-*hsp*65 method, three (2%) of the isolates could not be identified, while 149 (98%) were identified. Among the NTM isolates; 34 (22.4%) were *M.abscessus* type II, 32 (21.0%) were *M. abscessus* type I, 24 (15.7%) were *M. fortuitum* type I, and 16 (10.5%) were *M. avium* subsp. *avium* type I as the most encountered NTM species level. The other identified NTM species are shown in *Table 1*.

MALDI-TOF MS successfully identified 148 (97.4%) of the NTM isolates but failed to correctly identify four (2.6%) of them. Bruker mycobacteria library gave spectral scores higher than 2.0 for 45 (29.6%), between 1.6 and 2.0 for 98 (64.5%), and lower than 1.6 for nine (5.9%) NTM isolates. The isolates with low scores belonged to *M. fortuitum*, *M. avium*, *M. porcinum*, *M. arupense*, *M. farcinogenes-senegalense* group, and *M. intracellulare* (*Table 2*).

The results of MALDI-TOF MS were in agreement with the results of PRA-*hsp*65 method for 142 (93.4%) isolates. With PRA-*hsp*65 method, three isolates (2%) could not be identified. MALDI-TOF MS identified these isolates as *M. elephantis*, *M. neoaurum*, and *M. tokaiense* with scores of 1.76, 1.81, and 1.78, respectively. These results were verified with sequence analysis. MALDI-TOF MS presented discordant results compared to PRA-*hsp*65 for seven (4.6%) isolates.

In all, ten isolates (three isolates which could not be identified with PRA-*hsp*65 method, and seven isolates for which MALDI-TOF MS presented discordant results compared to PRA-*hsp*65) were sequenced. For six isolates the results of MALDI-TOF MS were in agreement with the results of the sequence analysis. MALDI-TOF MS incorrectly identified four (2.6%) of the isolates. The misidentified isolates were; *M. arupense* instead of *M. abscessus*, *M. intracellulare* instead of *M. paragordonae* , *M. porcinum* instead of *M. paragordonae*, and *M. peregrinum* instead of *M. szulgai* (*Table 3*).

DISCUSSION

Nowadays, there is a rising incidence of diseases caused by NTM species, especially in immunocompromised patients and those with underlying chronic pulmonary diseases. As conventional species-level identification of NTM isolates is a time-consuming and complicated process, molecular techniques are favoured in most clinical laboratories. Recently, MALDI-TOF MS became a method of choice for the identification of NTM species as simple, rapid, and cost effective assay. Very recently a new MALDI-TOF MS assay for phenotypic drug sensitivity, the MALDI Biotyper antibiotic susceptibility test rapid assay (MBT-ASTRA) was developed. This assay can be used for NTM species as well as *M. tuberculosis* complex members (Ceyssens *et al*., 2017).

In previous studies evaluating MALDI-TOF MS for the identification of NTM strains, the correct identification percentages varied according to the use of different culture media (Marekovic *et al*., 2015; Quinlan *et al*., 2015; Tudo *et al*., 2015), the differences in extraction protocols (El Khechine *et al*., 2011; Saleeb *et al*., 2011; Tudo *et al*., 2015) and the different versions of the library used (Rodriguez-Sanchez *et al*., 2016; Rodriguez-Temporal *et al*., 2017).

The manufacturer of MALDI-TOF MS reported that the spectra of mycobacteria grown on solid or liquid media showed no significant variation. Tudo *et al*. (2015) reported that no significant difference was observed between

Table 2 - *NTM isolates identified by MALDI-TOF MS.*

NTM species (n)	*MALDI-TOF MS score, (n)*			*Species incorrectly identified, (n)*
	≥2.0	*1.6-2.0*	*<1.6*	
M. abscessus (n=65)	16	49	-	-
M. fortuitum (n=29)	12	13	4	-
M. avium (n=16)	8	7	1	-
M. lentiflavum (n=7)	1	6	-	-
M. peregrinum (n=5)	2	3	-	1
M. simiae (n=5)	-	5	-	-
M. kansasii (n=4)	2	2	-	-
M. chelonae (n=4)	1	3	-	-
M. szulgai (n=2)	-	2	-	-
M. porcinum (n=2)	1	-	1	1
M. gordonae (n=2)	-	2	-	-
M. chimaera-intracellulare group (n=2)	2	-	-	-
M. arupense (n=1)	-	-	1	1
M. tokaiense (n=1)	-	1	-	-
M. celatum (n=1)	-	1	-	-
M. xenopi (n=1)	-	1	-	-
M. elephantis (n=1)	-	1	-	-
M. farcinogenes-senegalense group (n=1)	-	-	1	-
M. intracellulare (n=1)	-	-	1	1
M. mucogenicum (n=1)	-	1	-	-
M. neoaurum (n=1)	-	1	-	-
Total (n=152)	45 (29.6%)	98 (64.5%)	9 (5.9%)	4 (2.6%)

solid and liquid media showing correlation with reference methods by 70.8% and 75%, respectively. Marekovic *et al*. (2015) correctly identified 80% of the NTM isolates by using liquid medium with MALDI-TOF MS. The authors suggested that these high identification rates make the use of liquid medium with optimized extraction protocol more favourable than the use of solid media. On the contrary, Quinlan *et al*. (2015) reported a significantly higher identification rate from solid medium (76.2%) than liquid medium (52.3%). The authors suggested spectral acquisition failures probably due to the decreased available biomass/material on the sample spot.

The manufacturer of MALDI-TOF MS recommended the MycoEX protocol for the identification of mycobacteria. The MycoEX protocol consists of heating for inactivation of mycobacteria, use of zirconia/silica beads for mechanical disruption and use of formic acid and acetonitrile for protein extraction. Saleeb *et al*. (2011) added a grinding step with a micropestle for the extraction protocol of the specimens. The authors suggested that this procedure had

Table 3 - *NTM strains were not correctly identified by MALDI-TOF MS or PRA-hsp65 when compared by sequencing.*

Number of isolates (n=10)	*PRA-hsp65 ID*	*MALTI-TOF MS ID (score)*	*Sequencing ID*
1	*M.abscessus* type I	*M. arupense* (1.170)**	*M.abscessus*
1	*M.fortuitum* type I	*M. farcinogenes-senegalense* group (1.130)*	*M.senegalense*
1	*M.gordonae* type III	*M. intracellulare* (1.049)**	*M.paragordonae*
1	*M.lentiflavum* type I	*M. fortuitum* (1.350)*	*M.fortuitum*
1	*M.szulgai* type I	*M. fortuitum* (1.528)*	*M.fortuitum*
1	*M. peregrinum* type II	*M. porcinum* (1.520)**	*M.paragordonae*
1	*M.szulgai* type I	*M. peregrinum* (1.620)**	*M.szulgai*
1	No result	*M.elephantis* (1,760)*	*M.elephantis*
1	No result	*M.neoaurum* (1,810)*	*M.neoaurum*
1	No result	*M.tokaiense* (1,780)*	*M.tokaiense*

*Correctly identified by MALDI-TOF MS
**Not correctly identified by MALDI-TOF MS

dispersed the clumps and generated reproducible high quality spectra for all species of mycobacteria analyzed. Tudo *et al*. (2015) evaluated the two MycoEX protocols, MycoEX v2.0 and v3.0, released by the manufacturer in 2013 and 2014 respectively. They detected 57.3% and 73% correlations by comparing v2.0 and v3.0 protocols with the reference method, respectively. The study by El Kechine *et al*. (2011) reported that the addition of 0.5% Tween 20 during the inactivation phase increased the quality of the MALDI-TOF MS spectra.

The Mycobacteria Library 4.0 which covers 159 mycobacteria species with reference protein profiles of 880 strains is currently available. In this study, the Mycobacteria Library 3.0 containing 149 species with 853 reference entries was used. Rodriguez-Sanchez *et al*. (2016) analyzed 109 NTM isolates searching with v3.0 and v2.0 databases. The v2.0 database allowed a high-level confidence identification (score value ≥1.8) of 91 isolates (83.5%) versus 100 isolates (91.7%) by v3.0 database. In addition, the v3.0 database improved the score value of 45 (41.3%) NTM isolates. Rodriguez-Temporal *et al*. (2017) evaluated the identification of 240 NTM isolates by searching Mycobacteria Libraries v3.0 and v2.0. The application of v3.0 identified 15 (6.2%) NTM isolates more than the isolates identified by v2.0. The scores obtained using v3.0 were higher for 147 (61.2%) isolates than v2.0. It is very clear that updating of the MALDI-TOF MS database is necessary for the identification of NTM.

Rodriguez-Sanchez *et al*. (2016) analyzed 99 NTM isolates from clinical samples and ten reference strains using MALDI-TOF MS and the Mycobacteria Library 3.0. All of the isolates were correctly identified with scores ≥1.8 for 100 NTM isolates (91.7%). Tudo *et al*. (2015) analyzed 70 NTM isolates by searching the Mycobacteria Library 3.0. Among these isolates, 49 (70%) were correctly identified. In the study of Ge *et al*., (2016), 125 of 138 NTM isolates (90.6) were correctly identified. In this study, 148 (97.4%) of 152 NTM isolates were correctly identified by MALDI-TOF MS, and scores were ≥1.8 for 110 of these NTM isolates (72.4%).

In this study MALDI-TOF MS correctly identified 148 (97.4%) of the NTM isolates, but could not correctly identify four (2.6%) of them. The Bruker Mycobacteria Library v3.0 gave spectral scores higher than 2.0 for 45 (29.6%) isolates, between 1.6 and 2.0 for 98 (64.5%) isolates, and lower than 1.6 for nine (5.9%) isolates. These nine isolates with low scores were identified as *M. fortuitum* (four isolates), *M. avium*, *M. porcinum*, *M. arupense*, *M. farcinogenes-senegalense* group, and *M. intracellulare*. Among these, six isolates (four *M. fortuitum*, one *M. avium*, and one *M. farcinogenes-senegalense* isolates) were correctly identified by this method, but the identification of the other three isolates (*M. porcinum*, *M. arupense*, and *M. intracellulare*) by MALDI-TOF MS were not correct. By the sequence analysis, *M. porcinum* (1.520) and *M. intracellulare* (1.049) were identified as *M. paragordonae*, and *M. arupense* (1.170) was identified as *M. abscessus*. Another isolate, which was identified incorrectly as *M. peregrinum* with a score value of 1.620 was identified as *M. szulgai* by the sequence analysis. From these species, *M. paragordanae* is not covered by The Bruker Mycobacteria Library v3.0 database.

In this study, the results of MALDI-TOF MS were compared with the results of the PRA-*hsp*65 method. Of the 152 NTM isolates, three (2%) strains could not be identified by the PRA-*hsp*65 method as DNA failed to amplify with PRA primers in three experiments. The reason for this lack of amplification was not clear, but it could be caused by the presence of PCR inhibitors. These isolates were identified by MALDI-TOF MS as *M.tokaiense*, *M.neoaurum*, and *M.elephantis,* and these results were confirmed by sequence analysis. Previous studies reported that some isolates could not be identified by PRA-*hsp*65 method. Chimara *et al*. (2008) reported that 30 out of 434 (6.9%) NTM isolates representing 13 PRA-*hsp*65 patterns (*M. arupense*, *M. avium*, *M. cosmeticum*, *M. fortuitum*, *M. gordonae*, *M. mageritense*, *M. nonchromogenicum*, *M. sherrisii*, and *M. terrae*) were not available in databases. Da Silva *et al*. (2001) reported that 12 of 103 (11.7%) isolates were not identified with this method. Among these isolates, DNA from seven did not amplify with PRA primers, and in the other five isolates the pattern obtained could not be assigned to any pattern available in the databases.

MALDI-TOF MS presented discordant results compared to PRA-*hsp*65 for seven (4.6%) NTM isolates. Among these isolates, four of them were not identified correctly as discussed previously. However for three isolates the MALDI-TOF MS ID were correct. These were one isolate belonging to the *M. farcinogenes-senegalanse* group, and two isolates belonging to the *M. fortuitum* with score values of 1.130, 1.350, and 1.528, respectively.

In conclusion, we suggest that MALDI-TOF MS is a powerful technique capable of performing accurate, rapid, cost-effective, and easy identification of NTM isolates. If some NTM species are represented with low numbers of spectra, it may lead to insufficiency of the database. Further studies are required to validate the results in clinical practice.

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Conflict of interest

The authors declare that they have no conflict of interest.

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