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Evaluation of matrix-assisted laser desorption ionization-time of flight mass spectrometry in diagnosis of clinical *Nocardia* species

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Abstract

Background: The routine identification to the species level of *Nocardia* genus by conventional methods is a fastidious and time-consuming process owing to the limited biochemical reactivity of these microorganisms, often requiring 1 or more days to complete identification. Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) is a new technology for definitive and rapid species identification.
Methods: We evaluated the MALDI-TOF MS for the identification of 44 clinical isolates of *Nocardia* species in comparison to 16S ribosomal RNA (rRNA) gene sequencing. *Nocardia* isolates were identified by microbiological examination, phenotypical tests and MALDI-TOF MS and the results were compared by 16S rRNA gene sequencing.
Results: Of the 44 *Nocardia* strains, the identification of 28 isolates was determined with MALDI Biotyper database. According to this, 16 isolates (57.1%) of the strain log scores were ≥2. Two (7.1%) were identified to the species level (log scores of ≥2) as *Nocardia otitidiscaviarum*. The addition of a newly established *Nocardia* database (16 new *Nocardia* strains included to the original database) did significantly improve the scores. The results were 43 (97.7%) correct identification to the species level (log scores of ≥2).
Conclusions: This study showed that the identification of clinical *Nocardia* isolates by the Bruker MALDI Biotyper is highly reliable, whereas identification rates are generally lower than those for some Gram-negative bacteria and Gram-positive cocci. Based on our data, the identification rates can be improved by validated new database entries and the results can be confirmed with nucleic acid sequence analysis.

Keywords: identification; MALDI-TOF; *Nocardia*; 16S rRNA.

Introduction

Nocardia species are isolated from clinical specimens, especially those from immunocompromised patients. Infections of *Nocardia* species range from pulmonary nocardiosis to cutaneous nocardiosis, brain abscess and bacteremia [1]. As there are no comprehensive epidemiological studies related to *Nocardia* sp. in Turkey, the exact prevalence of the disease is still unknown. However, there are several case reports related to *Nocardia* spp. associated infections in Turkey [2, 3].

In clinical laboratories, *Nocardia* species are routinely identified by using Gram and modified acid-fast staining and biochemical tests. These tests are cumbersome, time-consuming processes and require trained staff [4]. 16S ribosomal RNA (rRNA) gene sequencing is considered to be the gold standard method for definitive identification of *Nocardia* species [5]. However, these methods are difficult to implement for routine bacterial identification.

Recently, matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) has been introduced for rapid, accurate, inexpensive and definitive identification of various groups of microorganisms [6–8]. There are several studies on the identification of various groups of microorganisms; however, few studies have investigated the performance of MALDI-TOF MS for *Nocardia* species [9].

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The aim of this study was to establish a reference database for clinically relevant *Nocardia* species and to compare the Bruker MALDI Biotyper system (Bruker Daltonics, Bremen, Germany) to the reference 16S rRNA gene sequencing.

Materials and methods

Patients and bacterial strains

Forty-four clinical isolates of *Nocardia* species and one reference strain (*Nocardia cyriacigeorgica* DSM 44484) were used in this study. The clinical *Nocardia* isolates had all been isolated from clinical samples of hospitalized adult patients (>18 years of age) between 2002 and 2012. Among the patients, 25 of them were female and 19 of them were male. Eighteen patients were from Central Anatolia, 12 patients were from the Aegean Region, six were from Marmara, four were from the Black Sea Region and four were from the Southeastern Anatolia Region. Clinical origins of the strains were as follows: 16 strains were isolated from the respiratory tract and two were isolated from the pleural fluid of 18 patients who suffered from pulmonary infection; seven strains were isolated from pus, five strains were from the tissue biopsy samples taken from 12 patients with cutaneous infections; eight strains were from brain abscess, one was from the cerebrospinal fluid samples obtained from nine patients with central nervous infection; among other samples, three of them were taken from patients with conjunctivitis and two of them were obtained from patients who had bacteremia. The study was conducted according to the ethical guidelines.

All of the *Nocardia* species were stored at −80 °C until the initiation of the study. The primary identification of isolates was made by Gram stain, modified acid-fast stain and phenotypical tests (lysozyme resistance, production of arylsulfatase, caseine, xanthine, hypoxanthine, tyrosine and starch hydrolysis, and acid production from rhamnose) and growth at 45 °C [10].

All strains were subcultured twice on Trypticase soy agar with 5% sheep blood (BBL, Becton Dickinson Microbiology systems, Cockeysville, MD, USA) and incubated for 48 h to 5 days at 37 °C prior to testing. The identification of strains was confirmed by microbiological examination and by biochemical tests.

MALDI-TOF MS

For the analysis of 44 *Nocardia* species by the Bruker MALDI Biotyper system, all isolates were cultured on Trypticase soy agar with 5% sheep blood (Becton Dickinson Microbiology Systems, Sparks, MD, USA) and incubated for 48 h at 37 °C. These isolates were extracted using the ethanol-formic acid extraction method [11]. Each sample was spotted in duplicate.

MALDI-TOF MS measurements were performed on a Microflex LT mass spectrometer (Bruker Daltonics GmbH, Bremen, Germany) equipped with a 60-Hz nitrogen laser. MALDI Biotyper 3.1 software program and a reference library of 4612 entries were used to analyze the spectra. For extension of the Bruker MALDI Biotyper database, reference spectra (metaspectra [MSP]) were established based on the Bruker standard procedure. MSP was calculated based on at least 20 mass spectra that passed the quality control successfully. Cut-off values reported by Blosser et al. [9] for the determination of *Nocardia* isolates were applied to determine the genus-level (1.7000–1.999) or the species-level (≥2000) identification.

DNA extraction and sequence analysis of 16S rRNA

DNA was extracted using the genomic DNA isolation kit (QIAamp DNA Mini Kit; Qiagen) according to the kit protocol. Universal primers: 27F; AGA GTT TGA TCM TGG CTC AG and 1492R; GGT TAC CTT GTT ACG ACT T were used to amplify a 1465-bp product of 16S rRNA [12]. The amplified polymerase chain reaction (PCR) products were purified using the QIAquick PCR purification kit (Qiagen) and the BigDye Direct Cycle Sequencing Kit (Applied Biosystems) was used in sequence analyses according to the manufacturer's instructions. After cycle sequencing, amplicons were purified with Sephadex G-50 (Sigma-Aldrich) and sequenced on Applied Biosystems 3500 Genetic Analyzer (Applied Biosystems, CA, USA). All sequences including the positive control strain were analyzed using Sequencing Analysis version 5.3 (Applied Biosystems, CA, USA) and compared with reference sequences in National Center for Biotechnology Information website with BLASTN. Discrepant identification results between the molecular and Bruker MALDI Biotyper methods were repeated twice.

Results

Phenotypic characterization

Forty-four clinical isolates were phenotypically characterized as *Nocardia* species based on colony morphology,

Gram stain, modified acid-fast stain and resistance to lysozyme. Sixteen *Nocardia* species were identified to the species level. Twelve strains were identified as *Nocardia farcinica* and four strains were identified as *Nocardia otitidiscaviarum*. The residual 28 strains were only identified to the genus level (Table 1).

16S rRNA sequencing of *Nocardia* strains

As a reference method all strains were analyzed by 16S rRNA gene sequencing. Sequence data of the amplified PCR products showed high similarity to reference sequences of strains in GenBank (National Center for Biotechnology Information [NCBI]) between 97% and 99% at the species level. The sequences of 44 *Nocardia* strains were deposited in GenBank (NCBI) under accession numbers as mentioned in Table 2. According to the results, *N. cyriacigeorgica* was the most frequently isolated species (56.8%) (Table 1).

MALDI-TOF MS identification of *Nocardia* strains

Of the 44 *Nocardia* strains, the first 28 were evaluated using the MALDI Biotyper 4612 database and only two of them (isolate numbers 16 and 28; *N. otitidiscaviarum*) were correctly identified at the species level. After DNA sequencing, these 28 (*N. cyriacigeorgica* [n=15], *N. farcinica* [n=10], *N. otitidiscaviarum* [n=3]) isolates were added to the database 4612 (Table 2).

Among 44 isolates, 16 of them were not evaluated by the database 4612. However, these isolates were identified by 16S rRNA sequencing analysis and one *Nocardia asteroides*, one *Nocardia neocaledoniensis* and one *Nocardia abscessus* species were also added to the database 4612.

Table 1: Phenotypic and 16S rRNA sequencing results of 44 *Nocardia* strains.

Phenotypic identification		16S rRNA sequencing	
Species	**Number (%)**	**Species**	**Number (%)**
Nocardia spp.	28 (63.6)	*N. cyriacigeorgica*	25 (56.82)
N. farcinica	12 (27.3)	*N. farcinica*	12 (27.28)
N. otitidiscaviarum	4 (9.1)	*N. otitidiscaviarum*	4 (9.09)
		N. asteroids	1 (2.27)
		N. neocaledoniensis	1 (2.27)
		N. abscessus	1 (2.27)
Total	44 (100)	Total	44 (100)

After these additions, the results were 43 (97.7%) identified correctly to the species level (log scores of ≥2). Only one *N. neocaledoniensis* isolate (isolate number 33) was misidentified as *N. asteroides* in this MALDI Biotyper extended database (Table 3).

Discussion

In clinical microbiology laboratories, *Nocardia* are traditionally identified using manual or automated phenotypic and biochemical methods. These methods are generally reliable for species-level identification but are often cumbersome and time-consuming.

Different approaches have been developed to accelerate the identification of clinically significant *Nocardia* when compared to traditional biochemical phenotyping. These include DNA probe hybridization, PCR and PCR-restriction fragment length polymorphism (RFLP) molecular analysis (PRA), DNA sequencing, pyrosequencing, multilocus sequence analysis and ribotyping [5, 13]. To fully utilize 16S rRNA sequencing in clinical microbiology, better guidelines are needed for interpretation of the identification results, and alternative molecular methods [14] are necessary for bacterial species that cannot be identified confidently by16S rRNA gene sequencing alone.

MALDI-TOF MS is a rapid bacterial identification technique that is increasingly used in microbiology laboratories [6]. Previous studies have compared the performance of MALDI-TOF MS systems and DNA sequence analysis techniques for identification of several types of bacterial pathogens; however, only a few studies have investigated the performance of MALDI-TOF MS for *Nocardia* species [4, 15, 16]. Verroken et al. [4] evaluated the methodology against 43 blind-coded clinical isolates of *Nocardia* species by phenotypical and full-length 16S rRNA gene sequencing. Of the studied *Nocardia* spectra aligned with the MALDI Biotyper database, 19 isolates (44%) were correctly identified, of which 10 (23%) were identified to the species level (log scores of ≥2) and nine (21%) were identified to the genus level (log scores between <2 and ≥1.7) [9]. The addition of their own established *Nocardia* database to the original database did significantly improve the scores, leading to 38 (88%) correct identification. The Bruker MALDI Biotyper system failed to identify *N. abscessus*, *Nocardia beijingensis*, *Nocardia brasiliensis*, *Nocardia carnea* and *Nocardia veterana*, yielded log scores of <1.7 and thus could not be identified. In the current study, only *N. neocaledoniensis* was misidentified as *N. asteroides*. There were 37 *Nocardia* strains and 35 species in the former database

with 4612 entries which was called 4612 database. After this study, 28 *Nocardia* strains and two new species (*N. asteroides* and *N. abscessus*) were added to the 4612 database and the database increased to 65 *Nocardia* strains and 37 species. Presently, the MALDI Biotyper database consists of over 7000 entries and called 7000 database, but *N. neocaledoniensis* still does not exist in this current database.

Correct identification to the species level for 12 different *Nocardia* species was reported by Farfour et al. [15] to be 91.3% (42/46 strains) using the Andromas MALDI-TOF MS system with the direct colony method. They concluded

Table 2: Results and scores of 44 *Nocardia* strains with the DNA sequencing, MALDI Biotyper and MALDI Biotyper extended database.

No	16S rRNA	Accession numbers	MALDI Biotyper 4612 database		MALDI Biotyper extended database	
			Result	Score	Result	Score
1	*N. cyriacigeorgica*	KP268886	*Nocardia* sp.	2.102	*N. cyriacigeorgica*[b]	2.647
2	*N. farcinica*	KP279642	*Nocardia* sp.	1.532	*N. farcinica*[b]	2.402
3	*N. cyriacigeorgica*	KP268887	*Nocardia* sp.	1.515	*N. cyriacigeorgica*[b]	2.314
4	*N. farcinica*	KP279646	*Nocardia* sp.	1.947	*N. farcinica*[b]	2.491
5	*N. farcinica*	KP279644	*Nocardia* sp.	1.296	*N. farcinica*[b]	2.321
6	*N. cyriacigeorgica*	KP268888	*Nocardia* sp.	2.137	*N. cyriacigeorgica*[b]	2.507
7	*N. farcinica*	KP279640	*N. brasiliensis*	1.599	*N. farcinica*[b]	2.254
8	*N. farcinica*	KP279643	*N. brasiliensis*	1.343	*N. farcinica*[b]	2.239
9	*N. cyriacigeorgica*	KP268889	*Nocardia* sp.	2.217	*N. cyriacigeorgica*[b]	2.47
10	*N. farcinica*	KP279647	*Nocardia* sp.	1.619	*N. farcinica*[b]	2.277
11	*N. cyriacigeorgica*	KP268882	*Nocardia* sp.	2.115	*N. cyriacigeorgica*[b]	2.593
12	*N. cyriacigeorgica*	KP268881	*Nocardia* sp.	2.174	*N. cyriacigeorgica*[b]	2.694
13	*N. cyriacigeorgica*	KP268883	*Nocardia* sp.	2.162	*N. cyriacigeorgica*[b]	2.396
14	*N. cyriacigeorgica*	KP268884	*Nocardia* sp.	2.255	*N. cyriacigeorgica*[b]	2.615
15	*N. cyriacigeorgica*	KP268890	*Nocardia* sp.	2.15	*N. cyriacigeorgica*[b]	2.516
16	*N. otitidiscaviarum*	KP268907	*N. otitidiscaviarum*	2.11[a]	*N. otitidiscaviarum*[b]	2.46
17	*N. cyriacigeorgica*	KP268891	*Nocardia* sp.	2.063	*N. cyriacigeorgica*[b]	2.506
18	*N. cyriacigeorgica*	KP268892	*Nocardia* sp.	2.13	*N. cyriacigeorgica*[b]	2.637
19	*N. cyriacigeorgica*	KP268893	*Nocardia* sp.	2.203	*N. cyriacigeorgica*[b]	2.627
20	*N. farcinica*	KP279648	*Nocardia* sp.	1.555	*N. farcinica*[b]	2.288
21	*N. otitidiscaviarum*	KP268908	*N. otitidiscaviarum*	1.804	*N. otitidiscaviarum*[b]	2.318
22	*N. farcinica*	KP279645	*Nocardia* sp.	1.378	*N. farcinica*[b]	2.359
23	*N. farcinica*	KP279649	*Nocardia* sp.	1.559	*N. farcinica*[b]	2.285
24	*N. cyriacigeorgica*	KP268894	*Nocardia* sp.	2.119	*N. cyriacigeorgica*[b]	2.605
25	*N. cyriacigeorgica*	KP268895	*Nocardia* sp.	2.135	*N. cyriacigeorgica*[b]	2.703
26	*N. cyriacigeorgica*	KP268880	*Nocardia* sp.	2.165	*N. cyriacigeorgica*[b]	2.583
27	*N. farcinica*	KP279641	*Nocardia* sp.	1.686	*N. farcinica*[b]	2.315
28	*N. otitidiscaviarum*	KP268904	*N. otitidiscaviarum*	2.033[a]	*N. otitidiscaviarum*[b]	2.274
29	*N. cyriacigeorgica*	KP268896	ND		*N. cyriacigeorgica*	2.073
30	*N. otitidiscaviarum*	KP268905	ND		*N. otitidiscaviarum*	2.656
31	*N. cyriacigeorgica*	KP268897	ND		*N. cyriacigeorgica*	2.334
32	*N. cyriacigeorgica*	KP268898	ND		*N. cyriacigeorgica*	2.355
33	*N. neocaledoniensis*	KP268903	ND		*N. asteroides*[b]	2.015
34	*N. cyriacigeorgica*	KR078341	ND		*N. cyriacigeorgica*	2.194
35	*N. cyriacigeorgica*	KP268899	ND		*N. cyriacigeorgica*	2.591
36	*N. abscessus*	KP268876	ND		*N. abscessus*[b]	2.232
37	*N. farcinica*	KP279650	ND		*N. farcinica*	2.358
38	*N. farcinica*	KP279651	ND		*N. farcinica*	2.19
39	*N. cyriacigeorgica*	KP268900	ND		*N. cyriacigeorgica*	2.738
40	*N. cyriacigeorgica*	KP268901	ND		*N. cyriacigeorgica*	2.177
41	*N. cyriacigeorgica*	KP268879	ND		*N. cyriacigeorgica*	2.504
42	*N. cyriacigeorgica*	KP268878	ND		*N. cyriacigeorgica*	2.038
43	*N. cyriacigeorgica*	KP268902	ND		*N. cyriacigeorgica*	2.606
44	*N. asteroides*	KP268877	ND		*N. asteroides*[b]	2.33

[a]Two *N. otitidiscaviarum* isolates were identified correctly in 4612 database. [b]Isolates that were added to the database. ND, not done.

Table 3: Identification results of 44 clinical *Nocardia* species with the MALDI Biotyper database and extended MALDI Biotyper database.

Log score	Isolate results determined with MALDI Biotyper 4612 database				Isolate results determined with MALDI Biotyper extended database			
	%	No. identified n = 28		No. misidentified	%	No. identified n = 44		No. misidentified
		Species level	Genus level			Species level	Genus level	
≥2	57.1	2[a]	14	0	100	43	1[c]	0
≥1.7 and <2	7.2	1	1	0	0	0	0	0
<1.7	35.7	2[b]	8	2[b]	0	0	0	0

[a]Only two strains were correctly identified as *N. otitidiscaviarum*. [b]Two strains were misidentified as *N. brasiliensis*. [c]One strain was identified as *N. neocaledoniensis* after 1465 base sequencing.

that for the rapid identification of pathogenic Gram-positive bacilli, these can be identified without an extraction step by MALDI-TOF MS.

Twenty-five clinical strains of *Nocardia* were studied in the evaluation of MALDI-TOF MS by Xiao et al. [17], but only eight of 25 (32%) isolates were identified to the genus level and 17 isolates were not identified (68%). After complementation with the "in-house" database, 95% were correctly identified to the species level (score ≥2.00). Many researchers used the Biotyper MS database in combination with the in-house database that they constructed, thus the identification rate reached to 90% [18] and 83.1% [19] from 42% and 53%, respectively, in the species level. Pasciak et al. [20] suggested that clinical laboratories processing various clinical strains can upgrade a commercial database to improve and accelerate the results obtained.

Carrasco et al. [21] examined 100 clinical *Nocardia* strains from different hospitals between 2006 and 2014 and compared the agreement between 16S rRNA full gene and MALDI-TOF MS identification of *Nocardia* species by using the current commercial database without in-house supplementation. These two methods provided the same identification for 76% of the strains, and the authors concluded that the Bruker database should be amplified. The MALDI-TOF MS platforms with the Bruker Biotyper library v.4.0 cannot be considered a reliable technique as a routine method for resolving *Nocardia* spp. identification. All these results show that the performance of the Biotyper is satisfactory using a customized database and in combination with the in-house database that is constructed, but there is still room for diagnostic improvement.

Another commercial MS system is VITEK MS (bioMérieux, Marcy l'Etoile, France). Girard et al. [22] evaluated a set of 164 *Nocardia* strains using this system, and their database led to 94% accurate identification to the species level. They concluded that the optimized VITEK MS database with suitable sample preparation protocol enables a rapid, accurate and robust identification of *Nocardia* spp.

Our analysis evaluated the performance of the Bruker MALDI Biotyper system for identification of *Nocardia* species that had been confirmed by 16S rRNA gene sequencing. In our study, among 44 *Nocardia* strains, at first only 28 isolates were evaluated with MALDI Biotyper database officially available at that time (database 4612). Sixteen isolates (57.1%) of the strains yielded log scores of ≥2. Only two (4.5%) were identified to the species level (log scores of ≥2) as *N. otitidiscaviarum*. Among these 28 isolates, all of them were added to the database 4612. The rest of the 44 isolates (n = 16) were identified by 16S rRNA sequencing and three of the *Nocardia* strains were also added to the database 4612. As a result, the addition of a total of 28 *Nocardia* strains has significantly improved the scores and after re-evaluation of all 44 strains by the extended database, the results were 43 (97.7%) correct identifications to the species level (log scores of ≥2).

Several databases and identification strategies have been developed, including the MALDI Biotyper (Bruker Daltonics, Bremen, Germany), the SARAMIS and VITEK MS (bioMérieux, Marcy l'Etoile, France) and the Andromas (Paris, France) systems. Previous reports using the Biotyper system suggested that this technique requires a preliminary extraction step to identify Gram-positive rods [4, 16, 23] but Farfour et al. [15] demonstrated that rapid identification of *Nocardia* species can be obtained without an extraction step by Andromas MALDI-TOF MS. Schulthess et al. [23] demonstrated that sample preparation using formic acid, compared to the direct transfer preparation method, improved the identification of Gram-positive rods by MALDI-TOF MS. Each MALDI-TOF MS identification system has its own specificities, including the way the data was built, the hardware itself and algorithms used to compare the mass spectrum of a sample with those of the database.

In conclusion, this study showed that the identification of several *Nocardia* species by the Bruker MALDI Biotyper is highly reliable whereas identification rates

are generally lower than those for some Gram-negative bacteria and Gram-positive cocci. The major limitation of the current study is that relatively few isolates representing a small number of species were examined. Based on our data, the identification rates can be improved significantly by validated new database entries and the results can be confirmed with nucleic acid sequence analysis.

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