Received: 7 March 2022 | Accepted: 1 September 2022

DOI: 10.1111/1751-7915.14146

MICROBIAL BIOTECHNOLOGY Applied Microbiology International

RESEARCH ARTICLE

Identification of *Nocardia* and non-tuberculous *Mycobacterium* species by MALDI-TOF MS using the VITEK MS coupled to IVD and RUO databases

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Funding information
Instituto de Investigación Sanitaria Gregorio Marañón, Grant/Award Number: 2021; Instituto de Salud Carlos III, Grant/Award Number: CPII19/00002, PI15/01073 and PI18/00997

Abstract

Identification of *Nocardia* and *Mycobacterium* species by matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) is still a challenging task that requires both suitable protein extraction procedures and extensive databases. This study aimed to evaluate the VITEK MS Plus system coupled with updated RUO (v4.17) and IVD (v3.2) databases for the identification of *Nocardia* spp. and *Mycobacterium* spp. clinical isolates. Sample preparation was carried out using the VITEK MS Mycobacterium/Nocardia kit for protein extraction. From 90 *Nocardia* spp. isolates analysed, 86 (95.6%) were correctly identified at species or complex level using IVD and 78 (86.7%) using RUO. Only two strains were misidentified as other species pertaining to the same complex. Among the 106 non-tuberculous *Mycobacterium* clinical isolates tested from a liquid culture medium, VITEK MS identified correctly at species or complex level 96 (90.6%) isolates in the IVD mode and 89 (84.0%) isolates in the RUO mode. No misidentifications were detected. Although the IVD mode was unable to differentiate members of the *M. fortuitum* complex, the RUO mode correctly discriminated *M. peregrinum* and *M. septicum*. The robustness and accuracy showed by this system allow its implementation for routine identification of these microorganisms in clinical laboratories.

INTRODUCTION

Nocardia spp. and *Mycobacterium* spp. are the causative agents of opportunistic infections in immunocompromised and immunocompetent individuals, mainly of pulmonary presentation but also cutaneous and soft-tissue infections (Conville et al., 2018; Falkinham, 2016). Moreover, isolation of non-tuberculous mycobacteria (NTM) has been increasing in last years in clinical microbiology laboratories (Alcaide et al., 2017). Rapid identification of clinical isolates of these two groups of microorganisms

at species level is essential due to different antimicrobial susceptibility patterns among them (Conville et al., 2018; Griffith et al., 2007).

Identification of these two groups of microorganisms has been traditionally performed by phenotypic and biochemical methods, which have been replaced by molecular techniques (Sinner et al., 2015). In the case of non-tuberculous *Mycobacterium* spp., they can be identified by commercial kits based on PCR-reverse hybridization, but only for a limited number of NTM species (Makinen et al., 2006). DNA sequencing services are then required, which may reveal laborious and costly.

The use of matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) has been widely implemented for microbial identification (Patel, 2015). However, some acid-fast microorganisms like *Nocardia* spp. and *Mycobacterium* spp. still represent a challenge. Due to the characteristics of their cell wall, they require a specific protein extraction procedure in order to obtain protein spectra suitable for identification by MALDI-TOF MS. Mycobacteria species also require an inactivation protocol due to their pathogenicity. The use of a commercial extraction kit can allow both extraction an inactivation. Several studies have evaluated different protein extraction protocols for *Nocardia* spp. and *Mycobacterium* spp. isolates (Girard et al., 2017; Rodriguez-Temporal et al., 2018; Saleeb et al., 2011). In addition, the updating of databases and implementation of in-house libraries can improve identification rate of these microorganisms but in the case of in-house databases, the results are not easy transposable to other laboratories (Rodriguez-Sanchez et al., 2016; Rodriguez-Temporal et al., 2017) (Marin et al., 2018).

The aim of this study was to evaluate the VITEK MS system using commercial and widely used In Vitro Diagnostic (IVD) and Research Use Only (RUO) databases for the identification of *Nocardia* spp. and *Mycobacterium* spp. with the help of a commercial kit for the protein extraction protocol.

EXPERIMENTAL PROCEDURES

Bacterial isolates

A total of 90 *Nocardia* isolates from 85 patients, representing 14 species (Table S1), and 106 non-tuberculous *Mycobacterium* isolates from 100 patients, encompassing 21 species (Table S2), were included in the study. They all sourced from clinical samples that were submitted to the Department of Clinical Microbiology and Infectious Diseases of the Hospital General Universitario Gregorio Marañón (Madrid, Spain) for identification between 1995 and 2021, and frozen at −80°C. *Nocardia* spp. isolates were previously identified by *16S rRNA* and/or *secA1* gene sequencing, thawed and cultured on Columbia sheep-blood agar (bioMérieux, Marcy l'Etoile, France) for 48 h at 37°C, the time required for colonies to be visible (Durand et al., 2020; Marin et al., 2018). *Mycobacterium* isolates were previously identified by 16S rRNA and/or *hsp65* gene sequencing (Rodriguez-Temporal et al., 2022), thawed and cultured in the BACTEC MGIT960 system following the manufacturer instructions (Becton Dickinson) until the tubes flagged positive.

Protein extraction and MALDI-TOF MS analysis

All isolates were processed with the VITEK MS *Mycobacterium*/*Nocardia* kit (bioMérieux) following the manufacturer's instructions. For *Nocardia* spp. strains, colonies were harvested with a cytology brush and transferred to a 1.5 ml Eppendorf tube with 500 μl of 70% ethanol and 0.5 mm glass beads. Then, the tubes were vortexed for 15 min for inactivation, followed by 10 min incubation at room temperature and the suspension was transferred to an empty tube, discarding the glass beads. Samples were centrifuged at 18,800 ***g*** for 2 min and the supernatant was discarded. Ten microliters of 70% formic acid were added to the pellet and the tubes were vortexed for 10 s. Then, 10 μl of acetonitrile were added and the tubes were vortexed again. Finally, the tubes were centrifuged at 14,000 rpm for 2 min, and 1 μl of supernatant was spotted in duplicate on the MALDI-TOF MS slide. After drying at room temperature, 1 μl of α-cyano-4-hydroxycinnamic acid (CHCA) matrix was added to each spot.

For *Mycobacterium* spp. isolates, 3 ml of liquid culture were transferred to a tube between 12–72 h after positivity. The tubes were centrifuged at 3000 *g* for 10 min, the supernatant was discarded, and the pellet was resuspended with 500 μl of 70% ethanol. Then, the suspension was transferred to a tube with glass beads and inactivated by vortexing for 15 min. After this step, the protein extraction procedure was the same as what was performed for *Nocardia* spp. isolates. Samples were analysed with the VITEK MS system (bioMérieux) with IVD (v.3.2) and RUO (v.4.17) modes. Spectra were acquired in the linear positive mode within the 2000–20,000 Da range. *Escherichia coli* strain (ATCC 8739) was used in each run for instrument calibration.

Identifications with values ≥99.0% and between 60.0% and 99.0% were considered as high and low-confidence results, respectively. Values lower than 60.0% indicated two or three probable identifications. In these cases, concordance at genus or complex level was evaluated. When a disagreement was found, the identification obtained by DNA sequencing was considered as the reference.

Ethics approval

The Ethics Committee from the University Hospital Gregorio Marañon approved this study (Code: MALDI-Micobacterias). The study was carried out using anonymized microbiological samples, not human products. Therefore, all the conditions to waive the informed consent have been met. The research procedures were conducted following the Declaration of Helsinki guidelines.

RESULTS

Identification of *Nocardia* isolates

The VITEK MS system yielded 84 (93.3%) isolates correctly identified at species level using the IVD mode and 77 (85.6%) isolates using the RUO mode (Table 1). With the RUO mode, 4 strains were correctly classified in comparison to reference spectra present in this database with a matching percentage lower than 60.0%: *Nocardia asiatica* (53.5%), *Nocardia cyriacigeorgica* (42.7%), *Nocardia farcinica* (53.2%) and *Nocardia pneumoniae* (54.3%). Regarding the misidentifications obtained, one isolate of *N. pneumoniae* was identified as *Nocardia abscessus* by the IVD mode with low-confidence level (67.7%) and one isolate of *Nocardia veterana* was identified as *Nocardia africana/nova* by both IVD (99.9% confidence level) and RUO (84.0% confidence level) modes. In the two cases, the identification obtained belonged to a closely related species pertaining to the same complex (*N. abscessus* complex and *N. nova* complex, respectively). Considering this, a total of 95.6% and 86.7% of *Nocardia* isolates were correctly identified at species or complex level using IVD and RUO modes, respectively. Among the different species included in the study, *Nocardia concava* and *N. pneumoniae* are not included in VITEK MS IVD database, and thus, they could not be identified.

Identification of *Mycobacterium* isolates

Among *Mycobacterium* isolates, IVD mode identified correctly 96 (90.6%) isolates at species or complex level, two of them with confidence <99.0%: *Mycobacterium fortuitum* group (confidence level 45.8% and 25.0%). On the other hand, RUO mode identified 89 (84.0%) isolates at species or complex level (Table 2), three of them with values below 60.0%: *Mycobacterium intracellulare* (44.0%) and *Mycobacterium kansasii* (55.2% and 55.9%). Overall, no misidentifications were obtained by VITEK MS. Four strains of *M. intracellulare* were identified at genus level by RUO mode (confidence levels of 80.0%, 84.0%, 92.0% and 96.0%). In this study, 21 isolates belonging to *M. fortuitum* complex encompassing 4 species were included (*M. fortuitum*, *Mycobacterium peregrinum*, *Mycobacterium porcinum* and *Mycobacterium septicum*). The IVD mode identified all of them as *M. fortuitum* group, whereas RUO identified separately *M. peregrinum* and

TABLE 1 Identification obtained for *Nocardia* isolates by IVD and RUO modes

		IVD			RUO		
Species	***N***	**Correct ID (%)**	**No ID (%)**	**Incorrect ID (%)**	**Correct ID (%)**	**No ID (%)**	**Incorrect ID (%)**
N. abscessus	14	14 (100)	0 (0)	0 (0)	10 (71.4)	4 (28.6)	0 (0)
N. asiatica	1	1 (100)	0 (0)	0 (0)	1 (100)	0 (0)	0 (0)
N. brasiliensis	1	1 (100)	0 (0)	0 (0)	1 (100)	0 (0)	0 (0)
N. carnea	1	1 (100)	0 (0)	0 (0)	1 (100)	0 (0)	0 (0)
N. cyriacigeorgica	34	34 (100)	0 (0)	0 (0)	33 (97.1)	1 (2.9)	0 (0)
N. concava[a]	1	0 (0)	1 (100)	0 (0)	0 (0)	1 (100)	0 (0)
N. farcinica	17	17 (100)	0 (0)	0 (0)	16 (94.1)	1 (5.9)	0 (0)
N. neocaledoniensis	1	1 (100)	0 (0)	0 (0)	1 (100)	0 (0)	0 (0)
N. nova	4	4 (100)	0 (0)	0 (0)	2 (50)	2 (50)	0 (0)
N. otitidiscavarium	5	4 (80)	1 (20)	0 (0)	4 (80)	1 (20)	0 (0)
N. pneumoniae[a]	2	0 (0)	1 (50)	1 (50)[b]	1 (50)	1 (50)	0 (0)
N. pseudobrasiliensis	1	0 (0)	1 (100)	0 (0)	0 (0)	1 (100)	0 (0)
N. veterana	6	5 (83.3)	0 (0)	1 (16.7)[b]	5 (83.3)	0 (0)	1 (16.7)[b]
N. wallacei	2	2 (100)	0 (0)	0 (0)	2 (100)	0 (0)	0 (0)
Total	90	84 (93.3)	4 (4.4)	2 (2.2)[b]	77 (85.6)	12 (13.3)	1 (1.1)[b]

[a]Not included in VITEK MS database.

[b]Identified as other close related species belonging to the same complex.

TABLE 2 Identification obtained for *Mycobacterium* isolates by IVD and RUO modes

Species	*N*	IVD			RUO			ID genus level (%)
		Correct ID (%)	No ID (%)	Incorrect ID (%)	Correct ID (%)	No ID (%)	Incorrect ID (%)	
M. abscessus	18	18 (100)	0 (0)	0 (0)	18 (100)	0 (0)	0 (0)	0 (0)
M. avium	12	11 (91.7)	1 (8.3)	0 (0)	11 (91.7)	1 (8.3)	0 (0)	0 (0)
M. chelonae	1	1 (100)	0 (0)	0 (0)	1 (100)	0 (0)	0 (0)	0 (0)
M. europaeum[a]	3	0 (0)	3 (100)	0 (0)	0 (0)	3 (100)	0 (0)	0 (0)
M. fortuitum[b]	13	13 (100)	0 (0)	0 (0)	12 (92.3)	1 (7.7)	0 (0)	0 (0)
M. gordonae	10	9 (90)	1 (10)	0 (0)	9 (90)	1 (10)	0 (0)	0 (0)
M. intracellulare	13	11 (84.6)	2 (15.4)	0 (0)	9 (69.2)	0 (0)	0 (0)	4 (30.8)
M. kansasii	5	5 (100)	0 (0)	0 (0)	5 (100)	0 (0)	0 (0)	0 (0)
M. lentiflavum	9	9 (100)	0 (0)	0 (0)	7 (77.8)	2 (22.2)	0 (0)	0 (0)
M. mageritense	2	2 (100)	0 (0)	0 (0)	1 (50)	1 (50)	0 (0)	0 (0)
M. malmoense	1	1 (100)	0 (0)	0 (0)	0 (0)	1 (100)	0 (0)	0 (0)
M. paragordonae[a]	1	0 (0)	1 (100)	0 (0)	0 (0)	1 (100)	0 (0)	0 (0)
M. peregrinum	3	3 (100)[b]	0 (0)	0 (0)	3 (100)	0 (0)	0 (0)	0 (0)
M. porcinum[b]	4	4 (100)[b]	0 (0)	0 (0)	4 (100)[b]	0 (0)	0 (0)	0 (0)
M. rhodesiae[a]	1	0 (0)	1 (100)	0 (0)	0 (0)	1 (100)	0 (0)	0 (0)
M. septicum	1	1 (100)[b]	0 (0)	0 (0)	1 (100)	0 (0)	0 (0)	0 (0)
M. shimoidei	1	0 (0)	1 (100)	0 (0)	0 (0)	1 (100)	0 (0)	0 (0)
M. smegmatis	3	3 (100)	0 (0)	0 (0)	3 (100)	0 (0)	0 (0)	0 (0)
M. szulgai	1	1 (100)	0 (0)	0 (0)	1 (100)	0 (0)	0 (0)	0 (0)
M. triplex	1	1 (100)	0 (0)	0 (0)	1 (100)	0 (0)	0 (0)	0 (0)
M. xenopi	3	3 (100)	0 (0)	0 (0)	3 (100)	0 (0)	0 (0)	0 (0)
Total	106	96 (90.6)	10 (9.4)	0 (0)	89 (84)	13 (12.3)	0 (0)	4 (3.8)

[a]Not included in VITEK MS database.
[b]Identified as *M. fortuitum* group.

M. septicum. Three species analysed are not included in VITEK MS database, and they were not identified: *Mycobacterium europaeum*, *Mycobacterium paragordonae* and *Mycobacterium rhodesiae*.

DISCUSSION

The implementation of the VITEK MS system allowed 95.5% and 90.6% correct species or complex level identification of *Nocardia* spp. from plates and NTM isolates from liquid medium, respectively. In both cases, the IVD database provided a higher number of correct identifications. Only two *Nocardia* spp. isolates were misidentified at the species level and none of the NTM isolates (specificity of 97.7% for *Nocardia* and 100% for NTMs at the species level; specificity of 100% for both groups of microorganisms at the complex level).

In this study, the VITEK MS system coupled with an updated database (IVD v3.2) identified successfully 95.5% of *Nocardia* spp. isolates at species or complex level. By comparing both identification modes used, IVD and RUO, the latter reached a lower number of isolates identified. Previous studies using VITEK MS with IVD mode also showed high identification rates (87.0%–91.0%) for *Nocardia* spp. species (Body et al., 2018; Durand et al., 2020; Girard et al., 2016, 2017; Wei et al., 2019). Although different numbers of isolates and species were included in these studies, they showed similar results, demonstrating the robustness of the system for accurate identification of *Nocardia* strains.

Regarding misidentifications, only two isolates were misidentified at species level, although in both cases they belonged to the same complex. One of them was a *N. pneumoniae* isolate identified as *N. abscessus* (*N. abscessus* complex), due to the fact that *N. pneumoniae* is not included in IVD database but it is present in the RUO mode. The other misidentification consisted of a *N. veterana* isolate identified as *N. africana*/*nova* (*N. nova* complex); the same misidentification has also been observed in previous studies (Body et al., 2018; Girard et al., 2016).

For non-tuberculous *Mycobacterium* spp. isolates, both modes combined (IVD and RUO) identified 86.7% of them, although the RUO identification rate was also lower than IVD. Other studies that have compared these two modes also obtained higher results using IVD mode, which could have relation with

different identification algorithms used by them (Leyer et al., 2017; Wilen et al., 2015).

Of special interest is the fact that all isolates from the study have been analysed from a liquid culture medium. Previous studies focused on liquid media reported heterogeneous identification rates, from 69.4% to 98.8% (Huang et al., 2018; Kehrmann et al., 2016; Moreno et al., 2018; Park et al., 2016). These different results could be due to different protein extraction protocols performed, and different numbers and diversity of mycobacterial species analysed. In our study, similar results were obtained using IVD database v3.2, reaching 90.6% of isolates identified.

Interestingly, no incorrect species assignation was obtained, which shows the high reliability of this system for NTM identification. Only a few misidentifications have been reported in literature, involving close related species such as *Mycobacterium avium* and *M. intracellulare* (Miller et al., 2018), *Mycobacterium immunogenum* and *M. abscessus* (Brown-Elliott et al., 2019) or *Mycobacterium scrofulaceum* and *Mycobacterium parascrofulaceum* (Luo et al., 2018). In the present study, although no *M. scrofulaceum* and *M. immunogenum* species were included, *M. avium* and *M. intracellulare* were correctly distinguished from each other. On the other hand, IVD mode was unable to differentiate members from *M. fortuitum* complex, as observed previously (Luo et al., 2018). However, RUO mode successfully identified *M. peregrinum* and *M. septicum* at the species level. This suggests that in the future, it could be possible to differentiate species from this complex by VITEK MS. Lastly, three of the species analysed (*M. europaeum*, *M. paragordonae* and *M. rhodesiae*) were not represented in the database and, as expected, they were unidentified.

In conclusion, the processing of *Nocardia* and *Mycobacterium* isolates with the VITEK MS Mycobacterium/Nocardia kit and their identification with the VITEK MS system demonstrated that this method is suitable for implementation in clinical microbiology laboratories for the robust, accurate and standardized identification of these microorganisms at the species level. Only 6.7% of the *Nocardia* isolates and 9.4% of the NTMs sent to the clinical microbiology laboratory for routine identification with VITEK MS may not to be reliably identified. In these cases, molecular methods must be applied to achieve a definitive identification. The limited number of isolates requiring confirmatory tests renders VITEK MS an efficient method for rapid identification of *Nocardia* and NTM isolates. Besides, the constant addition of less common species to its database will continue to benefit the identification accuracy obtained by this system and reduce the number or isolates requiring confirmatory testing.

AUTHOR CONTRIBUTIONS

DRT involved in formal analysis, data collection, validation, visualization, original draft preparation and review/ editing. MEZ involved in formal analysis, supervision, validation, visualization, original draft preparation and review/ editing. PB, MBS, MM and MM and MJRS involved in data collection, validation and review/editing. PM involved in writing and review/editing. BRS involved in conceptualization, project administration, formal analysis, supervision, validation, visualization, original draft preparation and review/ editing.

ACKNOWLEDGEMENT

This work was supported by the projects PI15/01073 and PI18/00997 from the Health Research Fund (FIS. Instituto de Salud Carlos III. Plan Nacional de I+D+I 2013–2016) of the Carlos III Health Institute (ISCIII, Madrid, Spain) partially financed by the European Regional Development Fund (FEDER) 'A way of making Europe'. BRS (CPII19/00002) is recipient of a Miguel Servet contract supported by the FIS. DRT has been funded by the IiSGM through its intramural program.

FUNDING INFORMATION

This work was supported by the projects PI15/01073 and PI18/00997 from the Health Research Fund (FIS. Instituto de Salud Carlos III. Plan Nacional de I+D+I 2013–2016) of the Carlos III Health Institute (ISCIII, Madrid, Spain) partially financed by the European Regional Development Fund (FEDER) 'A way of making Europe'. BRS (CPII19/00002) is recipient of a Miguel Servet contract supported by the FIS. DRT has been funded by the IiSGM through its intramural program.

CONFLICT OF INTEREST

BioMérieux SA provided a VITEK MS instrument for its evaluation as well as VITEK MS *Mycobacterium*/*Nocardia* kits for the preparation of isolates for MALDI-TOF MS analysis.

DATA AVAILABILITY STATEMENT

The protein spectra analysed in this project are available upon request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Rodríguez-Temporal, D., Zvezdánova, M.E., Benedí, P., Marín, M., Blázquez-Sánchez, M. & Ruiz-Serrano, M.J. et al. (2023) Identification of *Nocardia* and nontuberculous *Mycobacterium* species by MALDI-TOF MS using the VITEK MS coupled to IVD and RUO databases. *Microbial Biotechnology*, 16, 778–783. Available from: https://doi.org/10.1111/1751-7915.14146