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Identification of *Nocardia* Species by Matrix-Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry

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Matrix-assisted laser desorption ionization–time of flight mass spectrometry for identification of *Nocardia* species remains challenging. By identifying 83.1% (64 of 77) and 80% (8 of 10) to the species and complex levels, respectively, and 94.3% (82 of 87) to the genus level, we show that an approach using routine sample preparation, an up-to-date commercial database minimally augmented with custom spectra, and testing at an early stage of growth is promising.

Nocardia species are aerobic actinomycetes belonging to the family *Corynebacteriaceae*. They are Gram-positive, weakly acid-fast environmental saprophytes with diverse colony morphologies and are the most commonly isolated aerobic actinomycete human pathogens (1). *Nocardia* infections generally result either from trauma-related introduction of the organism or from inhalation, particularly in immunocompromised patients. Pulmonary nocardiosis is characterized by pneumonia and can progress to a cavitary disease that may resemble tuberculosis. Disseminated disease, central nervous system involvement, and indolent pulmonary disease with cavities or contiguous spread are among the indicators used to test for *Nocardia* species (1, 2).

Identification of *Nocardia* species is often performed by 16S rRNA gene sequencing (1, 3, 4), but matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS)-based identification has the potential to be a rapid and inexpensive alternative (5). Unfortunately, routine MALDI-TOF MS for identification of *Nocardia* species has proven difficult (6–8). Previous studies have stressed the need for enhanced sample preparation methods and/or considerably augmented reference spectrum databases to sufficiently identify *Nocardia* spp. (6, 8). In this study, we demonstrated that the age of *Nocardia* cultures plays an important role in the success of MALDI-TOF MS identification. In addition, we showed that additional extraction steps beyond those recommended by the manufacturer are not required and that relatively modest augmentation of the current database is sufficient for the identification of routinely encountered *Nocardia* spp. By optimizing testing conditions using these approaches, we were able to improve the performance of MALDI-TOF MS for identification of *Nocardia* species.

Clinical isolates ($n = 79$) and type strains ($n = 8$) of *Nocardia* species, selected on the basis of the frequency and diversity of isolates identified at ARUP Laboratories, were retrospectively tested by MALDI-TOF MS (Bruker Daltonics). These 87 isolates represented 25 unique *Nocardia* species identified to the species ($n = 77$) or complex ($n = 10$) level by partial 16S rRNA gene sequencing (Table 1). Reference spectra were created from the following additional 13 isolates identified to the species level by sequencing multiple genes (16S rRNA gene, *hsp65*, and *secA1*) (3, 9, 10): *N. abscessus*, *N. araoensis*, *N. asiatica*, *N. asteroides*, *N. beijingensis*, *N. blacklockiae*, *N. brasiliensis*, *N. brevicatena*, *N. pseudobrasiliensis*, *N. puris*, *N. transvalensis*, *N. vinacea*, and *N. wallacei*. These isolates were chosen to improve database diversity and help resolve identifications to the complex level by partial 16S rRNA gene sequencing. Isolates were cultivated in pure culture on Columbia sheep blood agar (Hardy Diagnostics) at 35°C and tested at 18 to 48 h, depending on the time required to achieve visible growth of colonies. Thirty-six isolates were also tested as mature colonies beyond 48 h. All isolates were tested by MALDI-TOF MS after routine formic acid-acetonitrile extraction, and mass spectra were acquired as previously described (7), except that each spectrum was a sum of 240 shots collected in increments of 40. Spectra were analyzed by using a commercial database (Bruker Biotyper v. 3.1, which contains 5,627 spectra, including 72 *Nocardia* sp. spectra) supplemented with the 13 custom *Nocardia* sp. reference spectra listed above. Currently, all of the *Nocardia* species in the Biotyper database are in the FDA-unclaimed category. MALDI-TOF MS scores of ≥ 1.9 for identification to the species and complex levels and ≥ 1.7 for identification to the genus level were used as described previously (7).

Isolates were initially tested by MALDI-TOF MS when well-defined, mature colonies appeared on the agar plate, as would be done for phenotypic or sequencing-based identification. However, we observed that even though *N. cyriacigeorgica* isolates were well represented in the Biotyper database (16 spectra), all seven isolates tested beyond 48 h of growth failed to be identified. These results prompted us to investigate the impact the growth stage has on MALDI-TOF MS scores for *Nocardia* spp. Thirty-six isolates initially tested beyond 48 h of growth were retested after 18 to 48 h of growth, when visible colonies just began to appear (Table 2). The results indicate that testing at an earlier growth stage significantly improved the identification scores ($P = 0.0002$, t test). Scores improved for 29 (80.5%) of 36 isolates, with an average increase of 0.39, while only 7 isolates (19.4%) saw decreased scores. The magnitude of this decrease ($\bar{x} = 0.06$) was similar to

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TABLE 1 MALDI-TOF MS identification results for 87 isolates

Organism(s) (no. of isolates tested)[a]	No. of isolates with:			No. of reference spectra[b]
	Species ID (≥1.9)	Genus-only ID (≥1.7)	No ID (<1.7)	
Nocardia abscessus (1)[c]	1			3
Nocardia abscessus complex (4)[d]	3	1		NA[g]
Nocardia africana (1)[c]	1			1
Nocardia aobensis (2)		2		1
Nocardia araoensis (2)	2			2
Nocardia asiatica (3)[c,e]	3			2
Nocardia asteroides (2)	2			4
Nocardia beijingensis (4)	3	1		1
Nocardia brasiliensis (3)[c]	2	1		2
Nocardia brevicatena (2)	2			1
Nocardia carnea (2)[c]	1		1	1
Nocardia cyriacigeorgica (13)	10	3		16
Nocardia farcinica (7)	7			12
Nocardia higoensis (2)[e]			2	1
Nocardia ignorata (1)			1	1
Nocardia niigatensis (1)	1			1
Nocardia nova (9)[c]	8	1		2
Nocardia otitidiscaviarum (3)[c]	3			6
Nocardia paucivorans (6)	6			2
Nocardia pseudobrasiliensis (4)	4			1
Nocardia puris (4)	4			1
Nocardia testacea (1)		1		1
Nocardia transvalensis complex (6)[f]	5		1	NA[g]
Nocardia veterana (1)[c]	1			1
Nocardia vinacea (1)	1			1
Nocardia wallacei (2)[e]	2			1
All 87 isolates (%)	72 (83)	10 (11)	5 (6)	

[a] Identification based on sequencing of the first ~500 bp of the 16S rRNA gene.
[b] Number of reference spectra in the Bruker Biotyper database, which contains 5,627 spectra, plus the 13 added during this study.
[c] One test isolate of this species was a type strain.
[d] *N. abscessus*, *N. asiatica*, and *N. arthritidis* belong to the *N. abscessus* complex (4).
[e] Identified to the species level by sequencing of the 16S rRNA gene, *hsp65, and secA1* (3, 9, 10).
[f] *N. transvalensis*, *N. wallacei*, and *N. blacklockiae* belong to the *N. transvalensis* complex (4, 14).
[g] NA, not applicable.

the variability observed between replicates rather than a significant change in score. Importantly, none of the identifications changed when scores decreased. In contrast, of the 29 isolates that saw increased scores with the analysis of younger colonies, 7 (24%) changed from incorrect to correct identifications. The reason for improved scores and identification at earlier stages of growth is unclear, but the improvement may be due to a reduced influence on the spectra of secondary characteristics, such as pigments and aerial mycelia, that may vary with time and by isolate (11). As these data were obtained by routine ethanol-formic acid-acetonitrile extraction (7), it is evident that testing of isolates by standard sample preparation methods, but at earlier stages of growth, can improve the effectiveness of *Nocardia* isolate identification to the species level by MALDI-TOF MS.

Among the isolates identified to the species level by sequencing, MALDI-TOF MS identified 83.1% (64 of 77) and 94.8% (73 of 77) to the species and genus levels, respectively (Table 1). Of the 10 isolates defined only to the complex level by sequencing, 80 and 90% were correctly identified to the complex and genus levels, respectively, by the MALDI-TOF MS method. There were no species or genus level misidentifications, but 5 (5.7%) of 87 isolates could not be identified (scores of <1.7). Of these five isolates, four were represented by only one reference spectrum in the database (Table 1). A qualitative review of data from these isolates showed high-quality spectra with many well-defined peaks, indicating that inadequate database coverage, rather than suboptimal extraction or data collection, was likely responsible for the low scores, as described previously (7, 12, 13). Interestingly, our data show that supplementation of the database with custom spectra is still important for improving performance. Had the default Biotyper database been used, only 53 and 62% of the isolates would have been identified to the species and genus levels, respectively. In fact, nearly 40% of the 87 isolates most closely matched one of our 13 custom spectra. Most, 29 of 34, matched with species level scores, yet only 1 (2.9%) would have been identified to the species level by using Bruker's default database. Overall, these results demonstrate that MALDI-TOF MS is effective for the identification of *Nocardia* species, but supplementation, even with small numbers of custom spectra, can yield substantial improvements in performance over the current commercial databases.

A study by Verroken et al. (8), using the Biotyper database containing 3,486 reference spectra, identified only 10 (23%) of 43 *Nocardia* isolates to the species level. When they significantly augmented the Biotyper database with 110 additional custom refer-

TABLE 2 Comparison of MALDI-TOF MS identifications when isolates were tested at <48 h and >48 h

Organism	MALDI-TOF MS ID at <48 h (reported result[a])	Score at <48 h[b]	MALDI-TOF MS ID at >48 h (reported result[a])	Score at >48 h[b]
Nocardia abscessus complex	*Nocardia asiatica*	1.968	*Nocardia asiatica* (no ID)	1.663
Nocardia abscessus complex	*Nocardia asiatica*	2.229	*Nocardia asiatica*	2.101
Nocardia aobensis	*Nocardia aobensis* (*Nocardia* sp.)	1.825	*Nocardia aobensis* (*Nocardia* sp.)	1.82
Nocardia beijingensis	*Nocardia araoensis* (*Nocardia* sp.)	1.791	*Pseudomonas jinjuensis* (no ID)	1.448
Nocardia brasiliensis	*Nocardia brasiliensis*	2.37	*Nocardia brasiliensis*	2.409
Nocardia carnea	*Nocardia asiatica* (no ID)	1.574	*Nocardia farcinica* (no ID)	1.365
Nocardia cyriacigeorgica	*Nocardia cyriacigeorgica*	2.385	*Nocardia farcinica* (no ID)	1.414
Nocardia cyriacigeorgica	*Nocardia cyriacigeorgica*	1.945	*Nocardia cyriacigeorgica* (no ID)	1.558
Nocardia cyriacigeorgica	*Nocardia cyriacigeorgica*	1.922	*Nocardia brasiliensis* (no ID)	1.245
Nocardia cyriacigeorgica	*Nocardia cyriacigeorgica* (*Nocardia* sp.)	1.811	*Streptococcus agalactiae* (no ID)	1.246
Nocardia cyriacigeorgica	*Nocardia cyriacigeorgica*	2.362	*Nocardia cyriacigeorgica* (no ID)	1.447
Nocardia cyriacigeorgica	*Nocardia cyriacigeorgica* (*Nocardia* sp.)	1.866	*Nocardia farcinica* (no ID)	1.41
Nocardia cyriacigeorgica	*Nocardia cyriacigeorgica* (*Nocardia* sp.)	1.853	*Salmonella sp* (no ID)	1.164
Nocardia farcinica	*Nocardia farcinica*	2.233	*Nocardia farcinica* (*Nocardia* sp.)	1.713
Nocardia farcinica	*Nocardia farcinica*	2.239	*Nocardia farcinica* (no ID)	1.578
Nocardia farcinica	*Nocardia farcinica*	2.292	*Nocardia farcinica* (*Nocardia* sp.)	1.833
Nocardia ignorata	*Nocardia asteroides* (no ID)	1.563	*Nocardia asteroides* (no ID)	1.599
Nocardia nova	*Nocardia nova*	2.217	*Nocardia nova*	2.375
Nocardia nova	*Nocardia veterana* (*Nocardia* sp.)	1.736	*Nocardia veterana* (no ID)	1.494
Nocardia nova	*Nocardia nova*	2.009	*Nocardia nova*	2.014
Nocardia nova	*Nocardia nova*	2.105	*Nocardia nova*	2.108
Nocardia nova	*Nocardia nova*	2.199	*Nocardia nova*	2.023
Nocardia nova	*Nocardia nova*	2.092	*Nocardia nova*	1.954
Nocardia nova	*Nocardia nova*	2.028	*Nocardia nova* (no ID)	1.631
Nocardia paucivorans	*Nocardia paucivorans*	2.488	*Nocardia paucivorans*	2.521
Nocardia pseudobrasiliensis	*Nocardia pseudobrasiliensis*	1.915	*Nocardia pseudobrasiliensis* (no ID)	1.628
Nocardia pseudobrasiliensis	*Nocardia pseudobrasiliensis*	2.122	*Nocardia pseudobrasiliensis* (*Nocardia* sp.)	1.864
Nocardia pseudobrasiliensis	*Nocardia pseudobrasiliensis*	2.245	*Nocardia pseudobrasiliensis*	1.907
Nocardia puris	*Nocardia puris*	2.362	*Nocardia puris*	2.352
Nocardia puris	*Nocardia puris*	2.527	*Nocardia puris*	2.386
Nocardia testacea	*Nocardia testacea* (*Nocardia* sp.)	1.741	*Nocardia puris* (no ID)	1.558
Nocardia transvalensis complex	*Nocardia veterana* (no ID)	1.643	*Nocardia farcinica* (no ID)	1.35
Nocardia transvalensis complex	*Nocardia wallacei*	2.152	*Nocardia wallacei* (no ID)	1.393
Nocardia transvalensis complex	*Nocardia wallacei*	2.699	*Nocardia wallacei* (*Nocardia* sp.)	1.852
Nocardia transvalensis complex	*Nocardia wallacei*	2.515	*Nocardia wallacei*	2.433
Nocardia vinacea	*Nocardia vinacea*	2.172	*Nocardia vinacea*	2.321
Overall avg		2.083		1.769

[a] If different from MALDI-TOF MS identification (ID).
[b] Isolates with scores of ≥1.7 but <1.9 were identified only to the genus level. Isolates with scores of <1.7 were considered unidentified (no ID).

ence spectra representing 13 species, identification to the species level increased to 79% (34 of 43). A more recent study by Hsueh et al. (6), using the Biotyper database containing 5,627 reference spectra without the addition of custom spectra, identified only 11 (15%) of 74 isolates to the species level. Both of these studies used a MALDI-TOF MS score threshold of ≥2.0 for identification to the species level. When the same threshold of ≥2.0 was applied to our data, the number of identifications to the species level dropped from 64 (83.1%) to 55 (71.4%) of 77 isolates, which is similar to that seen by Verroken et al., but only after the addition of 110 custom spectra to their database. This illustrates that even limited supplementation of the database can substantially improve MALDI-TOF MS performance for the identification of *Nocardia* spp.

Overall, these data show that a routine extraction method for isolates harvested at an early stage of growth can be used to successfully identify *Nocardia* spp. To achieve optimal performance, however, modest supplementation of the manufacturer's database with custom spectra was still required. Together, these improvements allow more rapid and accurate identification of *Nocardia* spp., which may be coupled with predicted susceptibility patterns to allow earlier implementation of appropriate antimicrobial therapy and improve patient care (4).

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