Rapid identification of clinically relevant *Nocardia* species using real-time PCR with SYBR Green and melting-curve analysis

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The objective of this study was to develop and evaluate a rapid new method of identifying clinically relevant *Nocardia* species. DNA extracted from different *Nocardia* strains was used in a real-time PCR assay with SYBR Green and melting-curve analysis to identify *Nocardia* species. Ten control strains and four bacterial strains of closely related genera were employed, and samples from 28 patients were used. All *Nocardia* strains were identified correctly, and there was no cross-reaction with strains from genera closely related to *Nocardia*. The sensitivity and specificity of the method were 90 and 100 %, respectively. This method can be used to rapidly detect *Nocardia* species in culture, without cross-reaction with other closely related genera.

INTRODUCTION

*Nocardia* species are aerobic and saprophytic actinomycetes (Fig. 1), and pathogenic species have been found in dust, sand, soil and swimming pools (McNeil & Brown, 1994). *Nocardiae* cause a variety of human infections, including cutaneous, pulmonary and systemic nocardiosis (McNeil & Brown, 1994).

*Nocardiae* are found extensively worldwide and make up an important component of the normal soil microflora, often being associated with water. They may also be associated with decomposing plant material, dust and air (Brown & McNeil, 2003; Saubolle, 2002).

The majority of *Nocardia* infections in the USA are acquired through inhalation (Brown & McNeil, 2003; Saubolle, 2002). A smaller number of infections are caused by the traumatic introduction of organisms percutaneously. Nocardial infections are not thought to be transmitted from person to person and are not usually acquired nosocomially (Saubolle, 2002). However, there have been rare reports of clusters of patients infected with identical strains of *Nocardia* while occupying beds in close proximity to one another on hospital wards. In such cases, nosocomial acquisition is probable (Brown & McNeil, 2003; Saubolle, 2002).

Nocardiosis is usually an opportunistic infection, and most commonly presents as pulmonary disease. The majority of patients with clinically recognized disease have underlying debilitating factors (McNeil & Brown, 1994; Brown & McNeil, 2003; Saubolle, 2002).

The majority of primary cases present as pulmonary disease, although traumatically induced local abscesses occur as well. Dissemination from the lungs may be manifested as bacteraemia, empyema, brain abscess, pericarditis, synovitis, or soft-tissue infection. Peritonitis and corneal ulcers have been described (McNeil & Brown, 1994; Brown & McNeil, 2003; Saubolle, 2002). Typically, nocardiosis is characterized by an acute inflammatory response terminating in necrosis and abscess formation; granulomas are not normally formed (Brown & McNeil, 2003; Saubolle, 2002; Burgert, 1999).

The clinical diagnosis of nocardiosis is difficult. Signs, symptoms and radiologic studies may suggest the diagnosis but are not pathognomonic. Serologic diagnosis is unreliable, and serologic tests are not available commercially. The evaluation of appropriate specimens by smear and culture
remains the principal method of diagnosis. Detection in smears and isolation of \textit{Nocardia} on primary and/or selective media are not usually difficult.

Accurate identification of \textit{Nocardia} species has become increasingly important as differences among species have emerged in terms of epidemiology, virulence and antibiotic susceptibility (Lerner, 1996). Optimal therapeutic strategies depend on rapid and accurate identification of \textit{Nocardia} species, including members of the \textit{Nocardia asteroides} complex. Molecular methods for identification, such as PCR, restriction enzyme analysis, and sequencing, offer a time-saving alternative to conventional methods that involve the assessment of growth characteristics and colony and microscopic morphology, and biochemical and susceptibility testing.

In this study, we developed an alternative PCR-based identification strategy targeting the gene encoding 16S rRNA. Although the sequence for this gene is largely phylogenetically conserved, there can 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 (Hou et al., 1997; Mateicka et al., 1997; Urakawa et al., 1997).

**METHODS**

**Bacterial strains.** Ten reference strains of \textit{Nocardia} were used (Table 1). Five of these were culture reference strains and the other five were reference genomic DNA controls. The five culture strains were purchased from the Japan Collection of Microorganisms (RIKEN). The genomic DNA controls were provided by Dr Andreas Roth (TIB MOLBIOL).

To test primer specificity, four bacterial strains of closely related genera were added. The isolates included five strains of \textit{Mycobacterium}, three strains of \textit{Rhodococcus}, three strains of \textit{Streptomyces} and three strains of \textit{Tsukamurella} (Table 1). All isolates were identified by conventional methods, including biochemical tests, whole-cell composition, and enzymic profiles (Boiron et al., 1993).

A total of 28 patient samples from biopsies, abscesses and cultures were used to test this assay. All isolates were identified by conventional methods, including biochemical tests, whole-cell composition, and enzymic profiles. The \textit{Nocardia} reference strains were cultured according to manufacturers’ protocols. All were plated on sheep blood agar media plates for at least 48 h at 37 °C. Isolated pure colonies were used for DNA extraction.

**DNA isolation.** A High Pure PCR Template Preparation kit (Roche Diagnostics) was used according to manufacturer’s protocols. The isolated DNA was eluted in 200 μl elution buffer and then stored at −80 °C for further processing.

Real-time PCR was carried out using the LightCycler 1.2 instrument (Roche Diagnostics).

**Primer selection.** The sequences of the 16S rRNA genes from a large number of isolates belonging to the genera \textit{Dietzia}, \textit{Gordona}, \textit{Mycobacterium}, \textit{Nocardia}, \textit{Rhodococcus}, \textit{Streptomyces}, and \textit{Tsukamurella} were found in the DDBJ/EMBL/GenBank database. They had been analysed in an earlier study (Boiron et al., 1993) with the \textsc{pileup} program of the Genetics Computer Group software package. The existence of regions exhibiting possible sequence signatures at the genus level was investigated in the same study. The results allowed the design of two primers with 39 extremities specific for the \textit{Nocardia} genus: the forward primer NG1 (5′-ACCGACCACAAGGGG-3′) was complementary to positions 966–982 on the antisense strand, and the reverse primer NG2 (5′-GTTGTAACCTCTTCGA-3′) was complementary to positions 386–405 on the sense strand. These primers have been published elsewhere (Laurent et al., 1999). All primers were synthesized by TIB MOLBIOL.

**Real-time PCR.** The LightCycler is a real-time PCR machine that allows both rapid PCR cycling and continuous monitoring of product formation (Wittwer et al., 1997). The formation of double-stranded PCR products is detected by SYBR Green I. SYBR Green I is an intercalating dye that fluoresces strongly when bound to double-stranded DNA; thus, when PCR products are formed, an

<table>
<thead>
<tr>
<th>Genus, species and strain</th>
<th>Reference no. and source</th>
<th>Number of strains</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{Nocardia} brevicatena (S437)</td>
<td>DSM 43024\textsuperscript{T}</td>
<td>1</td>
</tr>
<tr>
<td>\textit{Nocardia} farcinica (S644)</td>
<td>DSM 43131</td>
<td>1</td>
</tr>
<tr>
<td>\textit{Nocardia} asteroides (S68)</td>
<td>DSM 43757\textsuperscript{T}</td>
<td>1</td>
</tr>
<tr>
<td>\textit{Nocardia} carnea (S417)</td>
<td>DSM 43397\textsuperscript{T}</td>
<td>1</td>
</tr>
<tr>
<td>\textit{Nocardia} transvalensis (S694)</td>
<td>DSM 43405\textsuperscript{T}</td>
<td>1</td>
</tr>
<tr>
<td>\textit{Nocardia} farcinica (3088)</td>
<td>ATCC 3318</td>
<td>1</td>
</tr>
<tr>
<td>\textit{Nocardia} brasiliensis (3374)</td>
<td>ATCC 19296</td>
<td>1</td>
</tr>
<tr>
<td>\textit{Nocardia} otitidiscaurium (3377)</td>
<td>ATCC 14629</td>
<td>1</td>
</tr>
<tr>
<td>\textit{Nocardia} asteroides (33484)</td>
<td>ATCC 19247</td>
<td>1</td>
</tr>
<tr>
<td>\textit{Nocardia} nova (6044)</td>
<td>ATCC 33729</td>
<td>1</td>
</tr>
<tr>
<td>\textit{Nocardia} spp.</td>
<td>Patient sample (abscess, biopsies and sputum)</td>
<td>28</td>
</tr>
<tr>
<td>\textit{M. tuberculosis}</td>
<td>Patient sample</td>
<td>5</td>
</tr>
<tr>
<td>\textit{Streptomyces} sp.</td>
<td>Patient sample</td>
<td>3</td>
</tr>
<tr>
<td>\textit{Tsukamurella} sp.</td>
<td>Patient sample</td>
<td>3</td>
</tr>
<tr>
<td>\textit{Rhodococcus} spp.</td>
<td>Patient sample</td>
<td>3</td>
</tr>
</tbody>
</table>
increase in fluorescence is observed (Higuchi et al., 1993; Ririe et al., 1997). After PCR amplification, the LightCycler can monitor melting of the DNA with increasing temperature by measuring the decrease in fluorescence as SYBR Green I is released. For convenience, the negative derivative of fluorescence versus temperature is plotted to give a discrete melting peak. When the melting temperature of the PCR products is analysed in this way, it is not necessary to visualize the PCR products on an agarose gel.

The real-time PCR reactions were carried out in glass capillaries in volumes of 20 µl containing 2 µl template DNA, 0.8 µl MgCl₂ (4 mM), 2 µl forward and reverse primers (1 µM each), and 2 µl LightCycler FastStart DNA master SYBR Green (Roche Diagnostics). PCR amplification comprised an initial denaturation cycle at 95°C for 10 min, followed by 40 amplification cycles (with a temperature transition rate of 20°C s⁻¹) consisting of 95°C for 10 s, annealing at 55°C for 10 s, and extension at 72°C for 60 s. After amplification, a melting step was performed, consisting of 95°C for 0 s, cooling to 65°C for 60 s (with a temperature transition rate of 20°C s⁻¹), and finally a slow rise in the temperature to 95°C at a rate of 0·1°C s⁻¹ with continuous acquisition of fluorescence decline. Mg²⁺ titration experiments were done with patient samples and positive controls. The real-time PCR and the melting-curve analysis were performed as described above, except that the MgCl₂ concentration used during the assay was varied from 1 to 8 mM.

**Limits of detection.** After DNA extraction, a serial 10-fold dilution of Nocardia DNA was made, with bacterial concentrations ranging from 300 to 3 × 10⁸ bacteria per 2 µl. This series of 10-fold dilutions of Nocardia DNA was included in each amplification run to measure the detection limit of the method.

**RESULTS**

Typical amplification and melting curves depicting Tm detection for the various Nocardia species are shown in Figs 2 and 3. Control Nocardia species samples obtained from both cultures and genomic DNA were detected and correctly identified in the melting-curve analysis. All samples were detected after the 15 cycles. The mean melting-curve peak Tm was 89 ± 1°C. No amplification was identified in the negative sample (H₂O) or in the Mycobacterium tuberculosis sample. The NG1 and NG2 primers, which are specific for nocardial 16S rDNA, resulted in no product from either the negative control or the M. tuberculosis sample. The other bacterial strains of closely related genera (Streptomyces sp., Rhodococcus spp. and Tsukamurella spp.) were tested also, and were not detected by this assay (data not shown).

Concerning amplification of Nocardia DNA obtained from a pure culture, linearity was achieved over a six-log range of input DNA equivalent to 3 × 10⁸ to 300 bacteria. This meant that the detection limit could be as few as 300 bacteria per 2 µl.

Among the 28 patient samples, 20 were shown to be Nocardia spp. by conventional methods, including biochemical tests, whole-cell composition, and enzymic profiles. Eighteen samples were shown to be Nocardia spp. positive by our real-time PCR.

The sensitivity and negative predictive value of our PCR assay were good: 90 and 80%, respectively. The specificity and predictive positive value of the PCR were also excellent, both being 100% (Table 2).

There were only two samples which were positive by culture and negative by the real-time PCR. No sequence was determined for the 16S rDNA from these two samples, due to the lack of a sequencer in our department.
DISCUSSION

A definitive diagnosis of nocardiosis usually depends on the microbiological isolation of \textit{Nocardia} from clinical samples, such as pleural effusions or pus discharge. \textit{Nocardia} are not human commensals, and isolation from clinical samples should be regarded as evidence of active infection. The growth of the micro-organism is very slow, and it may be overlooked because of overgrowth of other rapidly growing aerobic bacteria in mixed flora. Prolonged treatment with various antibiotics may make it difficult to culture the micro-organism. When isolation of the micro-organism is not feasible, alternative means to identify pathogenic \textit{Nocardia} are required. PCR analysis of 16S rDNA is a useful tool in identifying \textit{Nocardia} spp.

In the current work, we used real-time PCR; this is relatively rapid, because amplification is carried out in capillary glass tubes, which are used only in the Lightcycler instrument. In addition, we were able to monitor PCR products in real-time and perform a simultaneous melting-curve and detection analysis. Real-time chemistries allow for the detection of PCR amplification during the early phases of the reaction. Measuring the kinetics of the reaction in the early phases of PCR provides a distinct advantage over traditional PCR detection (Wittwer \textit{et al}., 1997).

Our inability to identify two samples by the real-time PCR technology might have been due to PCR inhibition. We were unable to investigate this, because we do not have a sequencer in our department.

Identification of \textit{Nocardia} spp. by 16S rDNA analysis is useful for determining the most appropriate and effective treatment. It usually takes about 2 weeks for microbiological isolation and antibiotic susceptibility tests in cases of nocardiosis, but real-time PCR analysis of 16S rDNA can be completed in a few hours.

In the future, the real-time PCR method that we have developed could be adapted and developed to be performed directly with clinical samples, such as skin biopsy, blood, sputum or bronchoalveolar lavage specimens. The direct diagnosis of nocardiosis by real-time PCR of cerebrospinal fluid could replace the use of cerebral biopsy, which requires the use of an invasive technique. It would improve the speed of diagnosis, which is closely related to a favourable outcome for patients.

In summary, this study showed that real-time PCR amplification of 16S rDNA combined with melting-curve
analysis can be applied to the identification of strains of the genus *Nocardia*. Both the sensitivity and specificity of this method are excellent. The technique, as described here, was 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* 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.

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**REFERENCES**


