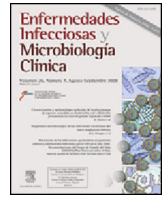




Enfermedades Infecciosas y Microbiología Clínica

www.elsevier.es/eimc



Original article

Comparative evaluation of the identification of rapidly growing non-tuberculous mycobacteria by mass spectrometry (MALDI-TOF MS), GenoType *Mycobacterium* CM/AS assay and partial sequencing of the *rpoB* gene with phylogenetic analysis as a reference method



José Javier Costa-Alcalde^{a,*}, Gema Barbeito-Castiñeiras^b, José María González-Alba^{c,d,e}, Antonio Aguilera^{a,f}, Juan Carlos Galán^{c,d,e}, María Luisa Pérez-del-Molino^a

^a Servicio de Microbiología, Hospital Clínico Universitario de Santiago de Compostela, Santiago de Compostela, A Coruña, Spain

^b Servicio de Microbiología, Hospital Clínico Universitario de Santiago de Compostela – Universidade de Santiago de Compostela, Santiago de Compostela, A Coruña, Spain

^c Servicio de Microbiología Hospital Universitario Ramón y Cajal and Instituto Ramón y Cajal de Investigación Sanitaria (IRYCIS), Madrid, Spain

^d CIBER en Epidemiología y Salud Pública (CIBERESP), Madrid, Spain

^e Unidad de Resistencia a Antibióticos y Virulencia Bacteriana (RYC-CSIC), Madrid, Spain

^f Departamento de Microbiología, Universidad de Santiago de Compostela, A Coruña, Spain

ARTICLE INFO

Article history:

Received 24 January 2018

Accepted 13 April 2018

Available online 2 June 2018

Keywords:

Non-tuberculous mycobacteria

MALDI-TOF MS

Partial *rpoB* gene sequencing

Phylogenetic analysis

Sequence analysis

ABSTRACT

Introduction: The American Thoracic Society and the Infectious Diseases Society of America recommend that clinically significant non-tuberculous mycobacteria (NTM) should be identified to the species level in order to determine their clinical significance. The aim of this study was to evaluate identification of rapidly growing NTM (RGM) isolated from clinical samples by using MALDI-TOF MS and a commercial molecular system. The results were compared with identification using a reference method.

Methods: We included 46 clinical isolates of RGM and identified them using the commercial molecular system GenoType[®] CM/AS (Hain, Lifescience, Germany), MALDI-TOF MS (Bruker) and, as reference method, partial *rpoB* gene sequencing followed by BLAST and phylogenetic analysis with the 1093 sequences available in the GeneBank.

Results: The degree of agreement between GenoType[®] and MALDI-TOF MS and the reference method, partial *rpoB* sequencing, was 27/43 (62.8%) and 38/43 cases (88.3%) respectively. For all the samples correctly classified by GenoType[®], we obtained the same result with MALDI-TOF MS (27/27). However, MALDI-TOF MS also correctly identified 68.75% (11/16) of the samples that GenoType[®] had misclassified ($p = 0.005$).

Conclusions: MALDI-TOF MS classified significantly better than GenoType[®]. When a MALDI-TOF MS score >1.85 was achieved, MALDI-TOF MS and partial *rpoB* gene sequencing were equivalent. GenoType[®] was not able to distinguish between species belonging to the *M. fortuitum* complex. MALDI-TOF MS methodology is simple, rapid and associated with lower consumable costs than GenoType[®]. The partial *rpoB* sequencing methods with BLAST and phylogenetic analysis were not able to identify some RGM unequivocally. Therefore, sequencing of additional regions would be indicated in these cases.

© 2018 Elsevier España, S.L.U. and Sociedad Española de Enfermedades Infecciosas y Microbiología Clínica. All rights reserved.

* Corresponding author.

E-mail address: xosexabier@gmail.com (J.J. Costa-Alcalde).

Evaluación comparativa de la identificación de micobacterias no tuberculosas de crecimiento rápido mediante espectrometría de masas (MALDI-TOF MS), GenoType® Mycobacterium CM/AS assay y la secuenciación parcial del gen *rpoB* con análisis filogenético como método de referencia

R E S U M E N

Palabras clave:

Micobacterias no tuberculosas
MALDI-TOF MS
Secuenciación parcial del gen *rpoB*
Análisis filogenético
Análisis de secuencias

Introducción: La American Thoracic Society y la Infectious Diseases Society of America recomiendan que las micobacterias no tuberculosas (MNT) clínicamente relevantes sean identificadas a nivel de especie para determinar su significado clínico. El propósito de este estudio fue a partir de MNT de crecimiento rápido (MCR) aisladas en muestras clínicas, evaluar su identificación mediante MALDI-TOF MS y un método molecular comercial, comparando estos resultados con la identificación obtenida usando un método de referencia.

Métodos: Se incluyeron 46 aislados clínicos de MCR. Estos aislados se identificaron mediante el método molecular comercial GenoType® Mycobacterium CM/AS (Hain, Lifescience, Alemania), MALDI-TOF MS (Bruker) y, como método de referencia, la secuenciación parcial del gen *rpoB* seguido de BLAST y análisis filogenético. Para el análisis filogenético se utilizaron 1.093 secuencias disponibles en el GeneBank.

Resultados: Entre GenoType® o MALDI-TOF MS, la concordancia respecto al método de referencia, secuenciación parcial de *rpoB*, fue 27/43 (62,8%) y 38/43 casos (88,3%), respectivamente. En todas las muestras que GenoType® clasificó correctamente con MALDI-TOF MS se obtuvo el mismo resultado (27/27). Pero además MALDI-TOF MS identificó bien 68,75% (11/16) de las muestras que GenoType® no clasificó correctamente ($p = 0,005$).

Conclusiones: MALDI-TOF MS clasificó significativamente mejor que GenoType®. Cuando MALDI-TOF MS alcanzó una puntuación >1,85, MALDI-TOF y la secuenciación parcial del gen *rpoB* fueron equivalentes. GenoType® no distinguió dentro del *M. fortuitum complex*. La metodología MALDI-TOF MS es simple, rápida y se asocia a un menor coste de consumibles que GenoType®. La secuenciación parcial del gen *rpoB* con BLAST y análisis filogenético no lograron identificar de manera inequívoca algunas MCR. Para estas MCR estaría indicado la secuenciación de regiones adicionales.

© 2018 Elsevier España, S.L.U. y Sociedad Española de Enfermedades Infecciosas y Microbiología Clínica. Todos los derechos reservados.

Introduction

The last twenty years have been characterized by an extraordinary increase of new *Mycobacterium* spp. Among over 150 officially recognized nontuberculous mycobacteria (NTM), only a few tens are familiar to clinicians.¹ NTM are widely present in the environment and commonly inhabit soil and water. This group of microorganisms are considered to be opportunistic pathogens which can cause pulmonary, skin, soft tissue, lymphatic and disseminated infections, as well as nosocomial outbreaks related to inadequate disinfection/sterilization of medical equipment. The incidence of diseases caused by NTM is on the rise due to, among other reasons, an increase in immunocompromised patients.² The American Thoracic Society and the Infectious Diseases Society of America recommend that clinically significant NTM should be identified to the species level in order to determine their clinical significance and select appropriate treatments.³

Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) allows the identification of organisms on the basis of unique spectral fingerprints produced by extracted proteins. In recent years, MALDI-TOF MS has irrupted as a rapid, powerful and relatively inexpensive tool for the identification of bacteria and yeast in the clinical laboratory setting.^{1,4}

The molecular approach, using both in-house and commercial systems, is the most frequently used method worldwide for the identification of mycobacterial species. In this work, two molecular approaches for mycobacterial identification are used: a molecular commercial system and partial RNA polymerase beta-subunit gene sequencing (*rpoB*).⁵

Genetic sequencing of conserved genes is the referent method for the identification of mycobacteria.¹ A number of targets useful for identification purposes have been detected within the genome of mycobacteria. Several studies have indicated that the

hypervariable region of the *rpoB* gene, located between 2300 and 3300 bp, is required for suitable identification at species level and this region is recommended as a starting point for identification.⁶

The aim of this study was to evaluate rapidly growing mycobacteria (RGM) identification from clinical samples by using MALDI-TOF MS technology and a molecular commercial system, both methodologies are used in the routine practice of our laboratory. For this purpose, we compared the results obtained by using the routine methodology with the results obtained by using gold standard methodology (partial *rpoB* gene sequencing phylogenetic analysis).

Material and methods

Clinical isolates

From January 2007 to April 2015, a total of 46 clinical isolates of RGM were obtained from patients belonging to the health area of Santiago de Compostela (458.759 inhabitants), Galicia, Northwest of Spain. Only 15 of these patients presented diagnosis criteria according to the American Thoracic Society (ATS).³ These mycobacterial isolates were firstly identified by using the molecular commercial system GenoType® CM/AS, Hain Lifescience, Nehren, Germany. Clinical isolates were preserved at -20°C in skimmed milk until they were used. Thereafter, each strain was inoculated into agar blood medium (Columbia-blood agar base, Becton Dickinson, New Jersey, USA) and incubated at 37°C in a 10% CO_2 incubator. After 5–7 days the growth achieved was enough to carry out the corresponding studies.

Clinical isolates were obtained as part of the routine activity of the Department of Microbiology and they were analyzed

anonymously in a retrospective manner. Thus, ethical approval and informed consent were not required.

GenoType[®] Mycobacterium CM/AS (Hain, Lifescience, Germany) (*GenoType*[®])

The *GenoType*[®] includes a multiplex PCR followed by reverse hybridization and line probe technology. This assay targets the 23S rRNA gene and is available as two kits: CM, which identifies 22 of the most frequently isolated species, and AS, which identifies an additional 13 species. The *GenoType*[®] assay was performed according to the manufacturer's instructions. The final identification was obtained by comparison between the line probe patterns and the evaluation sheet provided by manufacturers.

MALDI-TOF MS protein extraction protocol

It was used the inactivated mycobacteria bead preparation method provided by Bruker Daltonics GmbH (Bremen, Germany). With a sterile 10 μ L loop, mycobacterial colonies growing on solid medium (agar blood) were transferred into a 1.5 mL screw-cap microcentrifuge tub which contained 300 μ L of sterile water. Then, it was heated for 30 min at 100 °C in a thermoblock to inactivate the RGM. On average, colonies were 5–7 days old, depending on the growth rate of the mycobacteria. The tubes were allowed to cool at room temperature for 2 min. Then, 900 μ L of 100% ethanol were added. After vortexing, the tubs were centrifuged at 13,000 rpm for 2 min and the supernatant was discarded. The tubes were centrifuged again at 13,000 rpm for 2 min and all residual liquid were completely removed. The pellet was allowed to dry at room temperature for 5 min. The pellet was suspended in 25 μ L of acetonitrile, and approximately a volume of 50 μ L of 0.5 mm diameter glass beads was added to the pellet. The tubes were vortexed for 1 min, 25 μ L of 70% formic acid were added, and the samples were vortexed again for 1 min. The tubes were centrifuged at 13,000 rpm for 2 min, and the supernatant was used for analysis with MALDI-TOF MS.

All steps requiring open manipulation of mycobacteria before heat inactivation were performed inside a biological safety cabinet in a biosafety level 2 setup.

MALDI-TOF MS analysis

Strains were processed using a MALDI-TOF MS microflex LT (Bruker Daltonics GmbH, Bremen, Germany). Analyses were performed according to the manufacturer's instructions.

One microliter of supernatant was spotted in duplicate onto a steel target (MTP 384 polished steel target plate BC; Bruker Daltonics GmbH, Bremen, Germany) and air dried at room temperature. One microliter of matrix solution (saturate solution of alfa-cyano-4-hydroxycinnamic acid in 50% acetonitrile and 2.5% trifluoroacetic acid) was pipetted onto each of the spotted samples. After drying, the target was inserted into the MALDI-TOF MS. MALDI-TOF MS analysis was performed in automatic mode. Spectra were acquired at a laser frequency of 60 Hz across a mass/charge ratio (m/z) of 2000–20,000 Da.

The protein profile was obtained by the software FlexControl 3.4 (Bruker Daltonics GmbH, Bremen, Germany). Spectra were analyzed against those in the Bruker database Maldi Biotyper Mycobacteria Library 3.0 (853 references from 149 *Mycobacterium* species) using the Maldi Biotyper software (v 3.1; Bruker Daltonics). This assigned a logarithmic score ranging from 0 to 3 and determined the best match based on the m/z ratio and relative peak size of each ionized protein.

Partial *rpoB* gene sequencing

DNA extraction

It was performed using the DNA extraction method for solid culture included in the AnyplexTM MTB/NTM Real-time Detection V2.0 (Seegene, Seoul, Korea) according to the manufacturer's instructions.

rpoB amplification

5 μ L of extracted DNA were used for PCR in a 25 μ L final reaction volume by using the reagents provided by the GoTaq[®] PCR Core Systems I (Promega) kit: 5 U/ μ L GoTaq[®] DNA Polymerase, 5X Colorless GoTaq[®] Flexi Buffer (Mg-Free), 25 mM MgCl₂ and 10 mM PCR Nucleotide Mix. The direct and reverse primers were 10 μ M. The final concentrations were 0.025 U/ μ L GoTaq[®] DNA Polymerase, 4 mM MgCl₂, 0.2 mM dNTPs (each nucleotide), 0.2 μ M each primer. All the clinical strains were identified by partial *rpoB* gene sequencing using a previously described method.⁵ Briefly, flanking the most variable *rpoB* region, PCR primers MycoF (5'-GGCAAGGTCACCCGAAGGG-3'; base positions 2573–2592, the nucleotides have been numbered according to the *M. smegmatis* ATCC 14468 *rpoB* gene sequence) and MycoR (5'-AGCGGCTGCGGGTGATCATC-3'; base positions 3316–3337) amplified a 764-bp fragment from the *rpoB* gene region in clinical isolates. Conditions for *rpoB* gene amplification were pre-heated at 95 °C for 10 min, 35 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 90 s; finally, an elongation step of 72 °C for 7 min. Five microliters of PCR product were analyzed by ultraviolet fluorescence after having been stained with ethidium bromide in a 1% agarose gel.

rpoB partial sequencing

PCR products were purified by using Qiagen columns (Qiagen, Germany). Purified amplicons were sequenced by using the GenomeLabTM Dye Terminator Cycle Sequencing with the Quick Start kit according to the manufacturer's instructions (Beckman Coulter[®]) with the following program: 30 cycles of denaturation at 96 °C for 20 s, primer annealing at 50 °C for 20 s and extension at 60 °C for 4 min. The sequencing primers were the same as in the amplification. Products of sequencing reactions were read in an automatic sequencer (CEQTM 8000 Genetic Analysis System, Beckman Coulter[®]) by following the standard protocol of the supplier.

Those partial *rpoB* gene sequences obtained in that way were compared to those available in the GeneBank using BLAST software (NCBI) (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). After that, the most probable bacterial species were selected. Partial *rpoB* gene sequences that displayed at least 98% sequence identity when compared to those in the GeneBank were considered as identified species.⁵

Phylogenetic analysis

Phylogenetic analysis was carried out to improve the accuracy of partial *rpoB* gene sequencing. The partial *rpoB* gene sequence length ranged between 278 and 710 nucleotides. A set of reference sequences of *Mycobacterium*'s *rpoB* gene available in the GeneBank was obtained by using BLAST (Table S1 included as Supplementary material). The reference data set including 1093 sequences and nucleotide sequences were translated and aligned using the ClustalW algorithm implemented in MEGA. The sequences were divided according to the sequenced fragment for analysis. Phylogenetic trees were reconstructed by means of ML with PhyML⁷ 3.0 by using the general time reversible plus proportion of invariable sites plus gamma distribution parameter (GTR) plus I plus G evolutionary model selected with jModeltest⁸ 0.1 and a BIONJ starting

tree. Heuristic tree searches under the ML optimality criterion were performed using the nearest neighbor interchange (NNI) branch-swapping algorithm. The approximate likelihood ratio test (aLRT) based on a Shimodaira–Hasegawa-like procedure was used as a statistical test to calculate branch support.⁹ Only aLRT support values of >90% were considered statistically significant and displayed at the tree nodes.

Statistical analysis

The Kappa coefficient was used to study the degree of concordance between methods. Fisher's exact test was used to evaluate the agreement between GenoType[®] and MALDI-TOF. A *p* value <0.05 was considered statistically significant. The analyses were done with STATA 13.1 (StataCorp, USA).

Results

The results obtained are shown in Table 1 and Fig. 1 (Figures 2–8 are included as Supplementary material).

Phylogenetic and BLAST analyses were in agreement, but in 2 strains, phylogenetic analysis found no significant differences

between at least two species of *Mycobacterium* spp. (Table 1: strains 29 and 45). Strains were considered correctly classified if any of these results were obtained.

By using BLAST, samples 42 and 45 displayed a sequence identity less than 98%, when they were compared to those available in the GeneBank. The identification was in accordance with phylogenetic analysis. For sample 45, phylogenetic analysis found no significant differences between *M. duvalii* and *M. monacense*.

Three strains (6.5%) were not identified using partial *rpoB* gene sequencing (BLAST and phylogeny) because it was not possible to amplify them by PCR.

Eight strains were identified by partial *rpoB* gene sequence as *M. abscessus*. It was not possible classified these *M. abscessus* at subspecies level.

GenoType[®] and partial *rpoB* gene sequencing

Agreement between GenoType[®] and partial *rpoB* sequencing methods was observed in 27 of 43 cases (62.8%), Kappa value 0.534 (*p* < 0.001).

Table 1

Clinical samples, identification with GenoType[®], phylogenetical analysis, BLAST, % similarity with BLAST, identification with MALDI-TOF and MALDI-TOF score.

N° sample	Sample	GenoType [®]	Phylogenetical	BLAST	% similarity	MALDI-TOF	MALDI-TOF score
1	Sputum	<i>M. abscessus</i>	<i>M. abscessus</i>	<i>M. abscessus</i>	99.86	<i>M. abscessus</i>	2.019
2	Sputum	<i>M. abscessus</i>	<i>M. abscessus</i>	<i>M. abscessus</i>	100.00	<i>M. abscessus</i>	2.027
3	Bronchoalveolar lavages	<i>M. abscessus</i>	<i>M. abscessus</i>	<i>M. abscessus</i>	100.00	<i>M. abscessus</i>	2.162
4	Sputum	<i>M. abscessus</i>	<i>M. abscessus</i>	<i>M. abscessus</i>	99.86	<i>M. abscessus</i>	2.169
5	Bronchoalveolar lavages	<i>M. abscessus</i>	<i>M. abscessus</i>	<i>M. abscessus</i>	100.00	<i>M. abscessus</i>	2.174
6	Sputum	<i>M. abscessus</i>	<i>M. abscessus</i>	<i>M. abscessus</i>	100.00	<i>M. abscessus</i>	2.191
7	Sputum	<i>M. abscessus</i>	<i>M. abscessus</i>	<i>M. abscessus</i>	100.00	<i>M. abscessus</i>	2.225
8	Bronchial aspirates	<i>M. abscessus</i>	<i>M. abscessus</i>	<i>M. abscessus</i>	100.00	<i>M. abscessus</i>	2.273
9	Sputum	<i>M. abscessus</i>	^a	^a		<i>M. abscessus</i>	2.011
10	Sputum	<i>M. chelonae</i>	<i>M. chelonae</i>	<i>M. chelonae</i>	99.69	<i>M. chelonae</i>	1.87
11	Sputum	<i>M. chelonae</i>	<i>M. chelonae</i>	<i>M. chelonae</i>	100.00	<i>M. chelonae</i>	1.873
12	Sputum	<i>M. chelonae</i>	<i>M. chelonae</i>	<i>M. chelonae</i>	100.00	<i>M. chelonae</i>	1.93
13	Sputum	<i>M. chelonae</i>	<i>M. chelonae</i>	<i>M. chelonae</i>	100.00	<i>M. chelonae</i>	1.993
14	Skin biopsy	<i>M. chelonae</i>	<i>M. chelonae</i>	<i>M. chelonae</i>	100.00	<i>M. chelonae</i>	2.005
15	Skin biopsy	<i>M. chelonae</i>	<i>M. chelonae</i>	<i>M. chelonae</i>	100.00	<i>M. chelonae</i>	2.012
16	Skin biopsy	<i>M. chelonae</i>	<i>M. chelonae</i>	<i>M. chelonae</i>	99.69	<i>M. chelonae</i>	2.03
17	Sputum	<i>M. chelonae</i>	<i>M. chelonae</i>	<i>M. chelonae</i>	100.00	<i>M. chelonae</i>	2.041
18	Sputum	<i>M. chelonae</i>	<i>M. chelonae</i>	<i>M. chelonae</i>	99.86	<i>M. chelonae</i>	2.043
19	Sputum	<i>M. chelonae</i>	<i>M. chelonae</i>	<i>M. chelonae</i>	100.00	<i>M. chelonae</i>	2.057
20	Sputum	<i>M. chelonae</i>	<i>M. chelonae</i>	<i>M. chelonae</i>	100.00	<i>M. chelonae</i>	2.063
21	Sputum	<i>M. chelonae</i>	<i>M. chelonae</i>	<i>M. chelonae</i>	100.00	<i>M. chelonae</i>	2.098
22	Corneal ulcer exudate	<i>M. chelonae</i>	<i>M. chelonae</i>	<i>M. chelonae</i>	98.66	<i>M. chelonae</i>	2.117
23	Skin biopsy	<i>M. chelonae</i>	<i>M. chelonae</i>	<i>M. chelonae</i>	100.00	<i>M. chelonae</i>	2.131
24	Sputum	<i>M. chelonae</i>	<i>M. chelonae</i>	<i>M. chelonae</i>	100.00	<i>M. chelonae</i>	2.231
25	Sputum	<i>M. chelonae</i>	<i>M. chelonae</i>	<i>M. chelonae</i>	99.86	<i>M. chelonae</i>	2.338
26	Sputum	<i>M. chelonae</i>	^a	^a		<i>M. chelonae</i>	2.112
27	Sputum	<i>M. fortuitum</i>	<i>M. alvei</i>	<i>M. alvei</i>	99.08	<i>M. peregrinum</i>	1.717
28	Sputum	<i>M. fortuitum</i>	<i>M. alvei</i>	<i>M. alvei</i>	99.20	<i>M. boenickei/peregrinum</i>	1.757
29	Sputum	<i>M. fortuitum</i>	<i>M. alvei/peregrinum</i>	<i>M. alvei</i>	98.96	<i>M. conceptionense</i>	1.781
30	Sputum	<i>M. fortuitum</i>	<i>M. fortuitum</i>	<i>M. fortuitum</i>	99.20	<i>M. fortuitum</i>	2.046
31	Sputum	<i>M. fortuitum</i>	<i>M. fortuitum</i>	<i>M. fortuitum</i>	98.29	<i>M. fortuitum</i>	2.133
32	Sputum	<i>M. fortuitum</i>	<i>M. fortuitum</i>	<i>M. fortuitum</i>	100.00	<i>M. fortuitum</i>	2.265
33	Sputum	<i>M. fortuitum</i>	<i>M. mageritense</i>	<i>M. mageritense</i>	99.56	<i>M. mageritense</i>	1.715
34	Urine	<i>M. fortuitum</i>	<i>M. mageritense</i>	<i>M. mageritense</i>	100.00	<i>M. mageritense</i>	1.98
35	Sputum	<i>M. fortuitum</i>	<i>M. mageritense</i>	<i>M. mageritense</i>	99.22	<i>M. mageritense</i>	2.089
36	Bronchial aspirates	<i>M. fortuitum</i>	<i>M. mageritense</i>	<i>M. mageritense</i>	99.37	<i>M. mageritense</i>	2.125
37	Sputum	<i>M. fortuitum</i>	<i>M. mageritense</i>	<i>M. mageritense</i>	100.00	<i>M. mageritense</i>	2.227
38	Bronchial aspirates	<i>M. fortuitum</i>	<i>M. peregrinum</i>	<i>M. peregrinum</i>	100.00	<i>M. peregrinum</i>	1.978
39	Sputum	<i>M. fortuitum</i>	<i>M. peregrinum</i>	<i>M. peregrinum</i>	98.92	<i>M. peregrinum</i>	2.037
40	Sputum	<i>M. fortuitum</i>	<i>M. peregrinum</i>	<i>M. peregrinum</i>	100.00	<i>M. peregrinum</i>	2.269
41	Sputum	<i>M. fortuitum</i>	<i>M. peregrinum</i>	<i>M. peregrinum</i>	100.00	<i>M. peregrinum</i>	2.269
42	Sputum	<i>M. fortuitum</i>	<i>M. septicum</i>	<i>M. septicum</i>	95.01	<i>M. septicum</i>	1.704
43	Sputum	<i>M. fortuitum</i>	<i>M. setense</i>	<i>M. setense</i>	98.96	<i>M. alvei</i>	1.843
44	Sputum	<i>M. fortuitum</i>	^a	^a		<i>M. conceptionense</i>	1.89
45	Sputum	<i>Mycobacterium</i> sp.	<i>M. monacense/duvalii</i>	<i>M. duvalii</i>	91.91	<i>M. vaccae</i>	1.46
46	Pleural fluid	<i>Mycobacterium</i> sp.	<i>M. mucogenicum</i>	<i>M. mucogenicum</i>	100.00	<i>M. mucogenicum</i>	2.04

^a Not amplify by PCR.

particular way by researches, are necessary to identify the three subspecies: *rpoB*, *secA*, *sodA* and *hsp65* genes.^{17,18} The single gene-based approach is sensitive to the evolutionary history of that gene, but not necessarily of the species, and it may be limited by the lack of sufficient variation in a single gene sequence. Therefore, a multiple gene-based approach may lead to an accurate inference of phylogenetic tree for subspecies classification.¹⁹ Nevertheless some authors have described methods in which MALDI-TOF MS is used to differentiate *M. abscessus* complex at subsp. level.²⁰ We consider that it would be of great interest to expand the study for the identification of this species of mycobacteria and its subspecies by means of MALDI-TOF MS due to its vital clinical importance.

M. fortuitum complex comprises the following species: *M. fortuitum*, *M. peregrinum*, *M. senegalense*, *M. setense*, *M. mageritense*, *M. septicum*, *M. alvei*, *M. houstonense*, *M. boenickei*, *M. conceptiionense*, *M. porcinum*, *M. neworleansense* and *M. brisbanense*. In our study we found 18 *M. fortuitum* complex isolates but only six different species. According to the manufacturer's instructions, GenoType[®] differentiates *M. fortuitum* type 1, *M. fortuitum* type 2/*M. mageritense* and *M. peregrinum*/*M. alvei*/*M. septicum* but, in this study, all of them were identified as *M. fortuitum* by GenoType[®]. Despite the limitation implied by the number and variety of those studied species, when GenoType[®], MALDI-TOF MS and *rpoB* sequencing methods were compared, it was shown that MALDI-TOF MS was superior respect to GenoType[®] method to distinguish *M. fortuitum* complex species. With our results, GenoType[®] would not be able to distinguish *M. fortuitum* complex species. However, it would be necessary and interesting to have a greater amount of isolates and species of this complex available in order to be able to verify these results and obtain more solid conclusions. By using MALDI-TOF MS, 4/17 strains of *M. fortuitum* complex were misidentified. However, it must be pointed out that these four strains showed¹² a MALDI-TOF MS score <1.85.

M. mucogenicum was identified only in one sample with MALDI-TOF MS and partial *rpoB* sequencing method, while it was identified as *Mycobacterium* spp. with GenoType[®]. Han et al.²¹ described *M. mucogenicum* as the dominant RGM species responsible for bloodstream and catheter-related infections.

As we have pointed out previously, one limitation of our work would be the total number of different species included in the study. Another limitation of our study were the two strains with a partial *rpoB* gene sequencing similarity <98% with respect to the sequences of reference from BLAST. Besides, the phylogenetic analysis found no significant differences between at least two species of *Mycobacterium* spp.: *M. alvei/peregrinum* and *M. monacense/duvalii*. These strains could not be classified correctly due to the short length of the amplified fragment. These strains would be candidates to further study to establish their identification at species level. Interestingly, the 2 strains with partial *rpoB* gene sequencing with a similarity <98% showed MALDI-TOF MS scores <1.85 and one of them was identified with phylogenetic analysis. The sequencing of additional regions (complete 16S rRNA, *hsp65*, *sodA*, *dnaK* genes)^{22–24} would be indicated in these cases. It is known that sequence analysis of DNA isolated from clinical samples has also allowed the discovery of new bacterial pathogens.²⁵

When MALDI-TOF MS score was <1.85, the revision of these MALDI-TOF MS spectra is proposed with the aim of completing the *Mycobacterium* spp. MALDI-TOF data base.

Another interesting aspect would be the enlargement of the study with slowly growing NTM. However, this would present a greater difficulty due to the intrinsic characteristics of these NTM, as well as the wide diversity of existing species.

In summary, molecular methods are more costly and time-consuming and they may not be available in some clinical microbiology laboratory. Subsequently, our results, which were

obtained with a limited number of rapidly growing mycobacteria species, indicate that the MALDI-TOF MS analysis could be a quick identification method in the future. In addition, to identify new species and subspecies of *Mycobacterium* spp., as well as to identify correctly all clinically relevant *Mycobacterium* spp., it will be required the sequencing of additional genes of *Mycobacterium* spp. or whole genome sequencing and the subsequent phylogenetic analysis.

Conflict of interest

The authors report no conflict of interest. The authors alone are responsible for the content and writing of the paper.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.eimc.2018.04.012.

References

1. Tortoli E. Microbiological features and clinical relevance of new species of the genus *Mycobacterium*. Clin Microbiol Rev. 2014;27:727–52.
2. McGrath EE, Anderson PB. Increased prevalence of non-tuberculous mycobacteria infection. Lancet. 2007;370:28.
3. Griffith DE, Aksamit T, Brown-Elliott BA, Catanzaro A, Daley C, Gordin F, et al., ATS Mycobacterial Diseases Subcommittee; American Thoracic Society; Infectious Disease Society of America. An official ATS/IDSA statement: diagnosis, treatment, and prevention of nontuberculous mycobacterial diseases. Am J Respir Crit Care Med. 2007;175:367–416.
4. Clark AE, Kaleta EJ, Arora A, Wolk DM. Matrix-assisted laser desorption ionization-time of flight mass spectrometry: a fundamental shift in the routine practice of clinical microbiology. Clin Microbiol Rev. 2013;26:547–603.
5. Adékambi T, Colson P, Drancourt M. *rpoB*-based identification of nonpigmented and late-pigmenting rapidly growing mycobacteria. J Clin Microbiol. 2003;41:5699–708.
6. Adékambi T, Drancourt M, Raoult D. The *rpoB* gene as a tool for clinical microbiologists. Trends Microbiol. 2009;17:37–45.
7. Guindon S, Gascuel O. A simple, fast and accurate algorithm to estimate large phylogenies by maximum likelihood. Syst Biol. 2003;52:696–704.
8. Posada D. jModelTest: phylogenetic model averaging. Mol Biol Evol. 2008;25:1253–6.
9. Anisimova M, Gascuel O. Approximate likelihood ratio test for branches: a fast, accurate and powerful alternative. Syst Biol. 2006;55:539–52.
10. Russo C, Tortoli E, Menichella D. Evaluation of the new GenoType *Mycobacterium* assay for identification of mycobacterial species. J Clin Microbiol. 2006 Feb;44:334–9.
11. Mediavilla-Gradolph MC, De Toro-Peinado I, Bermúdez-Ruiz MP, García-Martínez L, Ortega-Torres M, Montiel Quezel-Guerraz N, et al. Use of MALDI-TOF MS for identification of nontuberculous *Mycobacterium* species isolated from clinical specimens. Biomed Res Int. 2015;8:54078.
12. Alcaide F, Amlerová J, Bou G, Ceyssens PJ, Coll P, Corcoran D, et al. How to identify non-tuberculous *Mycobacterium* species using MALDI-TOF mass spectrometry. Clin Microbiol Infect. 2017. <http://dx.doi.org/10.1016/j.cmi.2017.11.012> [in press].
13. Lee MR, Sheng WH, Hung CC, Yu CJ, Lee LN, Hsueh PR. *Mycobacterium abscessus* complex infections in humans. Emerg Infect Dis. 2015;21:1638–46.
14. Brown-Elliott BA, Nash KA, Wallace RJ Jr. Antimicrobial susceptibility testing, drug resistance mechanisms, and therapy of infections with nontuberculous mycobacteria. Clin Microbiol Rev. 2012;25:545–82.
15. Rubio M, March F, Garrigó M, Moreno C, Español M, Coll P. Inducible and acquired clarithromycin resistance in the *Mycobacterium abscessus* complex. PLOS ONE. 2015. <http://dx.doi.org/10.1371/journal.pone.0140166>.
16. Nie W, Duan H, Huang H, Lu Y, Bi D, Chu N. Species identification of *Mycobacterium abscessus* subsp. *abscessus* and *Mycobacterium abscessus* subsp. *bolletii* using *rpoB* and *hsp65*, and susceptibility testing to eight antibiotics. Int J Infect Dis. 2014;25:170–4.
17. Macheras E, Roux AL, Ripoll F, Sivadon-Tardy V, Gutierrez C, Gaillard JL, et al. Inaccuracy of single-target sequencing for discriminating species of the *Mycobacterium abscessus* group. J Clin Microbiol. 2009;47:2596–600.
18. Zelazny AM, Root JM, Shea YR, Colombo RE, Shamputa IC, Stock F, et al. Cohort study of molecular identification and typing of *Mycobacterium abscessus*, *Mycobacterium massiliense* and *Mycobacterium bolletii*. J Clin Microbiol. 2009;47:1985–95.
19. Tan JL, Khang TF, Ngeow YF, Choo SW. A phylogenomic approach to bacterial subspecies classification: proof of concept in *Mycobacterium abscessus*. BMC Genomics. 2013;13:879.
20. Fangous MS, Mougari F, Gouriou S, Calvez E, Raskine L, Cambau E, et al. Classification algorithm for subspecies identification within the *Mycobacterium abscessus*

- species, based on matrix-assisted laser desorption ionization-time of flight mass spectrometry. *J Clin Microbiol.* 2014;52:3362–9.
21. Han XY, Dé I, Jacobson KL. Rapidly growing mycobacteria: clinical and microbiologic studies of 115 cases. *Am J Clin Pathol.* 2007;128:612–21.
 22. Simmon KE, Kommedal Ø, Saebo Ø, Karlsen B, Petti CA. Simultaneous sequence analysis of the 16S rRNA and *rpoB* genes by use of RipSeq software to identify *Mycobacterium* species. *J Clin Microbiol.* 2010;48:3231–5.
 23. Zelazny AM, Calhoun LB, Li L, Shea YR, Fischer SH. Identification of *Mycobacterium* species by secA1 sequences. *J Clin Microbiol.* 2005;43:1051–8.
 24. Dai J1, Chen Y, Dean S, Morris JG, Salfinger M, Johnson JA. Multiple-genome comparison reveals new loci for *Mycobacterium* species identification. *J Clin Microbiol.* 2011;49:144–53.
 25. Slany M, Pavlik I. Molecular detection of non tuberculous mycobacteria: advantages and limits of a broad-range sequencing approach. *J Mol Microbiol Biotechnol.* 2012;22:268–76.