JOURNAL OF CLINICAL MICROBIOLOGY, Nov. 2010, p. 4015–4021 Vol. 48, No. 11
0095-1137/10/$12.00 doi:10.1128/JCM.01234-10
Copyright © 2010, American Society for Microbiology. All Rights Reserved.

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

A. Verroken,[1] M. Janssens,[2] C. Berhin,[1] P. Bogaerts,[1] T.-D. Huang,[1] G. Wauters,[2] and Y. Glupczynski[1]*

Department of Clinical Microbiology, UCL Mont-Godinne University Hospital, B-5530 Yvoir,[1] and Microbiology Unit, Faculty of Medicine, Catholic University of Louvain, B-1200 Brussels,[2] Belgium

Received 18 June 2010/Returned for modification 24 August 2010/Accepted 15 September 2010

The identification of *Nocardia* species, usually based on biochemical tests together with phenotypic *in vitro* susceptibility and resistance patterns, is a difficult and lengthy process owing to the slow growth and limited reactivity of these bacteria. In this study, a panel of 153 clinical and reference strains of *Nocardia* spp., altogether representing 19 different species, were characterized by matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS). As reference methods for species identification, full-length 16S rRNA gene sequencing and phenotypical biochemical and enzymatic tests were used. In a first step, a complementary homemade reference database was established by the analysis of 110 *Nocardia* isolates (pretreated with 30 min of boiling and extraction) in the MALDI BioTyper software according to the manufacturer's recommendations for microflex measurement (Bruker Daltonik GmbH, Leipzig, Germany), generating a dendrogram with species-specific cluster patterns. In a second step, the MALDI BioTyper database and the generated database were challenged with 43 blind-coded clinical isolates of *Nocardia* spp. Following addition of the homemade database in the BioTyper software, MALDI-TOF MS provided reliable identification to the species level for five species of which more than a single isolate was analyzed. Correct identification was achieved for 38 of the 43 isolates (88%), including 34 strains identified to the species level and 4 strains identified to the genus level according to the manufacturer's log score specifications. These data suggest that MALDI-TOF MS has potential for use as a rapid (<1 h) and reliable method for the identification of *Nocardia* species without any substantial costs for consumables.

Nocardia spp. are ubiquitous bacteria dispersed in vegetation, dust, soil, freshwater, and salt water that are isolated with increasing frequency from clinical specimens, especially those from immunocompromised patients (1).

The taxonomy of the genus *Nocardia* has undergone major changes during the last decades, and currently more than 50 species have been characterized by phenotypic and molecular methods, besides a number of unnamed genomospecies (2). Not all of them have been found in humans, and *Nocardia asteroides*, previously considered the species most frequently isolated from clinical specimens, has been shown to be heterogeneous and has been divided into several species (2). Moreover, several additional species of human origin have been recently described and reported (5, 6).

The routine identification of *Nocardia* strains to the species level by conventional phenotypical methods is a fastidious and time-consuming process owing to the limited biochemical reactivity of these organisms, often requiring 1 or more days to complete identification. Moreover, the available tests may be difficult to interpret and inconclusive and require dedicated trained staff. In order to overcome these drawbacks, molecular methods such as 16S rRNA gene sequencing and PCR-restriction fragment length polymorphism analysis of both the 65-kDa heat shock protein-encoding gene (*hsp65*) and the 16S rRNA gene have been recently advocated for *Nocardia* species identification (3). However, these methods remain accessible to reference laboratories only and are difficult to implement for routine bacterial identification in a clinical laboratory.

Matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) can analyze the protein composition of a bacterial cell and has emerged as a new technology for species identification. By measuring the exact sizes of peptides and small proteins, which are assumed to be characteristic for each bacterial species, it allows determination to the species level within a few minutes when the analysis is performed on whole cells, cell lysates, or crude bacterial extracts (8). Through the improvement of the technique, MALDI-TOF MS has proved over the recent years to be a rapid, accurate, easy-to-use, and inexpensive method for the universal identification of microorganisms (11). Until now, MALDI-TOF MS has been challenged for the identification of various groups of microorganisms, including Gram-positive bacteria, *Enterobacteriaceae*, Gram-negative nonfermenters, mycobacteria, anaerobes, and yeasts (8, 9, 11–13). In this respect, the use of MALDI-TOF MS as a tool for the identification of fastidious, slow-growing organisms such as *Nocardia* species, which are notoriously difficult to identify by conventional tests, in the routine laboratory appeared to us of major interest. One factor limiting the use of MALDI-TOF MS remains the limited availability of reference data sets for microorganisms that are infrequently isolated from clinical specimens, and it has been shown previously that the absence or the availability of only a small number of isolates of a given species

* Corresponding author. Mailing address: Department of Clinical Microbiology, Cliniques Universitaires de Mont-Godinne, Université Catholique de Louvain, 1 Avenue Docteur Gaston Therasse, B-5530 Yvoir, Belgium. Phone: 32-81-42-32-45. Fax: 32-81-42-32-46. E-mail: youri.glupczynski@skynet.be.

▽ Published ahead of print on 22 September 2010.

in the reference database may account for most of the cases in which no identification can be obtained by the MALDI-TOF MS method (11).

In this study, we therefore aimed to establish a large reference database for the MALDI-TOF MS-based identification of *Nocardia* species isolates. In a first step, we developed a simple modified extraction procedure based on boiling for 30 min, followed by ethanol-formic acid extraction, and we generated our own spectrum database issued from a large collection of clinical and reference *Nocardia* sp. isolates. Following the establishment of our reference database, we subsequently evaluated the methodology against 43 blind-coded clinical isolates of *Nocardia* species that were analyzed by phenotypical, biochemical, and enzymatic tests and by full-length 16S rRNA gene sequencing, which was used as the reference identification method.

MATERIALS AND METHODS

Bacterial strains. Overall, 153 *Nocardia* strains were collected for the study, of which 134 were from clinical specimens and 19 were reference strains of the most commonly isolated species. The clinical *Nocardia* isolates had all been isolated from clinical specimens in Belgium between 1990 and 2009 and were referred by several Belgian clinical laboratories to one of us (G.W.) for confirmation of identification by conventional biochemical and enzymatic tests and by sequencing of the entire16S rRNA gene according to previously published methods (16). Susceptibilities to 11 antimicrobial agents were furthermore determined by Etest for all *Nocardia* strains (5). The clinical origins of the strains were as follows: 38 strains were isolated from the respiratory tract, 20 were from wounds, 13 were from blood, 8 were from brain abscesses or cerebrospinal fluid, and 55 were of unknown origin. All of the *Nocardia* strains available in our collection were included in the study in order to avoid any strain selection bias. One hundred ten *Nocardia* isolates, comprising 91 randomly selected clinical strains identified in our laboratory and the 19 reference strains of the most relevant *Nocardia* species, were selected to generate our own spectrum database, while 43 blind-coded *Nocardia* clinical isolates representative of the various species available in our collection were subsequently analyzed for the challenge identification part of the study. The distribution of the different *Nocardia* species is listed in Table 1.

All of the strains were cultivated on Trypticase soy blood agar with 5% sheep blood (TSA; bioMérieux, Marcy-L'Etoile, France) and incubated for 48 to 72 h at 37°C. A subset of 10 *Nocardia* isolates was also cultivated on Sabouraud agar (bioMérieux, Marcy-L'Etoile, France) and on Löwenstein-Jensen medium (Bio-Rad, Marnes-la-Coquette, France). For each microorganism, approximately 10 colonies were scraped from the agar and added to 500 μl distilled water. A modified extraction procedure consisted of boiling the mixture for 30 min, followed by a 2-min centrifugation at 13,000 rpm (10,000 × *g*). The supernatant was removed, and the pellet was mixed with 300 μl distilled water, followed by the addition of 900 μl ethanol. Two series of centrifugation for 2 min at 13,000 rpm and complete supernatant removal were necessary to obtain a dried pellet. The pellet was suspended in 50 μl of formic acid (70%) and incubated for 15 min at room temperature. Finally, 50 μl of acetonitrile was gently added and the mixture was centrifuged for 2 min at 13,000 rpm. The supernatant was the final product of the extraction procedure, and it was subjected to MALDI-TOF MS analysis.

MALDI-TOF MS. MALDI-TOF MS measurements were performed on a microflex LT (Bruker Daltonik GmbH, Leipzig, Germany) provided with a 20-Hz nitrogen laser. Spectra were recorded in the positive linear mode with an accelerating voltage of 20 kV and analyzed over a mass range of 2,000 to 20,000 Da. Each series of measurements was preceded by calibration with a bacterial test standard (BTS 255343; Bruker Daltonik).

Establishment of a *Nocardia* database. One microliter of the final extraction product of the *Nocardia* strain was spotted eight times onto a steel target. Directly after drying in air, each spot was overlaid with 1 μl of matrix. The matrix used was a saturated solution of α-cyano-4-hydroxycinnamic acid dissolved in a basic organic solvent composed of 50% acetonitrile and 2.5% trifluoroacetic acid. Each spot was measured three times by the MBT_FC.par flexControl method and the MBT-autoX.axe autoExecute method. Every measurement resulted from six series of 40 laser shots at different positions on the spotted product. The 24 spectra obtained were closely analyzed in the flexAnalysis program with a major alertness for intrusive peaks. Finally, a minimum of 20 accurate spectra out of 24 were downloaded in the MALDI BioTyper software and assembled in order to generate a single mean spectrum accounting for the extracted *Nocardia* strain with the BioTyper MSP creation standard method. In order to appreciate the correlation between the organisms and visualize the clustering, a dendrogram was calculated with the MALDI BioTyper software. Distance values in a dendrogram are relative and always normalized to a maximal value of 1,000.

TABLE 1. Species distribution of 153 *Nocardia* isolates subjected to analysis by MALDI-TOF MS

Species	Reference strain(s) in own database (n = 19)[a]	No. of clinical isolates	
		In own database (n = 91)	Tested for identification (n = 43)
Nocardia abscessus	DSM44432^{T}[b]	5	4
Nocardia africana	DSM44491[b]		
Nocardia asiatica	DSM44668	1	
Nocardia asteroides	DSM43258, NCTC11293^{T}		
Nocardia beijingensis			1
Nocardia brasiliensis	NCTC11294^{T}	6	3
Nocardia carnea	DSM46071, DSM44558, DSM43397^{T}	2	1
Nocardia cyriacigeorgica	DSM44484^{T}[b]	11	5
Nocardia elegans		1	
Nocardia farcinica	DSM43665^{T}[b]	40	12
Nocardia niigatensis	DSM44670[b]	1	
Nocardia nova	DSM43207^{T}	21	15
Nocardia otitidiscaviarum	NCTC1934^{T}		
Nocardia paucivorans	DSM44386^{T}[b]	2	
Nocardia pseudobrasiliensis	DSM44290		
Nocardia puris	DSM44599^{T}		
Nocardia shimofusensis			1
Nocardia transvalensis	DSM43405^{T}[b]		
Nocardia veterana	DSM44445^{T}	1	1

[a] A superscript capital T indicates the type strain.
[b] *Nocardia* reference strain also included in the original software of the MALDI BioTyper database (Bruker Daltonik GmbH).

MALDI-TOF MS identification of *Nocardia* challenge strains. One microliter of the extraction product of the *Nocardia* clinical strain was spotted once onto the target, followed by the addition of 1 μl matrix on each spot and measurement by the MBT_FC.par flexControl method. The bacterial spectra acquired by MALDI-TOF MS were analyzed in the MALDI BioTyper 2.0 software incorporating the MALDI BioTyper database and our own established *Nocardia* database. A first score was obtained after submitting the downloaded raw spectra to the original MALDI BioTyper database version 3.0.2 bearing the spectra of 3,486 cellular organism, while a second score resulted from the association of the former database and our own *Nocardia* database. Identification results and best matching scores are displayed in Table 2. The score indicated is a logarithmic value resulting from the alignment of the peak list of the unknown raw spectrum and the best matching database spectrum. According to the specifications of the manufacturer, a high log score of ≥2 was required for identification to the species level and an intermediate log score lying between <2 and ≥1.7 was required for identification to the genus level. A low score of <1.7 was considered unreliable for identification. The raw spectra of the 43 *Nocardia* clinical isolates were obtained through a unique run.

RESULTS

Establishment of a *Nocardia* database. The 110 resulting *Nocardia* spectra were stored in the *Nocardia* node of the projects taxonomy tree linked to the MALDI BioTyper taxonomy tree database. The dendrogram generated from the 110 registered *Nocardia* spectra pointed out distinctive clusters consisting of nocardiae of the same species (Fig. 1). The 41 *Nocardia farcinica* spectra constituted a first cluster that could potentially be subdivided into two smaller groups and with an additional distinct spectrum of one single strain (*N. farcinica*

TABLE 2. Best-match identification results and scores of 43 blind-coded clinical *Nocardia* isolates with the MALDI BioTyper database alone and enriched with our own *Nocardia* database

Strain no.	Clinical isolate	MALDI BioTyper database		MALDI BioTyper database and own *Nocardia* database	
		Identification result	Score	Identification result	Score
101	*Nocardia farcinica*	*Nocardia farcinica*	1.491	*Nocardia farcinica*	2.385
102	*Nocardia nova*	*Nocardia nova*	1.965	*Nocardia nova*	2.356
103	*Nocardia beijingensis*	*Neisseria meningitidis*	1.260	*Nocardia abscessus*	1.586
104	*Nocardia abscessus*	*Nocardia abscessus*	1.616	*Nocardia abscessus*	1.616
105	*Nocardia nova*	*Nocardia nova*	1.979	*Nocardia nova*	2.382
106	*Nocardia nova*	*Nocardia nova*	2.233	*Nocardia nova*	2.547
107	*Nocardia abscessus*	*Nocardia abscessus*	1.523	*Nocardia abscessus*	2.318
108	*Nocardia nova*	*Nocardia nova*	2.013	*Nocardia nova*	2.560
109	*Nocardia nova*	*Nocardia nova*	1.878	*Nocardia nova*	2.403
110	*Nocardia brasiliensis*	*Nocardia species*	2.084	*Nocardia brasiliensis*	2.255
111	*Nocardia nova*	*Nocardia nova*	1.973	*Nocardia nova*	2.347
112	*Nocardia nova*	*Nocardia nova*	2.122	*Nocardia nova*	2.546
113	*Nocardia farcinica*	*Clostridium chauvoei*	1.43	*Nocardia farcinica*	2.421
114	*Nocardia abscessus*	*Nocardia abscessus*	1.746	*Nocardia abscessus*	2.107
115	*Nocardia nova*	*Nocardia nova*	2.227	*Nocardia nova*	2.571
116	*Nocardia cyriacigeorgica*	*Lactobacillus kalixensis*	1.110	*Nocardia cyriacigeorgica*	2.108
117	*Nocardia nova*	*Nocardia nova*	2.028	*Nocardia nova*	2.389
118	*Nocardia abscessus*	*Nocardia abscessus*	1.665	*Nocardia abscessus*	2.346
119	*Nocardia carnea*	*Pandoraea pulmonicola*	1.423	*Pandoraea pulmonicola*	1.423
120	*Nocardia nova*	*Nocardia nova*	1.988	*Nocardia nova*	2.257
121	*Nocardia farcinica*	*Nocardia farcinica*	1.322	*Nocardia farcinica*	2.293
122	*Nocardia cyriacigeorgica*	*Nocardia* sp.	1.17	*Nocardia cyriacigeorgica*	1.870
123	*Nocardia shimofusensis*	*Nocardia testacea*	1.73	*Nocardia testacea*	1.733
124	*Nocardia nova*	*Nocardia aobensis*	1.72	*Nocardia nova*	1.846
125	*Nocardia farcinica*	*Clostridium septicum*	1.24	*Nocardia farcinica*	2.255
126	*Nocardia farcinica*	*Nocardia farcinica*	1.38	*Nocardia farcinica*	2.266
127	*Nocardia farcinica*	*Kocuria rosea*	1.29	*Nocardia farcinica*	2.153
128	*Nocardia farcinica*	*Nocardia farcinica*	1.25	*Nocardia farcinica*	2.337
129	*Nocardia nova*	*Nocardia nova*	2.19	*Nocardia nova*	2.367
130	*Nocardia nova*	*Nocardia nova*	2.06	*Nocardia nova*	2.511
131	*Nocardia brasiliensis*	*Issatchenkia occidentalis*	1.2	*Issatchenkia occidentalis*	1.204
132	*Nocardia veterana*	*Arthrobacter polychrolactophilus*	1.25	*Nocardia nova*	1.689
133	*Nocardia farcinica*	*Alcaligenes faecalis*	1.29	*Nocardia farcinica*	1.988
134	*Nocardia brasiliensis*	*Nocardia species*	1.94	*Nocardia brasiliensis*	2.318
135	*Nocardia farcinica*	*Nocardia species*	1.297	*Nocardia farcinica*	2.406
136	*Nocardia farcinica*	*Lactobacillus oligofermentans*	1.297	*Nocardia farcincia*	2.045
137	*Nocardia farcinica*	*Clostridium septicum*	1.347	*Nocardia farcinica*	2.229
138	*Nocardia nova*	*Nocardia nova*	2.066	*Nocardia nova*	2.285
139	*Nocardia cyriacigeorgica*	*Streptococcus gordonii*	1.34	*Nocardia cyriacigeorgica*	2.113
140	*Nocardia cyriacigeorgica*	*Candida glabrata*	1.288	*Nocardia cyriacigeorgica*	2.097
141	*Nocardia cyriacigeorgica*	*Issatchenkia orientalis*	1.385	*Nocardia cyriacigeorgica*	2.117
142	*Nocardia farcinica*	*Issatchenkia orientalis*	1.379	*Nocardia farcinica*	2.031
143	*Nocardia nova*	*Nocardia nova*	2.144	*Nocardia nova*	2.322

20). A distance level of 450 separated the two most extreme *N. farcinica* spectra, whereas the subclustering reduced the maximal distance level in both groups to 150. A second cluster regrouped 11 *N. cyriacigeorgica* spectra despite a high distance level of 650 between the two most distant strains. In fact, two strains of *N. cyriacigeorgica* were distantly related to the others, namely, *N. cyriacigeorgica* 17 and reference type strain DSM44484^T. All of the spectra of the *N. africana*, *N. elegans*, *N. nova*, and *N. veterana* strains made up another cluster with a maximum distance level of 500.

The *N. abscessus*, *N. asteroides*, *N. brasiliensis*, *N. carnea*, and *N. niigatensis* species, respectively, united all of their recorded spectra into small groups with a maximum distance level below 200 within each group. Eventually, a small group unified two *N. paucivorans* spectra.

Seven remaining *Nocardia* spectra were located in clusters assembling the spectra of other species. The spectra of the reference type strains *N. pseudobrasiliensis* DSM44290^T, *N. otitidiscaviarum* NCTC1934^T, and *N. puris* DSM44599^T were found within the *N. cyriacigeorgica* cluster. Despite the grouping of the two *N. asiatica* strains (clinical strain *N. asiatica* 79 and type strain *N. asiatica* DSM44668^T), a distance level of only 350 separated them from the *N. abscessus* cluster. Finally, the *N. transvalensis* DSM43405^T and *N. paucivorans* DSM44386^T spectra were gathered together.

The spectrum of clinical strain *N. cyriacigeorgica* 34 had an

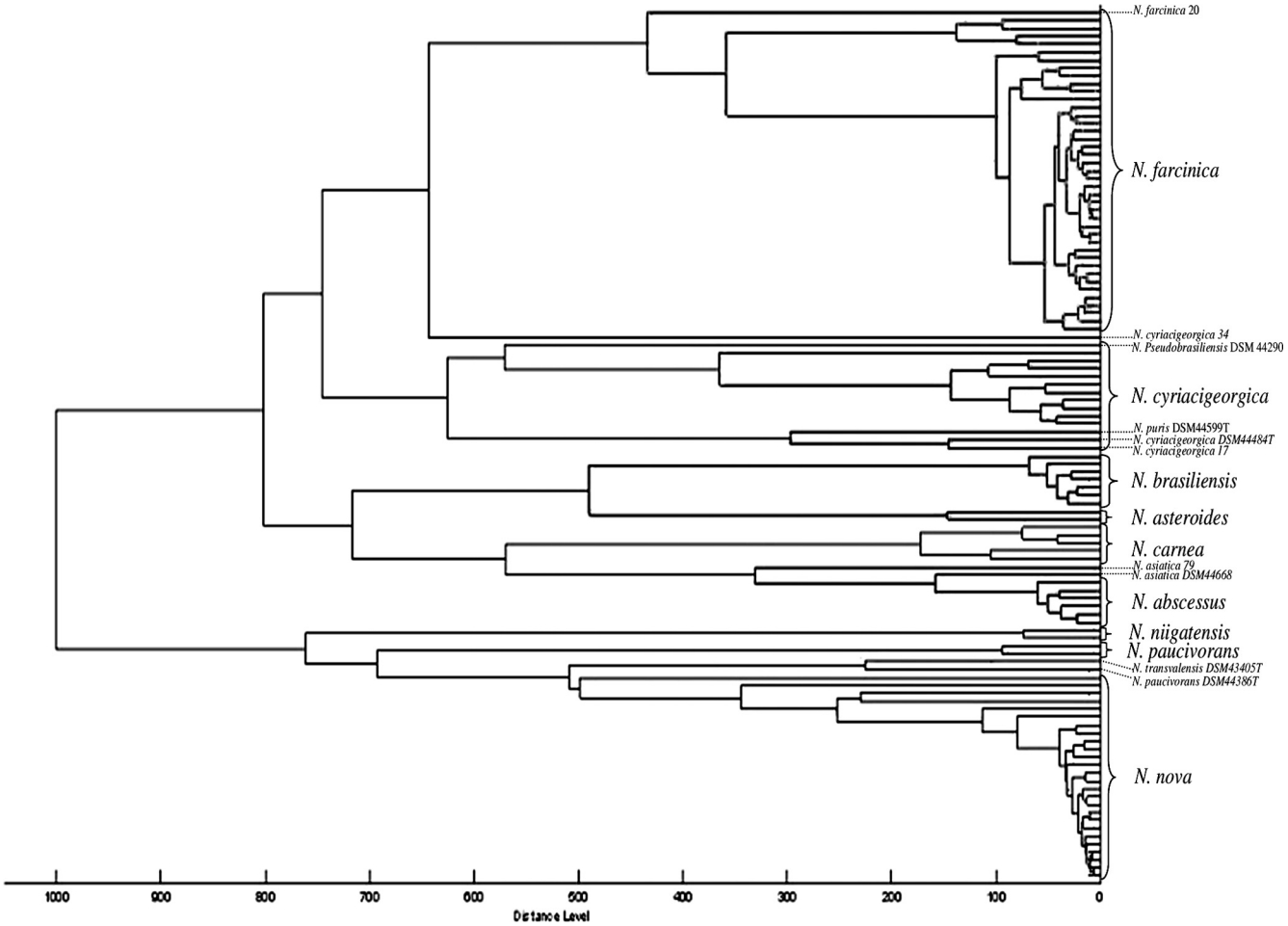


FIG. 1. Dendrogram of 110 *Nocardia* strains analyzed, including 91 clinical strains and 19 reference strains. Species clustering can be observed. Ten *Nocardia* strains are annotated on the dendrogram because of the unexpected positioning of their spectra.

isolated position in the dendrogram, with a distance level of >600 from the closest *Nocardia* spectrum.

MALDI-TOF MS identification of *Nocardia* challenge strains. Of the 43 acquired blind-coded *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, ≥2) and 9 (21%) were identified to the genus level (log scores between <2 and ≥1.7). The remaining 24 isolates (56%) yielded log scores of <1.7 and were thereby considered not identifiable.

Log scores are listed in Table 3. The strains with high log scores were all of *N. nova*, except one *N. brasiliensis* isolate that was identified as a *Nocardia* sp. by the MALDI BioTyper database. Of the nine strains with intermediate scores, five of *N. nova* and one of *N. abscessus* were correctly identified to the species level. Likewise despite low log scores, three strains of *N. abscessus*, five strains of *N. farcinica*, and one strain of *N. cyriacigeorgica* were correctly identified to the species level.

The addition of our own established *Nocardia* database to the original database did significantly improve the scores, lead-

TABLE 3. Best-match identification results of 43 clinical *Nocardia* isolates with the MALDI BioTyper database alone and enriched with our own *Nocardia* database

Log score	Isolate result determined with MALDI BioTyper database				Isolate result determined with MALDI BioTyper database supplemented with spectra issued from our home-made database			
	%	No. identified/total		No. misidentified/ total	%	No. identified/total		No. misidentified/ total
		Species level	Genus level			Species level	Genus level	
≥2	23	10/43			79	34/43		
≥1.7 and <2	21	7/43	2/43		9	3/43	1/43	
<1.7	56	9/43		15/43	12	1/43	2/43	2/43

ing to 38 (88%) correct identifications, of which 34 (79%) were to the species level (log scores of ≥2) and 4 (9%) were to the genus level (log scores between <2 and ≥1.7). Five isolates (12%), including one isolate each of *N. abscessus*, *N. beijingensis*, *N. brasiliensis*, *N. carnea*, and *N. veterana*, yielded log scores of <1.7 and thus could not be identified.

DISCUSSION

The identification of *Nocardia* species has always been considered a long and tedious task in the routine laboratory, and several publications have proposed tools in order to improve, accelerate, and ease the process. For instance, Wauters et al. recently proposed an algorithm including a combination of nine conventional and enzymatic tests for the rapid identification of the most common species (16). This scheme proved to be very efficient and highly discriminating for the accurate identification of more than 95% of the clinical isolates of *Nocardia* that belong to six different species.

Another identification approach was based on the antimicrobial susceptibility profiles within the genus *Nocardia*. In 1988, Wallace and Steele delineated six different susceptibility and resistance pattern types within 78 isolates of *N. asteroides* (14). This study led to the subsequent in-depth evaluation of *Nocardia* taxonomy by molecular methods that eventually resulted in the creation of distinct *Nocardia* species associated with described drug patterns (2). Glupczynski et al. consolidated the typical multidrug resistance patterns of five major clinical species, namely, *N. farcinica*, *N. nova*, *N. cyriacigeorgica*, *N. abscessus*, and *N. brasiliensis*, by determining MICs against 12 selected antimicrobial agents by Etest (5).

Further on, the expansion of molecular methods has distinctly contributed to the rapid and accurate identification of *Nocardia* species. PCR amplification of a 999-bp fragment of the 16S rRNA gene and subsequent restriction endonuclease analysis were described by Conville et al. (3), as they observed distinct restriction fragment length polymorphism patterns for all of the former drug pattern types of *N. asteroides* but not for *N. asteroides* drug pattern type IV. Another gene target explored for the identification of *Nocardia* species was the 16S-23S rRNA gene internal transcribed spacer (ITS) region containing different signatures for appropriate interspecies discrimination. Wang et al. (15) recently developed species ITS rRNA gene operon-specific probes enabling the identification of the five emerging pathogenic *Nocardia* species *N. beijingensis*, *N. blacklockiae*, *N. thailandica*, *N. elegans*, and *N. vinacea*. Altogether, it appears, however, that the use of molecular methods nevertheless requires the presence of a highly qualified staff and often sophisticated material specific to each technique.

MALDI-TOF MS has launched a new era in the routine microbiology laboratory. This proteomic method has proved its efficacy for the identification of various microorganisms (11, 13). Several evaluations have been reported for the identification of fastidious, slow-growing organisms such as Gram-negative nonfermenters, yeasts, or mycobacteria known to be difficult to identify by conventional tests in the routine laboratory (8, 9, 13). After establishing a reference database of 248 nonfermenting Gram-negative bacteria, Mellmann et al. analyzed 78 blind-coded clinical nonfermenters and obtained 85.9% correct identifications (8). Recently, van Veen et al. demonstrated the ability of the MALDI-TOF MS method to identify yeasts of clinical interest accurately (13). In a prospective study, 61 clinical yeast isolates belonging to seven different genera were analyzed following extraction. Overall, correct species identification was found for 85% of the microorganisms and only two minor misidentifications (identification correct to the genus but not the species level) were observed.

Regarding the identification of other fastidious microorganisms, a database of 37 *Mycobacterium* strains representing 13 species was created by Pignone et al. (9) allowing an in-depth analysis and comparison of the different spectra, which led to the conclusion that all of the strains produced reproducible, unique mass spectral profiles. The spectra analyzed arose from the direct deposit of the *Mycobacterium* strains. In our study, laser bombing of a direct deposit of *Nocardia* strains led to spectra of very poor quality or even to the absence of any spectra. While creating a new database requires the achievement of optimal spectra, we faced the challenge of obtaining *Nocardia* spectrum peaks with sufficient intensity. Because of the thickness and hydrophobicity of the *Nocardia* cell wall, which, like that of mycobacteria, contains a large amount of mycolic acids, pretreatment of the strains was mandatory prior to analysis by MALDI-TOF MS. It rapidly came out that the standard ethanol-formic acid extraction procedure alone was insufficiently efficient to allow the membrane proteins to be ionized by the matrix. A pretreatment that consisted of boiling the *Nocardia* colonies in 500 μl of distilled water for 30 min led to rupture of the cell wall and thus allowed us to solve this problem. A shorter boiling time of 10 min had no effect, while increasing the time beyond 30 min did not improve the quality of the spectra generated (data not shown). The above issue could also be resolved by plunging the solution of *Nocardia* colonies three times into liquid nitrogen at −196°C for 5 s. The boiling technique was preferred to the liquid nitrogen freezing method due to the less frequent availability of liquid nitrogen in routine laboratories and to the easier handling of the boiling technique.

A major advantage of MALDI-TOF MS is that it allows the rapid identification of nocardiae from cultured colonies with a very short turnaround time, only 1 h being necessary to perform the entire identification procedure, starting from the boiling procedure, followed by the ethanol-formic acid extraction and the analysis by MALDI-TOF MS of a *Nocardia* isolate cultured on TSA. *Nocardia* strains can also be isolated from culture media used for the growth of mycobacteria or fungi. Extraction of colonies of a selection of 10 *Nocardia* spp. grown on Sabouraud agar and on Löwenstein-Jensen medium yielded identification and score results similar to those obtained with cultures grown on TSA medium (data not shown). The development and establishment of our own *Nocardia* database expanded the MALDI BioTyper database by 110 *Nocardia* spectra. A dendrogram (Fig. 1) of those added spectra enabled us to visualize the clustering of spectra in species-specific groups. The 16S rRNA sequence similarity of the species *N. africana*, *N. elegans*, *N. nova*, and *N. veterana*, which form a species complex, could explain the clustering together of their respective MALDI-TOF spectra (2).

Likewise, the MALDI-TOF MS dendrogram pointed out 10 *Nocardia* strains because of the unexpected positioning of their

individual spectra. Starting from this observation, a panel of rapid biochemical tests developed by Wauters et al. (16) was repeated with those 10 *Nocardia* strains, including 6 reference type strains (*N. asiatica* DSM44668^T, *N. otitidiscaviarum* NCTC1934^T, *N. paucivorans* DSM44386^T, *N. pseudobrasiliensis* DSM44290^T, *N. puris* DSM44599^T, and *N. transvalensis* DSM43405^T) and 4 clinical strains (*N. asiatica* 79, *N. cyriacigeorgica* 17, *N. cyriacigeorgica* 34, and *N. farcinica* 20). The results confirmed the identification of all of the strains but clinical strain *N. farcinica* 20, which displayed a negative γ-glutamyl aminopeptidase test and which also clustered at a large distance from all of the other strains included in the *N. farcinica* cluster (Fig. 1). Total 16S rRNA gene sequencing was repeated with this last strain, and the isolate was best identified as *N. higoensis* with 99.5% sequence similarity at the level of the 16S rRNA sequence. Newly described in 2004 by Kageyama et al. (7), *N. higoensis* was isolated for the first time from a patient with lung nocardiosis in Japan. The reported susceptibility to tobramycin differentiating *N. higoensis* from *N. farcinica* strains was also confirmed for this strain (data not shown). The major distance level of 600 separating the *N. cyriacigeorgica* strain 34 spectrum from the other *Nocardia* spectra could not be explained, as the strain showed 99.9% 16S rRNA sequence similarity to *N. cyriacigeorgica* type strain DSM44484^T and displayed an antimicrobial susceptibility and resistance profile (resistance to ciprofloxacin and clarithromycin, susceptibility to ceftriaxone, imipenem, and tobramycin) matching the *N. cyriacigeorgica* drug resistance pattern.

Another interesting observation was that the *N. asiatica* DSM44668^T and *N. asiatica* 79 spectra clustered quite closely with the *N. abscessus* cluster at a distance level of 350. Newly described in 2004 by Kageyama et al. (6), *N. asiatica* has a significant 16S rRNA sequence divergence from *N. abscessus*, while only two biochemical tests (nitrate reduction and esculin decomposition) could distinguish the two species. Moreover, the rapid *Nocardia* species identification tests proposed by Wauters et al. (16) yielded the same phenotypic results for both species.

The *N. cyriacigeorgica* cluster was divided into two groups, with the first group formed by the clinical strain *N. cyriacigeorgica* 17 and reference type strain *N. cyriacigeorgica* DSM44484^T separated from the other strains with a distance level of 600. These two strains were furthermore joined by reference strain *N. puris* DSM44599^T. The second group included nine *N. cyriacigeorgica* clinical strains, as well as reference type strain *N. pseudobrasiliensis* DSM44290^T. Biochemical tests and antimicrobial susceptibility testing could not differentiate between the two *N. cyriacigeorgica* groups. Interestingly Roth et al. (10) also observed a microheterogeneity in the sequence of the 16S rRNA gene (sequence divergence of fewer than five bases) in several *Nocardia* species. This observation might account for the subclustering of the *N. cyriacigeorgica* group and the *N. nova* group observed in the MALDI-TOF MS dendrogram. Furthermore, the analysis of sequence similarities of the 16S rRNA genes of the species *N. puris* and *N. pseudobrasiliensis* with that of the *N. cyriacigeorgica* strain through the phylogenetic tree developed by Roth et al. (10) was inconclusive. Poor 16S rRNA gene sequence similarity between *N. paucivorans* and *N. transvalensis* was also observed, despite their close relatedness in the MALDI-TOF MS dendrogram. It should, however, be stressed that several species were insufficiently represented in our study, with only a single strain each being available for *N. puris*, *N. pseudobrasiliensis*, and *N. transvalensis*. Hence, an evaluation with a larger number of strains of those species is clearly necessary to confirm or reject the latter dendrogram observations.

It should be stressed that the original database included in the MALDI BioTyper software version 3.0.2 contains 36 *Nocardia* spectra accounting for 29 different species. Of note, the species that are most frequently isolated from clinical specimens were represented by, at most, two different spectra. The broadening of this database with our own *Nocardia* database, including a large panel of *Nocardia* isolates from clinical specimens and reference strains, was intended to improve the accuracy of the database for the identification of *Nocardia* spp. In fact, the failure of MALDI-TOF MS to reach identification is most often explained by an insufficient coverage of organisms by the database. For instance, Seng et al. (11) pointed out the gap in non-*Clostridium* anaerobe identification as no or only very few reference spectra were included for these organisms in the Bruker database. The same authors also showed that the inclusion of additional spectra and broadening of the database could solve this problem. On the other hand, misidentification with a log score value of ≥1.7 can rarely be resolved by expansion of the database. First reported by Seng et al. (11), two recent studies confirmed that nearly 50% of the *Streptococcus pneumoniae* isolates tested were misidentified as *Streptococcus parasanguinis* and that incorporation of additional spectra of those species into the database did not solve the problem (4, 13).

In this study, we were confronted with a lack of identification by MALDI-TOF MS and we could clearly confirm the benefit of an extended database, since correct identification to the species level with a score of ≥2 increased from 44% to 88% when our own *Nocardia* database was added to the original database of the manufacturer. Likewise, our created database essentially comprised *Nocardia* species most frequently found in human clinical specimens. The absence of identification (scores of <1.7) with the MALDI BioTyper database involved 24 strains belonging to seven species, including strains of major clinical significance, namely, *N. abscessus*, *N. cyriacigeorgica*, and *N. farcinica*. Among the three *Nocardia* strains that could only be identified to the genus level despite the addition of our own database, the *N. shimofusensis* and *N. beijingensis* strains were not represented in the databases and were subsequently added. One *N. brasiliensis* and one *N. carnea* strain each were misidentified despite their inclusion in both databases but with scores of <1.7 (Table 2).

Repeating the MALDI-TOF MS analysis with triple deposits of the same *Nocardia* culture isolate did not lead to better identification scores than using a single deposit (data not shown).

Notwithstanding the database, all of the results with scores of ≥2 yielded correct identification to the species level and results proved at least correct to the genus level for scores of ≥1.7 and <2. Consequently, misidentification was always associated with a low score of <1.7. In conclusion, the added *Nocardia* database clearly improves identification to the species level. We therefore recommend MALDI-TOF MS for rapid and accurate identification of the most commonly en-

countered *Nocardia* sp. isolates after pretreatment by 30 min of boiling and ethanol-formic acid extraction of the organism. Further expansion of the database with a larger number of isolates including the less common recently described *Nocardia* species is also clearly warranted.

ACKNOWLEDGMENTS

This work was funded in part by EU grant FP7-HEALTH-2009-SINGLE-STAGE TEMPOtest-QC, project 241742.

We have no conflicts of interest to report.

REFERENCES

1. **Ambrosioni, J., D. Lew, and J. Garbino.** 2010. Nocardiosis: updated clinical review and experience at a tertiary center. Infection **38:**89–97.
2. **Brown-Elliott, B. A., J. M. Brown, P. S. Conville, and R. J. Wallace, Jr.** 2006. Clinical and laboratory features of the *Nocardia* spp. based on current molecular taxonomy. Clin. Microbiol. Rev. **19:**259–282.
3. **Conville, P. S., S. H. Fischer, C. P. Cartwright, and F. G. Witebsky.** 2000. Identification of *Nocardia* species by restriction endonuclease analysis of an amplified portion of the 16S rRNA gene. J. Clin. Microbiol. **38:**158–164.
4. **De Bel, A., I. Wybo, D. Pierard, and S. Lauwers.** 2010. Correct implementation of matrix-assisted laser desorption ionization-time-of-flight mass spectrometry in routine clinical microbiology. J. Clin. Microbiol. **48:**1991–1992.
5. **Głupczynski, Y., C. Berhin, M. Janssens, and J. Wauters.** 2006. Determination of antimicrobial susceptibility patterns of *Nocardia* spp. from clinical specimens by Etest. Clin. Microbiol. Infect. **12:**905–912.
6. **Kageyama, A., N. Poonwan, K. Yazawa, Y. Mikami, and K. Nishmura.** 2004. *Nocardia asiatica* sp. nov., isolated from patients with nocardiosis in Japan and clinical specimens from Thailand. Int. J. Syst. Evol. Microbiol. **54:**125–130.
7. **Kageyama, A., K. Yazawa, A. Mukai, M. Kinoshita, N. Takata, K. Nishmura, R. M. Kroppenstedt, and Y. Mikami.** 2004. *Nocardia shimofusensis* sp. nov., isolated from soil, and *Nocardia higoensis* sp. nov., isolated from a patient with lung nocardiosis in Japan. Int. J. Syst. Evol. Microbiol. **54:**1927–1931.
8. **Mellmann, A., J. Cloud, T. Maier, U. Keckvoet, I. Ramminger, P. Iwen, J. Dunn, G. Hall, D. Wilson, P. Lasala, M. Kostrzewa, and D. Harmsen.** 2008. Evaluation of matrix-assisted laser desorption ionization-time-of-flight mass spectrometry in comparison to 16S rRNA gene sequencing species identification of nonfermenting bacteria. J. Clin. Microbiol. **46:**1946–1954.
9. **Pignone, M., K. M. Greth, J. Cooper, D. Emerson, and J. Tang.** 2006. Identification of mycobacteria by matrix-assisted laser desorption ionization-time-of-flight mass spectrometry. J. Clin. Microbiol. **44:**1963–1970.
10. **Roth, A., S. Andrees, M. Kroppenstedt, D. Harmsen, and H. Mauch.** 2003. Phylogeny of the genus *Nocardia* based on reassessed 16S rRNA gene sequences reveals underspeciation and division of strains classified as *Nocardia asteroides* into three established species and two unnamed taxons. J. Clin. Microbiol. **41:**851–856.
11. **Seng, P., M. Drancourt, F. Gouriet, B. La Scola, P. E. Fournier, J. M. Rolain, and D. Raoult.** 2009. Ongoing revolution in bacteriology: routine identification of bacteria by matrix-assisted laser desorption time-of-flight mass spectrometry. Clin. Infect. Dis. **49:**543–551.
12. **Spanu, T., E. De Carolis, B. Fiori, M. Sanguinetti, T. D'Inzeo, G. Fadda, and B. Posteraro.** 2 February 2010, posting date. Evaluation of matrix-assisted laser desorption ionization-time-of-flight mass spectrometry in comparison to *rpoB* gene sequencing for species identification of bloodstream infection staphylococcal isolates. Clin. Microbiol. Infect. [Epub ahead of print.] doi: 10.1111/j.1469–0691.2010.03181.x.
13. **van Veen, S. Q., E. C. J. Claas, and E. J. Kuijper.** 2010. High-throughput identification of bacteria and yeast by matrix-assisted laser desorption ionization-time of flight mass spectrometry in conventional medical microbiology laboratories. J. Clin. Microbiol. **48:**900–907.
14. **Wallace, R. J., Jr., and L. C. Steele.** 1988. Susceptibility testing of *Nocardia* species for the clinical laboratory. Diagn. Microbiol. Infect. Dis. **9:**155–166.
15. **Wang, X., M. Xiao, F. Kong, V. Sintchenko, H. Wang, B. Wang, S. Lian, T. Sorrell, and S. Chen.** 2010. Reverse line blot hybridization and DNA sequence studies of the 16S-23S rRNA gene intergenic spacer regions of five emerging pathogenic *Nocardia* species. J. Med. Microbiol. **59:**548–555.
16. **Wauters, G., V. Avesani, J. Charlier, M. Janssens, M. Vaneechoutte, and M. Delmée.** 2005. Distribution of *Nocardia* species in clinical samples and their rapid identification in the routine laboratory. J. Clin. Microbiol. **43:**2624–2628.