JOURNAL OF CLINICAL MICROBIOLOGY, June 2005, p. 2624–2628 Vol. 43, No. 6
0095-1137/05/$08.00+0 doi:10.1128/JCM.43.6.2624–2628.2005

Distribution of *Nocardia* Species in Clinical Samples and Their Routine Rapid Identification in the Laboratory

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Received 16 December 2004/Returned for modification 3 February 2005/Accepted 4 March 2005

Eighty-six *Nocardia* strains isolated from clinical samples in Belgium were identified by 16S rRNA gene sequencing. Eighty-three (96%) strains belonged to only six *Nocardia* species: *N. farcinica* (38 [44%]), *N. nova* (19 [22%]), *N. cyriacigeorgica* (13 [15%]), *N. brasiliensis* (6 [6.9%]), *N. abscessus* (5 [5.8%]), and *N. paucivorans* (2 [2.3%]). A gallery of nine conventional and enzymatic tests was developed for the rapid identification of the most common species isolated during this survey. Pyrrolidonyl aminopeptidase, γ-glutamyl aminopeptidase, α-mannosidase, and α-glucosidase were found to be highly discriminating and could be used to develop an identification scheme.

Nocardia species are isolated with an increased frequency from clinical specimens, especially in specimens from immunocompromised patients (19). The taxonomy of the genus has dramatically been revised during the last decade, and at least 30 valid species have been reported, besides a number of unnamed genomospecies (18). Not all of them have been found in humans, and *Nocardia asteroides*, previously most frequently isolated from clinical specimens, has proved to be heterogeneous and has been divided into several species (18). More recently, additional species of human origin have been described (6, 8, 9, 11, 12, 17, 28).

The routine identification of *Nocardia* strains at the species level is difficult in the laboratory. This, and the nomenclature changes, may explain that species distribution in clinical isolates has been poorly documented up to now, and even recent surveys still report the "*N. asteroides* complex" as the most frequent *Nocardia* species isolated in humans (7, 10, 16, 19). Identification studies have not been systematically carried out since the several recent taxonomic changes.

The aim of this study was to assess the species distribution of a large number of *Nocardia* isolates and to propose simple and rapid identification tests that may be helpful to identify the species most commonly encountered in clinical material.

MATERIALS AND METHODS

Bacterial strains. Eighty-six *Nocardia* strains isolated from clinical specimens in Belgium were collected for the study. Most strains were isolated during the past decade, but a few were received before 1990. They were isolated by several laboratories in different parts of the country. All the *Nocardia* isolates were included in the study to avoid any bias in the selection of the strains. Only one strain per patient was considered. The clinical origins of the isolates were as follows: 36 strains were isolated from the respiratory tract, 18 from pyogenic lesions and wounds, 8 from blood, 4 from brain abscesses, and 1 from cerebral fluid. Nineteen were of unknown origin.

The type strains of the most relevant species were included for phenotypic comparison as well as reference strains of some less common species. *Nocardia* species and strains are listed in Table 1.

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Sequencing of the 16S rRNA gene. The full-length 16S rRNA gene sequences (±1,400 nucleotides) of all strains were determined as described previously (24), and sequences were compared to those of the type strains deposited in the GenBank database.

Cellular fatty acids were analyzed by gas-liquid chromatography as outlined elsewhere (22).

Phenotypic characterization. Strains were cultured on tryptic soy blood agar plates at 35°C. They were examined for partial acid-fastness, presence of aerial hyphae, and resistance to lysozyme. Conventional tests for species identification, including tyrosine, xanthine, and hypoxanthine decomposition, growth at 45°C, and gelatin hydrolysis, were also performed (3). Furthermore, the following tests were used in this study.

(i) Urea hydrolysis. For urea hydrolysis, a heavy loopful of bacteria was suspended in 0.5 ml of a medium containing 1 g urea and 1 ml of a cresol red solution (0.5% [wt/vol]) in 100 ml phosphate buffer (0.005 M) (pH 6). Tubes were incubated at 35°C and examined after 1 and 2 days for a pink color shift indicating a positive reaction.

(ii) Casein hydrolysis. Casein hydrolysis was detected on plates containing 10% (vol/vol) skim milk in nutrient agar. A heavy streak or spot was inoculated, and plates were incubated at 35°C for 7 days. Clearing around the streak indicated a positive reaction.

(iii) Acid production from rhamnose. Acid production from rhamnose was detected on low-peptone phenol red (LPPR) agar with 1% rhamnose as previously described for ethylene glycol acidification (25).

(iv) Citrate alkalinization. Citrate alkalinization was performed on Simmons citrate agar. Slants were heavily inoculated with organisms grown on blood agar. Cultures were incubated at 35°C and examined daily for 5 days. Citrate utilization with alkali production resulted in a blue color of the slant.

(v) α-Glucosidase activity. α-Glucosidase activity was detected by the use of tablets containing the nitrophenyl conjugate of α-D-glucopyranoside (Rosco, Taastrup, Denmark). A heavy loopful of bacteria was suspended in 0.5 ml distilled water and dispersed as much as possible. No attempt was made to obtain a homogeneous suspension. The suspension with the reagent tablet was incubated at 35°C and read after 4 h. When α-glucosidase was present, the suspension turned yellow. When results were negative, the reaction was read again after overnight incubation.

(vi) α-Mannosidase. α-Mannosidase was detected in the same way, using nitrophenyl-α-D-mannopyranoside tablets (Rosco). Results were read after 4 h of incubation.

(vii) PYR and γ-GLU. A heavy bacterial suspension was made in 0.5 ml distilled water for each test as for α-glucosidase. For the pyrrolidonyl aminopeptidase (PYR) and γ-glutamyl aminopeptidase (γ-GLU) tests, substrate tablets of pyrrolidonyl-naphthylamide and γ-glutamyl-naphthylamide (Rosco) were added to the suspensions, respectively. After 4 h of incubation at 35°C, 1 drop of cinnamaldehyde reagent was added. A color shift to orange or pink indicated a positive reaction. The suspension remained yellow when the test was negative.

API ID 32 C strips (bio-Mérieux, Marcy l'Etoile, France) was also used for several strains.

TABLE 1. Biochemical test results of the 86 study strains and *Nocardia* reference strains[a]

Species	Strain(s)	No. of strains	Growth at 45°C	Casein	Urease	Rhamnose[b]	Citrate[c]	PYR	γ-GLU	α-Gluco-sidase	α-Manno-sidase
N. farcinica	DSM 43665^T		+	−	+	+	−	+	+	+	−
	Clinical strains	38	37	0	37	37	0	38	38	38	0
N. nova	DSM 43207^T		+	−	−	−	−	+	−	+	−
	Clinical strains	19	3	0	10	0	0	19	0	19	0
N. cyriacigeorgica	DSM 44484^T		+	−	−	−	−	−	+	+	−
	Clinical strains	13	13	0	0	0	0	0	13	13	0
N. brasiliensis	NCTC 11294^T		−	+[d]	+	−	−	−	+	+	+
	Clinical strains	6	0	6[d]	6	0	0	0	6	6	6
N. abscessus	DSM 44432^T		−	−	+	+[w]	+	−	+	+	−
	Clinical strains	5	0	0	5	0	5	0	5	5	0
N. paucivorans	DSM 44386^T		+	−	−	−	−	−	+	−	−
	Clinical strains	2	2	0	0	0	0	0	2	0	0
N. asiatica	DSM 44668^T		−	−	+	+	+	−	+	+	−
	Clinical strain	1	0	0	1	1	1	0	1	1	0
N. niigatensis	DSM 44670^T		−	−	−	−	−	+	−	+	−
	Clinical strain	1	0	0	0	0	0	1	0	1	0
N. camea	DSM 43397^T, 44558, 46071		−	−	−	−	−	−	+	−	−
	Clinical strain	1	0	0	0	1	0	0	1	0	0
N. otitidiscaviarum	NCTC 1934^T		+	−	+	−	−	−	+	+	−
N. africana	DSM 44491^T		+	−	−	−	−	+	−	+	−
N. veterana	DSM 44445^T		+	−	+	−	−	+	−	+	−
N. puris	DSM 44599^T		−	−	−	−	+	−	+	+	−
N. transvalensis	DSM 43405^T		−	−	+	−	+[w]	+	+	+	+
N. pseudobrasiliensis	DSM 44290^T		−	+[e]	+	−	−	−	+	+	+
N. asteroides	NCTC 11293^T, DSM 43258		−	−	+	−	−	−	+	+	−

[a] Test results for type strains are shown as follows: +, positive; +[w], weak reaction; −, negative. For clinical strains, the number of positive strains is shown.
[b] Acid production from rhamnose within 1 day.
[c] Citrate alkalinization on Simmons citrate agar within 3 days.
[d] Positive after 24 h.
[e] Positive after 5 days.

Antibiotic susceptibility tests for diagnostic purpose were performed to determine susceptibility patterns (21).

RESULTS

All strains exhibited partial acid-fastness, varying from weak to strong. Aerial hyphae were observed at least once in all strains, and growth was observed in the presence of lysozyme. The main cellular fatty acids were 16:0, 18:1^{9cis}, 18:0, and tuberculostearic acid, consistent with the pattern of *Nocardia* species. On the basis of these properties, the strains were presumably considered members of the genus *Nocardia*.

Sequencing of the 16S rRNA gene. The full-length sequences of almost all the strains showed a similarity rate of 99.8% to 100% with a named species of the genus *Nocardia* compared to the sequences of the GenBank database. One *N. farcinica* strain displayed only 99.2% homology, one *N. brasiliensis* 99.6%, one *N. cyriacigeorgica* 99.7%, and one *N. nova* 99.7% with the sequence of the respective type strain, but the phenotypic characteristics of these strains were consistent with those of the corresponding species.

The distribution of the species among the 86 clinical isolates, as determined by 16S rRNA gene sequencing (GenBank accession numbers within parentheses), was as follows: 38 (44.1%) *N. farcinica* (AF430033), 19 (22.0%) *N. nova* (AF430028), 13 (15.1%) *N. cyriacigeorgica* (AF430027), 6 (6.9%) *N. brasiliensis* (AF430038), 5 (5.8%) *N. abscessus* (AF430018), 2 (2.3%) *N. paucivorans* (AF179865), 1 (1.1%) *N. carnea* (AF430035), 1 (1.1%) *N. niigatensis* (AB092565), and 1 (1.1%) *N. asiatica* (AB162796).

Eighty-three strains (96.5%) belonged to only six species represented by at least two isolates. Only three strains each (3.5%) belonged to three less common species.

The clinical origin of the *N. farcinica* and *N. nova* isolates was very diversified. It is noteworthy that seven of the eight blood isolates and the four brain abscess strains were *N. farcinica*, which is considered by several authors as the most invasive *Nocardia* species (19, 20). All but two strains of *N. cyricigeorgica* were isolated from the respiratory tract. All *N. brasiliensis* strains were found only in superficial abscesses or wounds, in agreement with previous reports (19). The three documented strains of *N. abscessus* were from wounds or pus collections. The two *N. paucivorans* strains, as well as the *N. niigatensis* and *N. carnea* strains, were isolated from the respiratory tract. *N. asiatica* was found in a wound swab.

The clinical isolates were first examined by the conventional hydrolysis test of xanthine, hypoxanthine, and tyrosine. All these tests were negative except for tyrosine and hypoxanthine for the six *N. brasiliensis* strains.

Nine tests were selected because the results were achieved in a short time from 4 h to 1 or 2 days. Results of the 86 clinical isolates and those of the reference strains are reported in Table 1. As can be seen, most tests have a 100% or 0% positivity rate, resulting in a high discrimination of the six main species isolated in this study. It should be noted that test results of species represented by a small number of strains should not be considered definitive until more isolates are examined in the future.

Urea hydrolysis varies considerably depending on medium composition, inoculum size, and incubation time. This may explain some discrepancies in the literature reports (18, 26,

27). By using the method described here, urea hydrolysis was either positive within 24 h or negative after 2 days in all species investigated, except for *N. nova*, which exhibited variable results ranging from positive within a few hours to negative after more than 3 days.

Casein hydrolysis and gelatin hydrolysis were parallel, but positive results with the former were usually achieved after 24 h of incubation and clearing of the medium was clear-cut. Casein hydrolysis was positive only in *N. brasiliensis* and *N. pseudobrasiliensis*. The latter has not been isolated during this study, but the type strain was positive, in our hands, after 5 days in contrast to the rapid clearing by *N. brasiliensis*. Positivity within 24 h may therefore suggest *N. brasiliensis* in an initial screening procedure.

Citrate was assimilated by several species on identification systems using AUX medium (bioMérieux), e.g., *N. abscessus*, *N. brasiliensis*, and *N. asteroides* (type strain). However, in this study, alkalinization of Simmons citrate agar slants within 1 to 3 days was observed only in *N. abscessus* and in some rare species, such as *N. asiatica* and the type strain of *N. puris*, whereas the type strain of *N. transvalensis* was weakly positive after 3 to 4 days. The *N. brasiliensis* strains were negative except for two strains which exhibited slight and delayed alkalinization after more than 6 days. Therefore, rapid alkalinization of Simmons citrate agar was highly suggestive of *N. abscessus*, since only this species and the one strain of *N. asiatica* in this study were positive within 2 days among our 86 clinical isolates.

Similarly, rhamnose acidification on LPPR agar was not equal to rhamnose assimilation on AUX medium, for example in the API ID 32 C system. Using our method, acid production from rhamnose after overnight incubation occurred only in *N. farcinica* among the PYR-positive isolates. Only one out of 39 strains (including the type strain) of *N. farcinica* did not acidify rhamnose, even after prolonged incubation, but this strain did not assimilate rhamnose in the API ID 32 C system either. Other species, such as *N. abscessus*, may assimilate rhamnose, but in our study, they did not acidify it on LPPR agar, although the type strain was weakly positive after 1 or 2 days.

Although α-glucosidase was positive within 4 h in most species, the test was often weakly positive or even negative after this incubation time in *N. nova* strains, but all were positive after overnight incubation. Therefore, against the manufacturer's recommendations, the test was read again after 18 to 24 h. After this prolonged incubation time, only *N. paucivorans* and *N. carnea* were consistently negative.

α-Mannosidase was rapidly positive in all *N. brasiliensis* strains, confirming the results of Biehle et al. (1). However, we found that this enzyme was also present in the type strain of *N. pseudobrasiliensis* and in the type strain of *N. transvalensis*.

PYR and, to a lesser extent, γ-GLU were key tests in the initial screening of *Nocardia* species and even in the identification of the common clinical isolates. An advantage of these enzymatic reactions was the short incubation time with results being achieved within 4 h. Therefore, some species may be discarded, and the results of further investigations may be more accurate. PYR gave clear-cut results, and the test was positive in two common species, *N. farcinica* and *N. nova*, accounting together for about two-thirds of our isolates. These findings were in agreement with those reported by Biehle et al. (1). γ-GLU separated the two species, which could be presumably identified the same day.

DISCUSSION

When full-length sequences or partial sequences of more than 1,000 nucleotides are analyzed, 16S rRNA gene sequencing is considered a reliable tool for species identification in the genus *Nocardia* (5, 18).

It is noteworthy that 83 (96.5%) of our 86 clinical isolates belong to only six species, whereas more than 30 species have been validly described in 2003 (18). Since that time, several of the new species are represented by only one strain or a few strains, so their occurrence in humans still has to be assessed (6, 8, 11, 12, 17, 28).

In our survey, *N. farcinica* outnumbers all other species, accounting for about 44% of the isolates, followed by *N. nova* and *N. cyriacigeorgica*. *N. abscessus* and *N. brasiliensis* are less frequently found. *N. paucivorans*, represented by two strains, has only seldomly been isolated from humans (5, 26). From the three single isolates, *N. carnea* has only rarely been reported as a pathogen in humans (5), while *N. asiatica* and *N. niigatensis* have been described quite recently (8, 11). It should also be noted that no *N. asteroides* isolates sensu stricto (type strain cluster) have been collected during this protracted survey in Belgium. However, the small number of strains in some species and the limited geographical area where the study was carried out do not permit extrapolation of this distribution to other countries.

There are only a few reports dealing with the distribution of *Nocardia* species in clinical isolates using the present nomenclature. One reason may be the recent changes in taxonomy and the subdivision of the previous "*N. asteroides* complex" into several new species (18). Indeed, in most cases, the "*N. asteroides* complex" is considered by several authors as an entity without reference to newly described species, such as *N. abscessus* or *N. cyriacigeorgica* (7, 16, 19).

Kageyama et al. (10) reported 303 cases of nocardiosis in Japan between 1992 and 2001. About 72% of the strains belonged to the "*N. asteroides* group," including 81 strains of *N. farcinica*, which was the most frequent isolate, in agreement with our findings. It was followed by *N. asteroides*, *N. nova*, and *N. cyriacigeorgica*. Besides the "*N. asteroides* group," the main species was *N. brasiliensis* with 66 strains (10).

In the study carried out by Saubolle and Sussland (19) in the United States, only 34 of 455 isolates were reported to be *N. farcinica*, while 319 belonged to "the *N. asteroides* complex." These figures are in sharp contrast with our findings, but the species distribution within the "*N. asteroides* complex" is not related in detail and might include *N. farcinica*. Moreover, the authors emphasize the possibility of geographic variations (19).

Identification of *Nocardia* species by phenotypic characterization is often considered tedious and difficult and requires, at least for some tests, long incubation times. Besides the decomposition of tyrosine, xanthine, and hypoxanthine, it is mainly based on some conventional tests, such as urea hydrolysis, growth at 45°C, and assimilation of organic compounds on minimal media, such as the AUX medium used in the API ID 32 C system (15, 18). Susceptibility to some antibiotics has

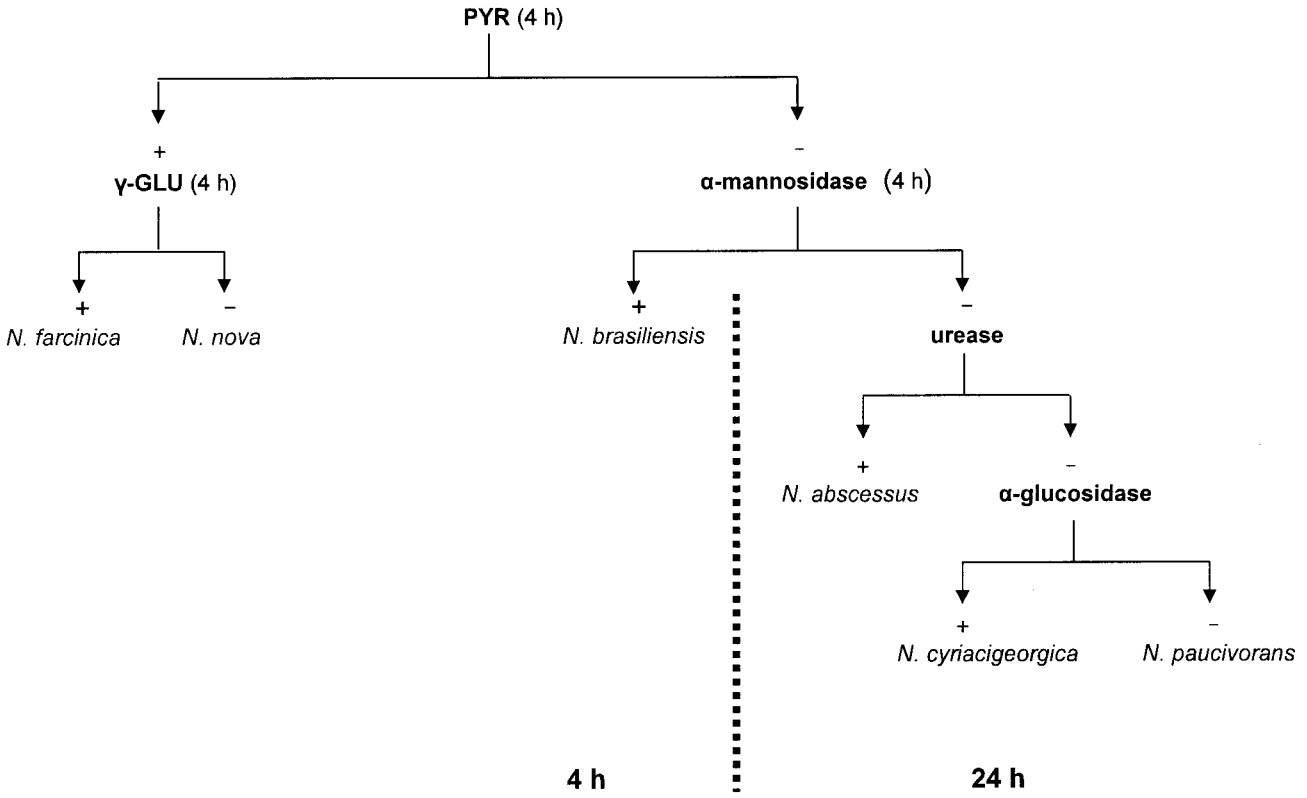

FIG. 1. Algorithm for the identification of the six most relevant species isolated in this study.

been proposed to subdivide the previous "*N. asteroides* complex," resulting in six patterns (I to VI) (21). In another study, a simplified battery of tests was proposed, allowing an identification within 3 to 4 days for most of the common species, but *N. asteroides* type I (i.e., *N. abscessus*) and type VI (i.e., *N. cyriacigeorgica*) remain difficult to distinguish (13). To overcome these difficulties, molecular methods have been proposed, resulting in a more reliable and rapid identification (14). Cloud et al. compared conventional and molecular methods and showed that with the Microseq 500 system, the average identification time dropped from 2 to 3 weeks to 1 to 3 days (4).

Enzymatic reactions in *Nocardia* were studied by Boiron and Provost using the API ZYM system (2). However, the species nomenclature in use at that time prohibits comparison with today's results. Moreover, pyrrolidonyl aminopeptidase and γ-glutamyl aminopeptidase are not included in the system used by these authors. It should also be noted that results of some enzymatic reactions, particularly α-glucosidase and α-mannosidase, may vary according to the conjugate of the substrate: nitrophenyl compounds (Rosco) may be more sensitive than naphthyl compounds present in the API ZYM system (23). Biehle et al. (1) described an identification scheme for five *Nocardia* species based on conventional tests and enzymatic reactions. The Microscan panels ANA and HNID were used, containing chromogenic substrates, e.g., nitrophenyl compounds for α-glucosidase and α-mannosidase and β-naphthylamide compounds for aminopeptidases, similar to our substrates, but readings were made after overnight incubation. They found that several enzymatic reactions are discriminating, and the total identification time for the five species was, on average, 5 days (1).

The small number of species involved in 96.5% of the isolates in our study would facilitate a simplified approach to routine identification of the most relevant species in the laboratory. In the scheme we propose, we have tried to select tests with short incubation times and which are easy to perform in the clinical laboratory. One of the drawbacks of *Nocardia* identification is the difficulty to obtain a homogeneous and/or standardized inoculum. Some authors use glass beads or other devices for this purpose (13). Even when using such methods, a fine particulate suspension is obtained, but some species, particularly *N. brasiliensis* and *N. abscessus*, are very difficult to resuspend (13). In our study no attempt was made to homogenize the inoculum to keep the test suitable for routine use, but a large inoculum size was used. It was roughly assessed visually, and a heavily loaded loop of bacteria was suspended in 0.5 ml of medium or water.

As can be seen in Table 1, only two identification profiles are shared by more than one species. *N. nova* is similar to *N. veterana* and *N. africana*. This is not surprising, since the three species are considered members of the "*N. nova* complex" (5). *N. niigatensis* also exhibits the same profile. However, this species can be easily differentiated by its resistance to imipenem, an uncommon feature in *Nocardia*, which is never present in *N. nova*. The *N. niigatensis* strains are also resistant to ampicillin and susceptible to piperacillin, while *N. nova* strains have the opposite pattern. *N. carnea* resembles *N. paucivorans*. However, the *N. paucivorans* strains grew at 45°C and the *N. carnea*

strains did not, in agreement with the findings of Roth et al. (18). It should be noted that our clinical isolate of *N. carnea* produced acid from rhamnose, whereas the three reference strains did not. Moreover, two other species, *N. abscessus* and *N. asiatica*, have very similar profiles in the proposed scheme, although the two *N. asiatica* strains (including the type strain) produced acid from rhamnose within 24 h, whereas all *N. abscessus* strains did not acidify this sugar even after several days, except for the type strain, which exhibited a weak positive reaction after 1 or 2 days. Furthermore, *N. abscessus* strains are very susceptible to amoxicillin-clavulanate, while *N. asiatica* strains are more resistant.

On the basis of a few rapid tests here described, an algorithm was constructed for the screening of the common species encountered in this survey (Fig. 1). It should be emphasized that the proposed identification scheme cannot be used for taxonomic purposes, since only a limited number of species have been studied. More extensive characterization based on existing schemes and molecular methods should be performed to identify the *Nocardia* species less commonly occurring in humans. Nevertheless, 96.5% of the 86 strains isolated during this study could be identified by this method, and this was achieved within 4 h for three species, *N. farcinica*, *N. nova*, and *N. brasiliensis*, accounting for more than 73% of our isolates. Therefore, it could be helpful for routine use in the clinical laboratory, either as screening or even as rapid identification of the most common species.

ACKNOWLEDGMENTS

We are grateful to J. Verhaegen, O. Denis, and G. Ieven for providing us with several strains.

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