Rapid Identification of Clinically Relevant *Nocardia* Species to Genus Level by 16S rRNA Gene PCR

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Two regions of the gene coding for 16S rRNA in *Nocardia* species were selected as genus-specific primer sequences for a PCR assay. The PCR protocol was tested with 60 strains of clinically relevant *Nocardia* isolates and type strains. It gave positive results for all strains tested. Conversely, the PCR assay was negative for all tested species belonging to the most closely related genera, including Dietzia, Gordona, Mycobacterium, Rhodococcus, Streptomyces, and Tsukamurella. Besides, unlike the latter group of isolates, all *Nocardia* strains exhibited one *MliI* recognition site but no *SacI* restriction site. This assay offers a specific and rapid alternative to chemotaxonomic methods for the identification of *Nocardia* spp. isolated from pathogenic samples.

*Nocardia* spp. are gram-positive, aerobic actinomycetes with a worldwide distribution in soil. At least six species are pathogenic for both humans and animals and may enter the body via inhalation of contaminated dust particles or via wounds contaminated with dust or soil (18). They are responsible for several infections including pulmonary, central nervous system, and cutaneous infections (1). An increasing number of cases have been reported since 1980, which supports the view of many investigators that the incidence of nocardiosis is on the rise (2). Nocardiosis is diagnosed by isolation and culture identification. However, colonial characteristics and cellular morphology are variable, and *Nocardia* spp. may be misidentified and confused with members of closely related genera such as *Dietzia* (22), *Gordona*, *Mycobacterium*, *Rhodococcus*, *Tsukamurella*, and even *Streptomyces*. Conventional identification to the genus level is based on chemotaxonomic characteristics, such as the presence of a major amount of meso-diaminopimelic acid, arabinose, galactose, and mycyclic acids with 46 to 60 carbons in cell walls (12). However, these techniques sometimes fail to fully differentiate some of the most closely related genera. Moreover, they are laborious, time-consuming, and expensive and can take 1 to 3 weeks to accomplish.

In this study, we developed an alternative PCR-based identification strategy targeted at the gene coding for 16S rRNA. Although the sequence for this gene is largely phylogenetically conserved, there may be variable sequences characteristic of particular organisms. The detection of these variable regions can therefore allow bacteria to be identified and differentiated from each other (13, 17, 26). The comparative analysis of the sequences of the complete 16S rRNA genes from 85 actinomycetes provided the basis for the design of diagnostic primers with a potential for the genus-specific detection of *Nocardia* by a PCR assay. We evaluated this approach for the fast, sensitive, and accurate identification of a wide range of *Nocardia* spp. isolated from pathogenic samples.

**MATERIALS AND METHODS**

**Bacterial strains.** One hundred seventeen clinical strains and eighteen reference strains of actinomycetes have been studied (Table 1). Cultures were obtained from the National Reference Center for Human Mycosis, Antifungal Agents, and Actinomycetes (Institut Pasteur, Paris, France) or from the American Type Culture Collection (ATCC; Rockville, Md.). All isolates were identified by conventional methods including biochemical tests, whole-cell compositions, and enzymatic profiles (3). The isolates included 5 strains of *Dietzia*, 8 strains of *Gordona*, 17 strains of *Mycobacterium*, 60 strains of *Nocardia* belonging to 8 different species, 20 strains of *Rhodococcus*, 20 strains of *Streptomyces*, and 5 strains of *Tsukamurella*. Cultures were maintained on Bennett’s agar medium at room temperature before being treated simultaneously.

**Primer selection.** The sequences of the 16S rRNA genes from a large number (*n* = 85) of isolates belonging to the genera *Dietzia*, *Gordona*, *Mycobacterium*, *Nocardia*, *Rhodococcus*, *Streptomyces*, and *Tsukamurella* were found in the DDBJ/EMBL/Genbank database. They were analyzed with the PILEUP program (9) of Genetics Computer Group software package (8). The existence of regions exhibiting possible sequence signatures to the genus level was investigated. The results allowed the design of two primers with 3’ extremities specific for the *Nocardia* genus (Fig. 1): forward primer NG1 (5’-ACCGGACCCAGGGG-3’) was complementary to positions 966 to 982 on the antisense strand, and reverse primer NG2 (5’-GGGTGAAACCTTTCGGA-3’) was complementary to positions 386 to 405 on the sense strand. Both primers were custom synthesized by Appligene (Ailkirch, France). Thus, the amplified product was to be 596 bp in length.

**DNA extraction, PCR amplification, and detection of PCR products.** Isolated colonies were subcultured for 3 to 5 days at 34°C on sterile cellulose acetate membranes (Millipore, Bedford, Mass.) that had been deposited on blood agar (bioMérieux, Marcy l’Étoile, France). DNA was extracted as described previously (21). A colony was suspended in 650 μl of extraction mixture containing 500 μl of sterile pyrolyzed water and 150 μl of Chelex solution (15% [wt/vol] Chelex 100 resin, 0.1% [wt/vol] sodium dodecyl sulfate, 1% [vol/vol] Nonidet P-40, 1% [vol/vol] Tween 80) and boiled for 30 min at 100°C. The samples were centrifuged for 5 min at 5,000 × *g*. The final supernatant containing the extracted DNA was recovered, and 15 μl of a 1:20 dilution aliquot was used for amplification. The 100-μl DNA amplification reaction mixture contained 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 1.5 mM MgCl2, 0.1% Triton X-100, each deoxynucleoside triphosphate at a concentration of 200 μM, 1 μM each primer, and 1 U of AmpliTaq Gold (Perkin-Elmer, Norwalk, Conn.). Amplified reactions were carried out in a DNA thermal cycler (Hybaid; Omni-gene, Teddington, United Kingdom). After initial denaturation at 94°C for 1 min, the reaction mixture was run through 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 20 s, and extension at 72°C for 1 min. Cycling was followed by a final extension incubation of 10 min at 72°C.

To ensure that no contaminating DNA would give positive results, one sample lacking a template was included in each series of reactions. After amplification, 12-μl samples were analyzed by electrophoresis for 2 h at 100 V in horizontal TAE (40 mM Tris-acetate, 2 mM Na2 EDTA; 2H2O) gels containing 2% agarose (Ultrapure; Bio-Rad Laboratories, Hercules, Calif.) and 0.5 μg of ethidium bromide per ml. The gels were then exposed to UV light to visualize the amplified products and photographed. The reproducibility of the reaction was tested at intervals of different numbers of days with freshly extracted DNA.

The identity of the amplified products was further investigated by restriction

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Differences in base sequences are indicated with boldface characters.

Because of the slow growth of *Nocardia*, we used a direct DNA extraction method from a single colony (isolated in primary culture or from samples received by the reference laboratory) using the Chelex method in less than 40 min. This DNA extraction method from a single colony (isolated in primary culture or from samples received by the reference laboratory) using the Chelex method in less than 40 min. This

The presence of regions within 16S rDNA sequences of *Nocardia* was investigated. The comparative analysis of the 16S rDNA sequences from 85 strains belonging to the genus was found to be effective in recovering sufficiently purified DNA for amplification even if DNA cannot be visualized. By reducing the time and labor associated with the conventional DNA isolation procedure with the rapid-release method, the entire PCR assay and restriction endonuclease cleavage can be completed within 2 days.

PCR, which allows the specific and sensitive amplification of a preselected DNA region, has been intensively applied to the species identification of numerous organisms (7, 11, 14, 23, 27). Its use requires the detailed examination of the molecular genetics of organisms, especially the identification of the DNA sequences that are specific for the organism tested. The target regions usually are pathogenicity or virulence genes. On the basis of the homologies of a heat shock protein (Hsp65) gene from *Nocardia* strains and rapidly growing *Mycobacterium* strains, Lungu et al. (16) developed such an approach: an RFLP analysis of DNA amplified from the Hsp65 gene enabled them to distinguish these two genera. In this case, however, no extensive study concerning other actinomycetes was done. Moreover, because the primers were not specific, only endonuclease digestion gave specificity to this test. Finally, the profiles obtained were sometimes not easy to interpret since the number of patterns was large for rapidly growing *Mycobacterium* and since fragment sizes were difficult to analyze.

Little is known about DNA from the *Nocardia* genus. The 16S rRNA gene (rDNA) sequences are the only extensive sequences that have been documented (6, 24). Thus, it is now established that the primary structure of rRNA is composed of regions with highly conserved sequences together with other regions composed of variable or hypervariable sequences, i.e., signatures, that are of special interest for identification to the genus or species level (4, 5, 10). The similarities in nucleotides of rRNA preparations provide a means of establishing relationships among representatives of diverse bacterial taxa. In the late 1970s, Mordarski et al. (19, 20) used DNA-rDNA pairing experiments to show the phylogenetic relationship between a genus belonging to the actinomycetes and the clustering of each genus. Recently, a comprehensive phylogenetic analysis of the genus *Nocardia* by 16S rDNA gene sequencing (6, 24) confirmed these results and indicated that the genus *Nocardia* (after several taxonomic modifications) constitutes a homogeneous taxon that can be distinguished from the other mycobacterial-containing organisms. In addition, ribotyping with a probe coding for a part of 16S RNA had demonstrated a species-level specificity for isolates belonging to the *N. asteroides* complex (15). Therefore, 16S rDNA appeared to be a potential interesting target in the development of new molecular techniques for *Nocardia* genus identification.

**RESULTS AND DISCUSSION**

Because of the slow growth of *Nocardia*, we used a direct DNA extraction method from a single colony (isolated in primary culture or from samples received by the reference laboratory) using the Chelex method in less than 40 min. This

<table>
<thead>
<tr>
<th>Genus and species</th>
<th>No. of isolates*</th>
<th>Reference strains used</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Nocardia asteroides</em></td>
<td>17</td>
<td>ATCC 19247</td>
</tr>
<tr>
<td><em>Nocardia farcinica</em></td>
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<td>ATCC 3318</td>
</tr>
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<td>11</td>
<td>ATCC 33727</td>
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<td>ATCC 19296</td>
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<tr>
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<td><em>Nocardia transvalensis</em></td>
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<td>Mycobacterium spp.</td>
<td>8</td>
<td></td>
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</table>

* National Reference Center for Human Mycosis, Antifungal Agents, and Actinomycetes (Institut Pasteur, Paris, France).

**TABLE 1. Strains used in the study**

![Fig. 1. Comparison of the DNA sequences of the 16S rRNA genes from members of the *Nocardia* genus and other non-*Nocardia* genera of aerobic actinomycetes. Differences in base sequences are indicated with boldface characters.](image-url)
was noted at position 523 of the DNA sequences of non-
expected, no restriction fragments were produced following en-
restriction site was observed in Nocardia
ments of 278 and 318 bp. On the other hand, no
Mln
mycetes (Fig. 1). As shown in Fig. 3, all
Nocardia
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fied fragment allowed the clear differentiation of members of
nucleotide positions 704 (Nocardia
amplified fragment. If a non-
ond level of control to verify the specificity of the 596-bp
uct was obtained in control experiments with DNA from any of
the primers was tested against a diverse array of
zyme. It has been used for the identification of different species
of aerobic actinomycetes, including the Nocardia species, with
an accuracy of 98.6% (25, 28). However, since the scheme
described for the identification of actinomycete species pre-
presented several subgroups (several profiles with several bands
for which sizes were not easy to estimate), the interpretation of
the result for a clinical strain was difficult within the actinomy-
cete group. On the contrary, if we knew in advance whether the
strains belonged to the Nocardia genus, use of interpretation
by the technique of Steinrube et al. (25) made identification of
the different species easier. Therefore, the technique de-
to actinomycetes (including data for 25 Nocardia strains) re-
vealed a high degree of homology. However, all Nocardia
strains examined exhibited the genus-specific GA at nucleotide
positions 404 and 405 and CC at nucleotide positions 966 and
967. Primers NG1 and NG2 were constructed by using these
sequences containing the Nocardia-specific signature and the
 corresponding preceding 5' area (Fig. 1). Although this area
was highly conserved among actinomycetes, the sequence of
two nucleotides specific for Nocardia was sufficient for the
design of diagnostic primers.

Figure 2 shows an example of the results of a PCR assay with
the primers that were designed. The general applicability of
the primers was tested against a diverse array of Nocardia
strains (including type strains). A 596-bp amplimer was ob-
served in all Nocardia strains, whereas no amplification prod-
uct was obtained in control experiments with DNA from any of
the other actinomycetes strains (Fig. 2). These results demon-
strated that all strains of Nocardia spp. tested can be distin-
guished from representative members of the genera Dietzia,
Gordona, Mycobacterium, Rhodococcus, Streptomyces, and
Tsukamurella by using the selected primer combination.

Restriction endonuclease analysis was performed as a sec-
ond level of control to verify the specificity of the 596-bp
amplified fragment. If a non-Nocardia isolate shows an ampli-
ified product despite theoretical and observed primer specific-
ity, a second level of check exists. Two recognition sites at
nucleotide positions 704 (MnlI) and 523 (SacI) on the ampli-
fied fragment allowed the clear differentiation of members of
the Nocardia genus from other genera of the aerobic actino-
mycetes (Fig. 1). As shown in Fig. 3, all Nocardia strains ex-
hibited one MnlI recognition site, producing two DNA frag-
ments of 278 and 318 bp. On the other hand, no SacI
restriction site was observed in Nocardia strains, and as ex-
pected, no restriction fragments were produced following en-
donuclease analysis; conversely, a potential SacI restriction site
was noted at position 523 of the DNA sequences of non-
Nocardia strains. In case of nonspecific amplification of the 16S
rRNA gene fragment, the SacI restriction site may produce
two fragments of 137 and 459 bp, and these differentiate an
isolate from Nocardia species. In fact, even though we never
observed nonspecific amplification, we retained the restriction
donuclease analysis step to increase the security of genus
identification.

On the basis of our results, we could establish a simple and
practical scheme for the identification of genus Nocardia
through actinomycetes: (i) 596-bp fragment amplification with
an MnlI restriction site but no SacI restriction site indicates the
DNA of a strain belonging to the genus Nocardia; (ii) no
amplification fragment indicates the DNA of a strain not be-
longing to the genus Nocardia; and (iii) amplification of a
596-bp fragment with a SacI restriction site and/or without an
MnlI restriction site indicates the possible nonspecific amplifi-
caction, requiring confirmation of the identification by conven-
tional methods.

PCR amplification with two primers (primers TB11 and
TB12) enabled amplification of a 440-bp fragment coding for a
part of a 65-kDa heat shock protein. This fragment was di-
gested for restriction endonuclease analysis with five enzymes
(BstEII, HaeIII, MspI, HinfI, and BsaI11). The results allowed
establishment of a practical identification scheme according to
the numbers and sizes of the bands generated with each en-
zyme. It has been used for the identification of different species
of aerobic actinomycetes, including the Nocardia species, with
an accuracy of 98.6% (25, 28). However, since the scheme
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by the technique of Steinrube et al. (25) made identification of
the different species easier. Therefore, the technique de-

FIG. 2. Agarose gel electrophoresis of PCR products of actinomycetes strains by using primers NG1 and NG2. Lanes: 1 to 4, N. asteroides sensu stricto; 5 to 8, N. farcinica; 9 to 12, N. nova; 13 to 15, N. brasiliensis; 16, N. otitidiscavi-
orum; 17, N. carnea; 18, N. transvalensis; 19, N. brevicatenae; 20, Gordona bron-
chiadis; 21, Streptomyces spp.; 22, Rhodococcus equi; 23, Mycobacterium tubercu-
losis; 24, N. asteroides ATCC 19247; M, 100-bp DNA ladder (Boehringer

FIG. 3. RFLP patterns of PCR products from strains belonging to Nocardia
genus, obtained by using endonucleases SacI (A) and MnlI (B). Lanes: 1 and 2,
N. asteroides sensu stricto; 3, N. farcinica; 4, N. nova; 5, N. brasiliensis; 6, N.
otitidiscaviorum; 7, N. carnea; 8, N. transvalensis; 9, N. brevicatenae; 10, PCR
products of N. asteroides ATCC 19247 without digestion; M, 100-bp DNA ladder
(Boehringer Mannheim GmbH). pb, base pairs.
scribed here complements the technique of Steingrube et al. (25). The genus- and species-specific techniques could be combined to accomplish the identification of *Nocardia* clinical isolates within 48 h after receipt of cultures in the reference laboratories. Further studies are needed to develop different systems of identification of all genera belonging to the pathogenic aerobic actinomycetes after the selection of primers specific for each one.

In the future, the PCR method that we developed could be performed directly with clinical samples such as skin biopsy, blood, sputum, or bronchoalveolar lavage specimens. The direct diagnosis of nocardiosis by PCR of cerebrospinal fluid could replace the use of cerebral biopsy, which requires the use of an aggressive technique. It would improve the speed of diagnosis that is closely related to the favorable course for the patients (in terms of mortality, relapses, and aftereffects).

In short, this study showed that the enzymatic amplification of 16S rDNA combined with restriction analysis of the amplitrimer can be applied to the identification of strains of the genus *Nocardia*. The technique, as it is described here, is conceived to enable the rapid identification of isolates from pure cultures. The development of such a rapid, simple, and valid assay for detection of members of the genus *Nocardia* combined with the technique described by Steingrube et al. (25) for the *Nocardia* species identification will facilitate rapid diagnosis and prompt the initiation of the appropriate chemotherapy. Moreover, it will facilitate epidemiological studies of the human carriage, environmental contamination, and/or soil distribution of these bacteria.

**ACKNOWLEDGMENT**

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**REFERENCES**