Creation of an In-House Matrix-Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry *Corynebacterineae* Database Overcomes Difficulties in Identification of *Nocardia farcinica* Clinical Isolates

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Nocardiosis is a rare disease that is caused by Gram-positive actinobacteria of the *Nocardia* genus and affects predominantly immunocompromised patients. In its disseminated form, it has a predilection for the central nervous system and is associated with high mortality rates. Therefore, prompt identification of the pathogen is critical. Matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry is a relatively novel technique used for identification of microorganisms. In this work, an upgraded MALDI-TOF Biotyper database containing *Corynebacterineae* representatives of strains deposited in the Polish Collection of Microorganisms was created and used for identification of the strain isolated from a nocardial brain abscess, mimicking a brain tumor, in an immunocompetent patient. Testing with the API Coryne system initially incorrectly identified *Rhodococcus* sp., while chemotaxonomic tests, especially mycolic acid analysis, enabled correct *Nocardia* identification only at the genus level. Subsequent sequence analysis of 16S rRNA and *secA1* genes confirmed the identification. To improve the accuracy of the results, an in-house database was constructed using optimized parameters; with the use of the database, the strain was eventually identified as *Nocardia farcinica*. Clinical laboratories processing various clinical strains can upgrade a commercial database to improve and to accelerate the results obtained. This is especially important in the case of *Nocardia*, for which valid microbial diagnosis remains challenging; reference laboratories are often required to identify and to survey these rare actinobacteria.

Nocardia represents Gram-positive actinobacteria that are etiological agents of the rare disease nocardiosis, which affects predominantly immunocompromised patients. Nocardiosis usually presents itself as a solitary lesion located in lung, skin, or brain (primary nocardiosis), but sometimes disseminated nocardiosis with multiple sites is observed. *Nocardia* spp. show a marked predilection for the central nervous system (CNS) (1).

Nocardiosis of the CNS constitutes about 40% of all cases of disseminated nocardioses and is associated with high mortality rates. The true incidence of *Nocardia* infections is difficult to assess, because of problems with the identification of these bacteria. Many different phenotypic, chemotaxonomic, or genotyping methods are available, but each method possesses drawbacks; for this reason, fast reliable identification of *Nocardia* isolates is very important. Because *Nocardia* spp. differ in antibiotic susceptibility, correct identification of clinical strains is critical for adequate treatment.

Matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry (MS) has been introduced for microbial identification in the past few years. Its reliability, accuracy, and cost-effectiveness have been well described in the literature (2–4). This method can be used to identify microorganisms from all domains of life, i.e., *Archaea*, *Bacteria*, and *Eukarya* (5), but above all it is useful in clinical microbiology.

In the MALDI-TOF MS technique, a unique mass spectral fingerprint is produced from extracted bacterial proteins. It is then compared with an existing database and, based on the degree of matching, identified to the genus or species level.

Currently, three reference databases for identification of microorganisms are available, namely, Saramis (bioMérieux, France), Andromas (Paris, France), and Biotyper (Bruker Daltonics, Germany), which were built around different identification strategies. The stock database can be updated, thus creating an “in-house database” by adding known species to improve the precision of identification.

This article describes the use of an upgraded MALDI-TOF Biotyper database containing representatives of the suborder *Corynebacterineae*, deposited in the Polish Collection of Microorganisms (PCM), for identification of clinically important strains of actinobacteria. This newly created database was used for identification of

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the strain isolated from a nocardial brain abscess mimicking a brain tumor in an immunocompetent patient with no underlying risk factors.

MATERIALS AND METHODS

Bacterial strains. Strains belonging to the *Corynebacterineae* suborder that were used for creation of the in-house MALDI database were obtained from the Polish Collection of Microorganisms (PCM) at the Institute of Immunology and Experimental Therapy (Wrocław, Poland) (see Table S1 in the supplemental material). The clinical strain R679, which was isolated from the brain abscess (see below), was also studied.

The actinobacterial strains were cultivated on solid media, i.e., glucose-yeast extract agar (medium 79) (6), nutrient agar, brain heart infusion (BHI) agar, and blood agar, at 37°C for 24 h to 48 h. The *Mycobacterium* spp. were cultivated on Lowenstein-Jensen agar slants at 37°C for 2 to 14 days, depending on the strain. For determination of the optimal time of cultivation, actinobacterial strains were incubated at 37°C for 24 h to 14 days.

Sample preparation for MALDI-TOF MS. For a direct transfer method, material from a fresh 24-h or 48-h colony was picked up with a toothpick, smeared on an MTP 384 polished steel target plate, coated with a matrix solution of α -cyano-4-hydroxycinnamic acid (HCCA) in 50% acetonitrile with 2.5% trifluoroacetic acid (TFA), and dried at room temperature.

For the protein extraction method, the standard procedure recommended by the manufacturer (M1) was used, with some modifications. The procedure was as follows. One loop of bacterial mass (approximately 10 μ l) was suspended in 300 μ l of Milli-Q water using a micropestle, and the suspension was vortex-mixed for 30 s. Next, 900 μ l of pure ethanol was added, and the suspension was mixed for 1 min using a vortex mixer. The cell suspension was centrifuged with a Sigma 1–15K centrifuge at 13,000 rpm for 2 min, the supernatant was discarded, and then the pellet was centrifuged again to remove the remaining ethanol. The pellet was dried in a laminar cabinet and resuspended in 70% formic acid (5 to 50 μ l) with thorough mixing, and then acetonitrile in the same volume as formic acid was added. After centrifugation (13,000 rpm for 2 min), 1 μ l of protein extract was applied to a steel target, dried at room temperature, and coated with 1 μ l of matrix solution (HCCA). The following procedure modifications were applied: use of 0.1-mm zirconia/silica beads (Carl Roth, Karlsruhe, Germany) to aid extraction with formic acid, according to the report by Saleeb et al. (7) (modification M2), heating of the bacterial mass at 100°C for 30 min in a water bath (modification M3), and both heating and zirconia/silica beads as additions to the standard procedure (modification M4). The modified procedure with Tween 20 described by El Khéchine et al. (8) was also utilized (modification M5). A chart depicting the extraction methods is presented in Fig. S1 in the supplemental material.

MALDI-TOF MS analysis. All analyses were performed with an Autoflex III mass spectrometer (Bruker Daltonics, Germany) using the Biotyper 3.0 software and database, containing 4,613 entries. The clinical strain R679 was also analyzed with an Ultraflex system (Bruker Daltonics, Germany), using the same software and database. Before each measurement, calibration of the mass spectrometer with Bruker Bacterial Test Standard was performed according to the manufacturer's recommendations. Spectra were recorded in the linear positive mode at a laser frequency of 200 Hz, in a mass range of 2,000 to 20,000 Da. The sum spectra of 1,000 laser shots were acquired in portions of 200 laser shots from 5 different spot positions with 40% laser intensity. To generate the in-house database, 12 raw spectra of good quality were chosen and transferred to the peak list by the Biotyper 3.0 software. Identification criteria used in the analysis, formulated by the manufacturer, were as follows: score value below 1.699, identification unreliable; score value of 1.700 to 1.999, probable genus identification; score value of 2.000 to 2.299, reliable genus

identification, probable species identification; score value of 2.300 to 3.000, highly probable species identification.

Optimal conditions for creation of in-house MALDI-TOF MS database. In order to improve the quality of MS spectra, different cultivation media and times of cultivation were evaluated. The effects of modifications of the extraction procedure were also analyzed. The species used in these analyses were *Corynebacterium glutamicum* PCM 1945, *Gordonia terrae* PCM 2591, *Nocardia nova* PCM 2607^T, *Rhodococcus rhodochrous* PCM 2452^T, *Tsukamurella paurometabola* PCM 2453^T, and *Mycobacterium smegmatis* PCM 659.

Identification of clinical strain R679 isolated from brain abscess. (i) Biochemical identification. Biochemical testing of the clinical strain R679 was performed using commercially available API Coryne test strips (bioMérieux) for manual identification. The API Coryne test strip system contains 20 dehydrated substrates for enzymes such as pyrazinamidase, pyrrolidonyl arylamidase, β -galactosidase, alkaline phosphatase, α -glucosidase, *N*-acetylglucosaminidase, and β -glucuronidase. It also allows for testing of nitrate reduction, gelatin, urea, and esculin hydrolysis, and carbohydrate (glucose, ribose, D-xylose, mannitol, maltose, lactose, sucrose, and glycogen) fermentation. The test was set up according to the manufacturer's instructions, and samples were incubated at 36°C for 24 h. Catalase activity was determined by adding a drop of hydrogen peroxide (3%) to isolated colonies.

(ii) Antimicrobial susceptibility testing. Susceptibility to amikacin, ceftriaxone, clarithromycin, minocycline, trimethoprim-sulfamethoxazole (TMP-SMX), imipenem, amoxicillin-clavulanate, moxifloxacin, ciprofloxacin, and linezolid was assessed by the broth microdilution method, according to the current CLSI recommendations (9). Susceptibility testing was performed by the Special Bacteriology Reference Laboratory (SBRL) of the Bacterial Special Pathogens Branch, Centers for Disease Control and Prevention (Atlanta, GA).

(iii) Chemotaxonomic identification. Sugars and the diaminopimelic acid (DAP) isomers of peptidoglycan in a whole-cell hydrolysate of the clinical isolate were detected according to the method described by Lechevalier and Lechevalier (10) and *N*-glycolylated muramic acid by the colorimetric method (11). Phospholipid and glycolipid profiles were determined by thin-layer chromatography (TLC) (12). The fatty acid methyl esters were determined by gas-liquid chromatography (GLC)-MS and mycolic acids by TLC (13). Purified mycolic acid methyl esters from clinical isolate R679, *Nocardia asteroides*, and *Nocardia farcinica* were analyzed with an Ultraflex MALDI-TOF MS system (Bruker Daltonics, Germany), using 2,5-dihydroxybenzoic acid as a matrix, in reflectron positive-ion mode.

(iv) 16S rRNA and *secA1* gene sequencing. Chromosomal DNA was isolated from the clinical strain (14), and the 16S rRNA gene was amplified in three independent PCRs using specific primers (15); the PCR products obtained were cloned using the pGem-T Easy vector system and subsequently sequenced (16). Identification of the phylogenetic neighbors was initially carried out by BLASTN program matching against a database containing type strains with validly published prokaryotic names and representatives of uncultured phylotypes, i.e., the EzTaxon-e database (17). The top 30 sequences with the highest scores were then selected for the calculation of pairwise sequence similarity using a global alignment algorithm, which was implemented on the EzTaxon-e server. The *secA1* gene sequence was obtained using the following modified primers: *secA1*_F, 5'-GACAGYGAGTGGATGGGCGSGTGCACCG-3'; *secA1*_R, 5'-GC GGACGATGTAGTCCTTGTC-3' (18); products of three independent PCRs were sequenced. The phylogeny of the 16S and *secA1* genes was analyzed as described by Paściak et al. (19), using MEGA6 software (20).

Nucleotide sequence accession numbers. The 16S rRNA and *secA1* gene sequences of clinical isolate R679 from a brain abscess were submitted to GenBank under accession numbers [KC478309](#) (1,457 bp) and [KP662923](#) (489 bp), respectively.

FIG 1 Expanded view of MALDI-TOF mass spectra for samples obtained by the direct transfer method and by the extraction protocol (M1) for *Corynebacterium*, *Tsukamurella*, and *Nocardia*. (a) *C. glutamicum* PCM 1945, direct transfer method, score value of 1.907. (b) *C. glutamicum* PCM 1945, M1 method, score value of 2.537. (c) *T. paurometabola* PCM 2453^T, direct transfer method, score value of 1.920. (d) *T. paurometabola* PCM 2453^T, M1 method, score value of 2.178. (e) *N. nova* PCM 2607^T, direct transfer method, score value of 1.456. (f) *N. nova* PCM 2607^T, M1 method, score value of 1.964. Colonies grown on glucose-yeast extract agar (medium 79) were incubated at 37°C for 48 h.

RESULTS

Generation of in-house *Corynebacterineae* database. (i) Comparison of direct transfer and extraction methods. The first step in setting up the MALDI in-house database was to choose the method for sample preparation, which would be used for the creation of protein fingerprints. One representative strain from each genus was selected and cultivated on glucose-yeast extract agar (medium 79) at 37°C for 48 h. MALDI-TOF mass spectra were obtained for samples prepared by either the direct transfer method or the extraction method (M1), the latter as recommended by the manufacturer. The results obtained with these two sample preparation methods were compared in Fig. 1. Since the extraction method yielded better mass spectra and higher score values, it was used exclusively in further analyses. In comparison with other *Corynebacterineae* representatives, especially from the *Corynebacterium* or *Tsukamurella* genera, the score values of *Nocardia* spp. in the MALDI-TOF Biotyper system were always lower, regardless of the method of sample preparation (Fig. 1).

(ii) Elaboration of optimal conditions for MALDI-TOF in-house database construction. Depending on the culture medium, small differences in the mass spectra obtained were seen. For *Corynebacterium*, *Rhodococcus*, and *Tsukamurella* genera, the

best media were glucose-yeast extract agar (medium 79) and blood agar, while BHI medium was the worst (see Table S2 in the supplemental material). For construction of the database, two kinds of solid media were selected according to the results obtained for the genus (Table 1). The M2 modification of the extraction procedure, i.e., the use of zirconia/silica beads for extraction with formic acid, gave the best results in the case of *Gordonia* and *Nocardia* representatives (see Table S2 in the supplemental material). Another modification, M5 with Tween 20, proved to be most effective with *Mycobacterium* spp. (see Table S2 in the supplemental material). Thermal inactivation of the biomass, as used in the M3 and M4 modifications, worsened the score values in all cases.

The next step was to determine the optimal cultivation time for a given medium and extraction method. Satisfactory results were achieved after 2 days of cultivation for *N. nova* and *C. glutamicum*, and mass spectra obtained for 14-day incubations are presented in Fig. S2 and S3 in the supplemental material. Most of the *Mycobacterium* spp. studied belonged to the fast-growing mycobacteria and 72-h incubations were sufficient for their identification, with the exception of *Mycobacterium scrofulaceum*, for which 10 days of incubation was needed to obtain the necessary yield.

The best parameters determined for the creation of an in-

TABLE 1 Optimal parameters selected for creation of in-house MALDI-TOF MS *Corynebacterineae* database

Genus	No. of strains	Extraction method	Cultivation time (h)	Media
<i>Corynebacterium</i>	12	M1	72	Medium 79, nutrient agar
<i>Gordonia</i>	4	M2	72	Medium 79, blood agar
<i>Mycobacterium</i>	18	M5	72 ^a	Medium 79, blood agar, Löwenstein-Jensen medium
<i>Nocardia</i>	7	M2	48	Medium 79, blood agar
<i>Rhodococcus</i>	13	M1	24	Medium 79, blood agar
<i>Tsukamurella</i>	6	M1	48	Medium 79, nutrient agar

^a Cultivation time for fast-growing mycobacteria; for slow-growing mycobacteria, the time was ≥ 10 days.

house database are presented in Table 1. The same optimal conditions were used for all representatives of genera. For the *Nocardia* genus, the best results were obtained with solid media, i.e., medium 79 (6) and blood agar, with 48 h of incubation and extraction modification M2. The MALDI-TOF mass spectra of the *Nocardia* strains studied are presented in Fig. 2.

The construction of an in-house database allowed for a significant increase in the reliability of identification for the majority of tested *Nocardia* strains. Score values are listed in Table 2. The summary of identification of *Corynebacterineae* representatives of PCM species in the Biotyper database, using the optimal parameters determined, is presented in Table S3 in the supplemental material.

Case illustration. A 53-year-old woman presented to the emergency department due to rapidly progressing hemiparesis. Computerized axial tomography (CAT) followed by magnetic resonance imaging (MRI) revealed a relatively large pathological

mass (approximately 50 mm in diameter) located in the right temporoparietal region and surrounded by significant edema. With a tentative diagnosis of glioblastoma multiforme, the patient was admitted to the neurosurgical department for surgical treatment. Intraoperatively, the mass was determined to be an abscess. It was evacuated, and a specimen was sent for microbiological examination.

The culturing of pus on 5% sheep blood in tryptic soy agar (TSA) for 24 h yielded rough orange colonies. The API Coryne identification strip (bioMérieux) profile obtained identified the isolate as *Rhodococcus* with 84.4% probability (identification profile 3151004); the other possible identifications were *Gordonia*, *Dietzia*, or *Nocardia*. The strain was catalase, α -glucosidase, and esculin hydrolysis positive. The results of other tests (reduction of nitrates, pyrazinamidase, pyrrolidonyl arylamidase, alkaline phosphatase, β -glucuronidase, β -galactosidase, *N*-acetyl- β -glucosaminidase, urease, hydrolysis of gelatin, and fermentation of D-glucose, D-ribose, D-xylose, D-mannitol, D-maltose, D-lactose,

FIG 2 Expanded view of MALDI-TOF mass spectra representing the *Nocardia* genus and the clinical isolate. (a) *N. asteroides* PCM 2138. (b) *N. asteroides* PCM 2254^T. (c) *N. brasiliensis* PCM 2566^T. (d) *N. cyriacigeorgica* PCM 2638^T. (e) *N. farcinica* PCM 2173^T. (f) Clinical isolate R679. (g) *N. mikamii* PCM 2700^T. (h) *N. nova* PCM 2607^T. Colonies grown on glucose-yeast extract agar (medium 79) were incubated at 37°C for 48 h. Spectra were acquired using extraction protocol M2 and HCCA matrix solution.

TABLE 2 Score values for *Nocardia* representatives with Biotyper and in-house *Corynebacterineae* databases

Reference strain	Analyte name	Organism (best match) in Biotyper database	Score value	
			Biotyper database	In-house database
<i>Nocardia asteroides</i> PCM 2138	2138_79_48h	<i>Nocardia</i> sp.	1.532	2.673
	2138_blood_48h	<i>Nocardia</i> sp.	1.474	2.656
<i>Nocardia asteroides</i> PCM 2254 ^T	2254_79_48h	<i>Arthrobacter ramosus</i>	1.430	2.679
	2254_blood_48h	<i>Enterococcus faecium</i>	1.474	2.685
<i>Nocardia farcinica</i> PCM 2173 ^T	2173_79_48h	<i>Nocardia asiatica</i>	1.361	2.771
	2173_blood_48h	<i>Nocardia farcinica</i>	1.330	2.766
<i>Nocardia</i> sp. PCM 2308	2308_79_48h	<i>Pseudomonas veronii</i>	1.336	2.428
	2308_blood_48h	<i>Arthrobacter sulfureus</i>	1.395	2.499
<i>Nocardia brasiliensis</i> PCM 2556	2556_79_48h	<i>Nocardia</i> sp.	2.062	2.696
	2556_blood_48h	<i>Nocardia</i> sp.	2.038	2.648
<i>Nocardia cyriacigeorgica</i> PCM 2638 ^T	2638_79_48h	<i>Nocardia cyriacigeorgica</i>	1.357	2.669
	2638_blood_48h	<i>Nocardia cyriacigeorgica</i>	1.356	2.549
<i>Nocardia mikamii</i> PCM 2700 ^T	2700_79_48h	<i>Nocardia kruczakiae</i>	1.764	2.748
	2700_blood_48h	<i>Nocardia kruczakiae</i>	1.819	2.662
<i>Nocardia nova</i> PCM 2607 ^T	2607_79_48h	<i>Nocardia nova</i>	1.995	2.746
	2607_blood_48	<i>Nocardia nova</i>	2.094	2.704

D-saccharose, and glycogen) were negative. The following empirical antibiotic therapy was introduced: 480 mg of TMP-SMX three times a day, 100 mg of doxycycline once a day, and 500 mg of ciprofloxacin twice a day.

On day 7 after surgery, the patient's condition began to deteriorate and a recurrent abscess was found in a CAT scan. The patient underwent surgical drainage of the newly formed abscess. Intraoperative cultures again revealed *Rhodococcus* by API Coryne strip identification. After the second operation, the patient initially made a good recovery; after another 3 weeks, however, she presented with a new neurological deficit. The CAT and MRI scans revealed multiple new abscesses, surrounded by marked edema and significant midline shift. The patient underwent another operation, and the microbiological diagnosis was consistent with previous findings. Microbial cultures of blood samples (BD Bactec) incubated for 5 and 10 days under aerobic and anaerobic conditions yielded negative results. Eventually the patient was discharged with an order for oral TMP-SMX (480 mg) treatment twice a day for a prolonged period. Her neurological deficit eventually improved significantly, and imaging studies were negative for recurrence.

Phenotypic and chemotaxonomic identification. The isolate identified by the API Coryne system as *Rhodococcus* in the diagnostic laboratory of the treating hospital was sent to a reference laboratory for confirmation. This strain had a coccoid morphology in old cultures, resembling *Rhodococcus*, but branching rods were seen in young cultures (24 h) obtained from liquid media (see Fig. S4 in the supplemental material). After another 24 h, the rods started to fragment into smaller cells. The colony color on glucose-yeast extract agar was salmon pink and turned orange during cultivation. The strain was susceptible to amikacin, trimethoprim-sulfamethoxazole (TMP-SMX), amoxicillin-clavulanate, and linezolid, showed intermediate resistance to minocycline, imi-

penem, and moxifloxacin, and was resistant to ceftriaxone, clarithromycin, and ciprofloxacin by broth microdilution methodology, using interpretive criteria for *Nocardia* and other actinomycetes (9) (see Table S4 in the supplemental material).

The panel of chemotaxonomic tests was performed, and *meso*-DAP was detected in whole-cell hydrolysates, as were arabinose and galactose as diagnostic sugars. In addition, glucose and mannose were identified. *N*-Glycolylated muramic acid in peptidoglycan of the isolate was detected by the colorimetric method. The straight-chain saturated or monounsaturated fatty acids C16:0, C18:0, and C18:1, as well as tuberculostearic acid (10-methyl-branched C18:0), were present. In the lipid extract, the diagnostic phospholipid phosphatidylethanolamine and major glycolipids were identified. Mycolic acid analysis of *Nocardia asteroides*, *N. farcinica*, and the clinical isolate by MALDI-TOF MS showed the same range of molecular ions, with heterogeneity of the chain lengths of mycolic acids (Fig. 3). The calculated chain lengths of these nocardiomycolic acids ranged from 52 to 59 carbons, with the main peak belonging to mycolic acid, with a chain length of 55 carbon atoms and 4 double bonds (the observed value of the sodiated molecular ion was m/z 851.56, and the calculated value was m/z 851.81). The mycolic acid identification was crucial, as it classified the isolate as being in the *Nocardia* genus. Representatives of the *Corynebacterineae* suborder possess long-chain branched hydroxy acids of various chain lengths, which are characteristic for the genus; for *Nocardia* spp., the number of carbons is between 48 and 60 (21). Based on chemotaxonomy, the clinical isolate was identified as *Nocardia* sp.

MALDI-TOF MS Biotyper identification. The clinical strain was then identified by MALDI-TOF MS first using the Bruker Biotyper database. The direct smear method yielded poor spectra, and thus identification was not possible. The extraction procedure optimized for *Nocardia* (M2) was then used (Fig. 2f). The identi-

FIG 3 MALDI-TOF mass spectra of mycolic acid methyl ester. (a) *N. farcinica* PCM 2173^T. (b) Clinical isolate R679. The matrix was 2,5-dihydroxybenzoic acid in chloroform-methanol, and positive-ion mode was used.

fication result was *N. farcinica*, with a score value of 1.63, which implied poor test reliability according to the manufacturer. The same sample checked with the Ultraflex mass spectrometer demonstrated an increased identification score of 1.72.

The final identification of the clinical strain using the optimal parameters and our in-house *Corynebacterineae* database is presented in Table 3. The strain was identified as *N. farcinica* with a score of 2.27 or *N. asteroides* with a score of 2.12. Both values

TABLE 3 Identification of clinical isolate R679 in Biotyper 3.0 and in-house *Corynebacterineae* databases

Database and analyte	Organism (best match)	Score value
Biotyper 3.0 (4,613 entries)		
R679_79_M2	<i>Clostridium septicum</i>	1.580
	<i>Nocardia</i> sp.	1.433
R679_blood_M2	<i>Nocardia farcinica</i>	1.591
	<i>Nocardia</i> sp.	1.630
In-house <i>Corynebacterineae</i> database		
R679_79_M2	<i>Nocardia asteroides</i> PCM 2138 (2138_blood_48h)	2.089
	<i>Nocardia asteroides</i> PCM 2138 (2138_blood_48h)	2.123
R679_blood_M2	<i>Nocardia farcinica</i> PCM 2173 (2173_blood_48h)	2.270
	<i>Nocardia asteroides</i> PCM 2138 (2138_blood_48h)	2.121

indicated much higher match probability than with the stock Biotyper database.

Molecular identification. To ascertain the identification of the isolate and to verify the MALDI-TOF identification results, 16S rRNA and *secA1* gene amplification and sequencing were performed (16). The 16S rRNA gene sequence (1,457 bp) of the clinical isolate (GenBank no. [KC478309](#)) showed the greatest similarity (99.85% [1,455/1,457 bp]) to *N. farcinica* ATCC 3318^T (GenBank no. [Z36936](#)), followed by *Nocardia higoensis* IFM 10084^T (GenBank no. [AB108778](#)) (99.48% [1,441/1,457 bp]) and *Nocardia shimofusensis* IFM 10311^T (GenBank no. [AB108775](#)) (99.40% [1,398/1,414 bp]). To complete identification and to follow CLSI recommendations (16), the sequence of the *secA1* gene was determined. The similarities of the *secA1* sequences (489 bp) (GenBank no. [KP662923](#)) were as follows: 98.59% (464/469 bp) to *N. farcinica* ATCC 3318^T (GenBank no. [DQ360274.1](#)), 94.71% (445/467 bp) to *N. shimofusensis* IFM 10311^T (GenBank no. [EU178751.1](#)), and 94.21% (445/467 bp) to *N. higoensis* IFM 10084^T (GenBank no. [EU178747](#)). On the basis of 16S rRNA and *secA1* sequencing results, we concluded that the isolate should be assigned to *N. farcinica*. Phylogenetic analysis showed that the phylogenetic trees prepared for the 16S rRNA and *secA1* genes by different methods had highly similar branches.

The clinical isolate strain was deposited in the Polish Collection of Microorganisms (*N. farcinica* PCM 2712).

DISCUSSION

Nocardia is a rare cause of intracranial abscesses, representing only 1% to 2% of all such lesions (22), and is rarer still in immunocompetent patients (23). However, the number of the case reports concerning brain abscesses caused by *N. farcinica*, although small, is increasing. The literature search from 1966 to 2011 published by Kumar et al. (24) included 41 cases of *N. farcinica* brain abscesses, and 4 additional cases were reported recently (25–28). Nearly one-half of the cases involved primary brain abscesses. The studies by Wang et al. (29) of 132 nocardiosis patients with underlying cancer disease showed that *N. farcinica* was responsible for most of the brain and skin infections. The authors concluded that, in view of the isolation frequency, the invasiveness, and the lowest susceptibility to antimicrobials, *N. farcinica* is the most pathogenic of the *Nocardia* species (29). The mortality rates for nocardial abscesses are higher than those for most other brain abscesses, being 55% in immunocompromised patients and 20% in immunocompetent patients (30, 31).

Brain abscesses can mimic brain tumors in terms of both clinical presentation and diagnostic imaging results. Menkü et al. (23) reviewed 10 cases of primary nocardiosis in immunocompetent patients. In 6 cases, the lesions were initially diagnosed as neoplasms. The consequences of such misdiagnosis can be grave. Corticosteroids are routinely used with success to ameliorate peritumoral brain edema, but this treatment can lead to rapid progression when the lesion is of infectious etiology. In addition, early administration of empirical antibiotics can improve overall outcomes.

In the case described above, the clinical strain was susceptible to TMP-SMX, the drug of choice for the treatment of nocardiosis (32). Appropriate identification and assessment of the antibiotic sensitivity pattern allow targeted therapy, which is of critical importance considering the high mortality rates. The mortality rates remain high with TMP-SMX monotherapy,

especially for patients with CNS infections and other severe or disseminated cases. Stamm et al. (33) described a fatal case of an immunocompromised patient with *Nocardia brasiliensis* pneumonia and empyema that was refractory to therapy with sulfisoxazole, trimethoprim, and sulfamethoxazole and was complicated by disseminated infection with *N. asteroides*. Both *Nocardia* strains were susceptible to trimethoprim and sulfonamides *in vitro*. There are controversial reports concerning the susceptibility of *Nocardia* spp. to TMP-SMX. However, an *in vitro* multicenter study performed in the United States revealed that only 2% of 552 studied *Nocardia* clinical isolates were found to have resistance to TMP-SMX (34). In cases of patients with allergy or intolerance to sulfonamides, alternative therapy should be introduced (32). It appears that effective regimens for the treatment of nocardiosis should include linezolid (35, 36).

The present work highlighted the difficulties encountered in *Nocardia* identification, i.e., (i) the early diagnosis based on MRI results suggested brain cancer, (ii) identification by the automated system as *Rhodococcus* sp. was incorrect, and (iii) identification at the species level was difficult. Even after using a standard extraction procedure and the MALDI Biotyper database, some additional work was required to improve the reliability of identification.

The results on urease and glucose fermentation obtained for the clinical isolate R679 in the API Coryne system were different from those found for *N. farcinica* (37). *Nocardia* and *Rhodococcus* possess similar enzymatic activities in 20 tests detected in API ZYM profiles; the only difference between *N. farcinica* and *R. rhodochrous* was phosphohydrolase activity (38). The API Coryne system is the most commonly used method and is well suited for identification of *Corynebacterineae* in routine laboratory practice, but it is not recommended for identification of aerobic actinomycetes, including *Nocardia*. Limitations of this system for identification in the *Corynebacterium* genus have been described elsewhere (39). For acid-fast Gram-positive rods from the *Rhodococcus*, *Gordonia*, *Tsukamurella*, and *Nocardia* genera, the identification usefulness of the API Coryne system is limited (40). In the present work, the chemotaxonomic analysis confirmed that the isolate belonged to the *Corynebacterineae* suborder, and the most useful mycolic acid analysis showed that the isolate was a member of the *Nocardia* genus.

The taxonomy of *Nocardia* is changing rapidly, mainly due to developments in the field of bacterial identification. At present, 104 validly published species of the *Nocardia* genus are listed at the LPSN (list of prokaryotic names with standing in nomenclature) website (<http://www.bacterio.net/index.html>). The phenotypic, chemotaxonomic, and genotyping methods used by researchers for the identification of *Nocardia* isolates are numerous (Table 4). Historically, identification initially relied mostly on morphology and biochemical reactions. To this day, phenotypic methods for *Nocardia* identification are difficult, laborious, and time-consuming, often requiring batteries of tests before the final results are obtained. Advances in the automation of biochemical tests resulted in increased rates of processing. In order to increase test reliability, however, the existing databases must be improved to follow changes in taxonomy and the introduction of new species. Various molecular methods have been developed to improve the identification process (Table 4). However, DNA sequencing methods are still expensive, labor-intensive, and unavailable in many laboratories. In the case of *Nocardia* spp., relative genome

TABLE 4 Advantages and disadvantages of general methods used in *Nocardia* identification, with selected examples

Method(s)	Advantages	Limitations	Disadvantages	Reference(s)
Phenotyping methods				
Aerial hyphae, Gram and acid-fast stain reaction, biochemical characteristics	Discriminative at species level in some cases		Laborious, time-consuming	
Commercial identification systems	Relatively fast	Not all nocardial species are included		37
API 20C AUX system and biochemical tests plus susceptibility patterns	75 nocardial isolates were identified at species level by using battery of tests	Overlapping susceptibility patterns and API 20C results; combinations of all different biochemical tests are important for reliable identification		
Vitek system			Compared to sequencing results, Vitek II system was unable to identify <i>N. farcinica</i> clinical isolate	45
API Coryne system		Misidentification as <i>Rhodococcus</i> sp.		This work
Susceptibility patterns	Determination of susceptibility for appropriate treatment	Misinterpretation of bacterial identification		
Antimicrobial susceptibility testing		Susceptibility testing results for <i>N. kruczakiae</i> were essentially identical to those obtained for <i>Nocardia africana</i> , <i>N. nova</i> , and <i>Nocardia veterana</i> at “ <i>N. nova</i> complex” level	Use of susceptibility testing alone would be inadequate for reliable identification of <i>N. kruczakiae</i> isolates	47
Chemotaxonomic methods				
DAP determination, fatty acid methyl ester analysis, mycolic acid analysis	Identification at genus level; methods important for establishing new species	Alternative methods to exclude some genera	Needs special equipment (HPLC) ^a ; investigators must standardize systems to obtain reproducible and differential patterns	48
Genotyping and molecular biology methods				
16S rRNA sequencing	Ubiquitous, functionally constant, and conserved molecular marker in publically available databases	Costly procedure (sequencing is several times more costly than biochemical identification); sometimes incorrect identification, duplicate entries, and generally poor data quality	Not discriminative between highly related species	49, 50
PCR with species-specific primers	Relatively fast and sensitive	Results should be confirmed with restriction enzymes or sequencing; inability to recognize novel species; extensive in-house validation	Multiple copies of 16S RNA gene Lack of polymorphism of 16S rRNA gene	51, 52
Multilocus sequence analysis	Species cluster assignment; in future, could replace DNA-DNA hybridization	Routine application could be too cumbersome for clinical laboratories	Requires positive and negative controls	53
DNA-DNA hybridization	“Gold standard”	Difficult to perform; depends on experimental conditions		54
Whole-genome sequencing	Helpful in description of new <i>Nocardia</i> species Much information on different genes		Few nocardial genomes have been sequenced	47 55, 56
Proteomics-based method				
MALDI-TOF MS	Fast and reliable	Databases should contain more <i>Nocardia</i> species	Preparation of samples for extraction procedure requires more attention	44
	Low cost of simple analysis	In-house databases are helpful for <i>Nocardia</i> identification		57
	Identification of <i>N. farcinica</i> clinical isolate causing bacteremia		Score value of 1.879 at genus level	

^a HPLC, high-performance liquid chromatography.

ambiguity renders the precision of 16S RNA gene identification insufficient. According to CLSI recommendation, 99.6% identity of 16S rRNA sequences is required for genus and species identification. For *Nocardia*, the alternative DNA target *secA1* provides

better resolution at the species level. Even the multilocus sequence typing technique using four genes does not provide adequate discriminating power to identify novel *Nocardia* species (41). Proteomics-based methods are expected to be used more often in the

future, due to much improved specificity and efficiency of mass spectroscopic techniques.

MALDI-TOF mass spectrometry is an alternative to classical microbial and genetic identification techniques. This technique gives excellent results for Gram-negative bacteria even when the direct colony method is utilized. For characterization by MALDI-TOF MS, *Nocardia* spp., like *Mycobacterium* spp., require an additional extraction step before protein mass fingerprinting (7). This is due mainly to the thickness and strong hydrophobicity of the cell wall. We have noticed greater cell hydrophobicity in *Nocardia* than in *Corynebacterium* cells. The procedure is relatively fast, compared to molecular and phenotypic methods. The studies performed by Seng et al. (42) showed that MALDI-TOF MS reduced the identification times 55-fold and 169-fold and the costs 5-fold and 96-fold, compared with conventional phenotypic identification and gene sequencing, respectively.

In the case presented, the identification results obtained with the Biotyper MALDI-TOF system were correct (as corroborated by 16S rRNA and *secA1* sequencing results), but the score value obtained afforded identification only to the genus level. Hsueh et al. (43) studied 74 *Nocardia* isolates and found that only 14.9% of them ($n = 11$) were correctly identified to the species level by the Bruker Biotyper system. The authors concluded that the Bruker Biotyper MALDI-TOF system is ineffective for identification of *Nocardia* and other unusual Gram-positive rods because of current database limitations. Verroken et al. (44) checked 43 clinical isolates with the MALDI Biotyper database, and all score values obtained for *N. farcinica* clinical isolates were below 1.491. This was the reason the authors developed their own *Nocardia* database expanding the existing MALDI Biotyper database, which significantly improved the match scores.

The same approach was used in our case. Reliable identification of the clinical isolate was possible only after the generation of an in-house database, expanding the stock Bruker Biotyper database. To our surprise, the latter did not include the species *N. asteroides*, which, for many years before genotypic methods were introduced, was thought to be the most common agent of nocardiosis (see Table S5 in the supplemental material). Currently, nocardiae from the former *N. asteroides* complex, i.e., *N. farcinica* and *Nocardia cyriacigeorgica*, are responsible for most nocardiosis cases; the incidences of these two strains vary geographically. Therefore, the creation of an in-house database for the reference *Nocardia*, *Corynebacterium*, *Gordonia*, *Rhodococcus*, *Mycobacterium*, and *Tsukamurella* species was mandatory. Our database contains 6 species not represented in the Biotyper database, namely, *Gordonia amarae*, *N. asteroides*, *Nocardia mikamii*, *M. scrofulaceum*, *Tsukamurella pulmonis*, and *Tsukamurella tyrosinosolvens*. The Bruker Biotyper database used in this study (DB 4613) contains 70 different *Corynebacterium* species, 7 *Gordonia* species, 42 *Mycobacterium* species, 32 *Nocardia* species, 27 *Rhodococcus* species, and two *Tsukamurella* species. The optimal conditions were selected to acquire the best MALDI-TOF spectra that were least affected by the cultivation conditions, were reproducible, and were unique for a given bacterial strain.

We can conclude that, by upgrading commercial databases, it is possible to accelerate and to improve the accuracy of strain identification. This is especially important in the case of *Nocardia*, for which valid microbial diagnosis remains challenging; reference laboratories are often required to identify and to survey these rare actinobacteria.

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