A High Proportion of Novel Mycobacteria Species Identified by 16S rDNA Analysis Among Slowly Growing AccuProbe-Negative Strains in a Clinical Setting

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Key Words: Mycobacterium; Novel; Clinical; 16S rDNA

Abstract

Sequencing of the 16S ribosomal DNA (rDNA) for identification of nontuberculous mycobacteria (NTM) has contributed to the establishment of more than 35 new species during the last decade. Increasingly, NTM are accepted as potential or proven pathogens. We identified, by 16S rDNA sequence analysis, slowly growing NTM isolates negative by AccuProbe (Gen-Probe, San Diego, CA) that previously were identified by using conventional biochemical techniques, to determine the accuracy of reporting AccuProbe-negative NTM prior to sequence-based identification. Of 82 strains, 30 were deemed novel. An attempt was made to determine the clinical importance of previously misidentified novel species. Clinical cases are described for a number of strains previously identified as Mycobacterium terrae complex, Mycobacterium scrofulaceum, and Mycobacterium avium complex. As sequence-based identification methods become more commonplace in clinical microbiology laboratories, there is a need to understand the significance of previously undescribed species, which often mimic and subsequently are identified as well-established species.

Many nontuberculous mycobacteria (NTM) are ubiquitous in the environment and may colonize and occasionally cause infection in humans. Mycobacterium avium complex, Mycobacterium kansasii, and members of the Mycobacterium fortuitum complex are causative agents for most NTM infections and currently have a well-understood environmental epidemiology.1 Other NTM species rarely cause infection, despite their prevalence in the environment. For example, few cases of Mycobacterium gordonae infections have been reported, and whether some isolates were identified accurately remains uncertain.2 NTM infections have various clinical manifestations that occur largely in immunocompromised patients1,3: catheter-related infections, disseminated disease, lymphadenitis, and pulmonary disease. NTM species also may cause substantial pulmonary and extrapulmonary disease, including cutaneous and deep soft tissue infections, in immunocompetent people.4 While less common NTM species are being accepted increasingly as credible pathogens, there remains a large gap in the full understanding of their pathogenic potential. As these species have different susceptibility patterns, correct and early diagnosis may prove to be of utmost importance in helping to decrease the morbidity associated with NTM infections.

Conventional phenotypic identification of NTM species is based on a panel of biochemical tests, pigmentation, and growth characteristics.5,6 Owing to the inherent drawbacks of conventional testing, including slow turnaround time and limited accuracy, most mycobacteriology laboratories have adopted alternative methods for species identification of clinical strains: DNA probes (AccuProbe, Gen-Probe, San Diego, CA), high-performance liquid chromatography,7 polymerase chain reaction (PCR)-restriction enzyme analysis of the hsp65 gene,8 or sequencing of the 16S ribosomal DNA (rDNA).9
The use of the 16S ribosomal RNA (rRNA) gene became a “gold standard” for identification of bacteria during the late 1980s. Shortly thereafter, the 16S rDNA sequence was determined for established species of mycobacteria. This also revealed the existence of previously undescribed species, with Mycobacterium cookii, Mycobacterium confluens, and Mycobacterium genavense being the first species established, based in part on unique 16S rRNA gene sequences. Since then, sequencing of the 16S rRNA gene has been an effective tool contributing to the establishment of more than 35 new species and subspecies. The existence of this large number of NTM species suggests that with a limited panel of biochemical tests, genetically distinct NTM species might have similar or identical biochemical profiles. As more laboratories choose to use sequence-based methods that are more rapid, cost-effective, and easier to master than previously considered, they will find that many more species exist than are currently established.

During the past 20 years, NTM from the Provincial Mycobacteriology Laboratory, Winnipeg, Canada, have been identified by using conventional phenotypic methods. We hypothesized that a proportion of these isolates were not identified accurately, and we anticipated the detection of novel species. This study was designed to provide clinical details describing NTM infection caused by these new species, to add to the current literature.

Materials and Methods

A total of 4,400 NTM have been isolated at the Provincial Mycobacteriology Laboratory since 1981. These were identified mostly by using commercial DNA probes detecting the 3 most common NTM species groups (M. avium complex, M. kansasii, and M. gordonae), followed by an algorithm of biochemical tests and growth characteristics in probe-negative isolates. NTM isolates of known or potential clinical significance were stocked and listed by species name and isolation site. Of those, all probe-negative, slow-growing isolates, resulting in a total of 82 strains from January 1981 to December 1999, were selected for study.

From pure subcultures, DNA lysates were prepared as described previously. The 16S rRNA gene was amplified by PCR. Sequencing of the PCR product was performed with 1 forward reaction using primer pA to permit analysis of the first 500 base pairs, permitting the identification of most mycobacterial species. Sequences were deemed novel when 4 or more variations were observed in the 500-base-pair region from a type strain. Sequencing was performed using the ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA) and analyzed using SeqMan, version 5.03 (DNAStar, Madison, WI). Sequences were compared against 3 sequence databases: in-house and RIDOM (both quality-controlled mycobacterial 16S rDNA sequence databases consisting of type strains, other reference strains, and clinical specimens) and GenBank.

Determination of clinical syndrome or disease associated with each organism was achieved through analysis of the clinical data for the patient from whom the NTM organism was isolated. The American Thoracic Society has established criteria for diagnosing NTM pulmonary disease: (1) 2 positive cultures and 1 positive smear from sputum or bronchial washing cultures within 12 months; or (2) 3 positive sputum or bronchial washing cultures; or (3) 1 bronchial washing culture with a 2+ or more acid-fast bacillus smear or 2+ or more growth on solid media; or (4) isolation of NTM from a transbronchial or lung biopsy specimen or a biopsy specimen showing histopathologic features of mycobacteria with a minimum of 1 sputum or bronchial washing specimen positive for an NTM.

Results of sequence-based identification by 16S rDNA of the 82 isolates were categorized into 3 groups: (1) 29 strains (35%) were identified correctly as an established species by growth rates, pigmentation, and biochemical tests and with a 100% sequence identity by 16S rDNA; (2) 23 strains (28%) were identified incorrectly by biochemical tests, but the resulting sequence corresponded with a 100% sequence identity to a known species; and (3) 30 strains (37%) that did not correspond with a 100% sequence identity to an established species and, therefore, were identified as novel (Table 1). Strains correctly identified by conventional and sequence-based methods included Mycobacterium marinum (n = 10), Mycobacterium xenopi (n = 8), Mycobacterium simiae (n = 4), Mycobacterium gastri and Mycobacterium terrae (n = 2 each), and Mycobacterium malmoense, Mycobacterium flavescens, and M. genavense (n = 1 each).
Of the 30 unique strains, 20 isolates showed an identical match with sequences deposited in GenBank and briefly described previously, yet not fully characterized or named as a species. These were 5 isolates corresponding to MCRO 33 (GenBank accession No. AF152559), 7 isolates corresponding to MCRO 6 (X93032), and 8 isolates corresponding to MCRO 16 (X93027). Finally, 10 other isolates did not match any established species, did not correspond to the sequence of a noncharacterized species listed on a public database, or, to our knowledge, were not previously published.

Of these unique isolates, the clinical records were not available for 24 of them. These consisted of 14 strains isolated from sputum, 2 from lung biopsies, and 1 each from a uterine cervix mass, a brain biopsy, pleural fluid, a cervical lymph node, urine, peritoneal fluid, a mouth ulcer, and a bronchial washing. The medical records available for the remaining 6 cases are described in the following sections.

**Case 1658**

A 2-year-old girl was examined in early February 1994 because of a closed neck abscess in the right parotid area after...
### Table 2
Isolates Previously Misidentified by Conventional Testing (Group 2; n = 23)*

<table>
<thead>
<tr>
<th>Conventional Identification</th>
<th>16S rDNA Identification</th>
<th>Source (Year Isolated)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mycobacterium szulgai</td>
<td>Mycobacterium xenopi</td>
<td>NS (1983)</td>
</tr>
<tr>
<td>Mycobacterium terrae complex</td>
<td>Mycobacterium engbaekii*</td>
<td>NS (1983)</td>
</tr>
<tr>
<td>Mycobacterium scrofulaceum</td>
<td>Mycobacterium gordonae subspecies II*</td>
<td>NS (1983)</td>
</tr>
<tr>
<td>M. scrofulaceum</td>
<td>Mycobacterium lentiflavum</td>
<td>NS (1987)</td>
</tr>
<tr>
<td>M. szulgai</td>
<td>Mycobacterium kansasii subspecies II*</td>
<td>NS (1989)</td>
</tr>
<tr>
<td>Mycobacterium flavescens</td>
<td>M. gordonae</td>
<td>NS (1989)</td>
</tr>
<tr>
<td>M. terrae complex</td>
<td>Mycobacterium shimoidei</td>
<td>NS (1989)</td>
</tr>
<tr>
<td>M. terrae complex (n = 2)</td>
<td>Mycobacterium fortuitum</td>
<td>NS (1989, in 2 specimens)</td>
</tr>
<tr>
<td>Mycobacterium simiae (n = 2)</td>
<td>Mycobacterium abscessus/Mycobacterium chelonae</td>
<td>Urine (1991); sputum (1991)</td>
</tr>
<tr>
<td>MAIS intermediate (n = 3)</td>
<td>M. lentiflavum</td>
<td>Skin tissue (1991, in 2 specimens); urine (1994)</td>
</tr>
<tr>
<td>M. xenopi</td>
<td>Mycobacterium gordonii</td>
<td>Sputum (1991)</td>
</tr>
<tr>
<td>M. simiae</td>
<td>M. marinum</td>
<td>Cytology brush (1991)</td>
</tr>
<tr>
<td>M. scrofulaceum</td>
<td>Mycobacterium interjectum subspecies II*</td>
<td>Sputum (1992)</td>
</tr>
<tr>
<td>Mycobacterium marinum</td>
<td>M. kansasii subspecies III or VII*</td>
<td>NS (1992)</td>
</tr>
<tr>
<td>M. szulgai</td>
<td>M. kansasii subspecies Vc*22</td>
<td>Bronchial washing (1993)</td>
</tr>
<tr>
<td>MAIS intermediate</td>
<td>M. simiae</td>
<td>Sputum (1994)</td>
</tr>
<tr>
<td>M. xenopi</td>
<td>Mycobacterium heckeshornense</td>
<td>Sputum (1995)</td>
</tr>
<tr>
<td>Mycobacterium celatum</td>
<td>Mycobacterium branderii</td>
<td>Sputum (1997)</td>
</tr>
<tr>
<td>M. flavescens</td>
<td>Mycobacterium chromogen†</td>
<td>Sputum (1999)</td>
</tr>
</tbody>
</table>

MAIS, Mycobacterium avium-intracellulare-scrofulaceum; NS, not stated; rDNA, ribosomal DNA.

*The 16S rDNA sequence corresponded to 100% sequence identity with the type strain of an established species or to a subspecies previously described.

† Nonvalidated species name.

striking her cheek against a corner of a table. Following the accident, the swelling increased and the skin in the area became blue. Results of ultrasound imaging of the area were consistent with a hematoma, and, thus, the mass was observed. On March 2, a fever developed, and the patient was treated with amoxicillin; 2 days later, she had increasing tenderness at the site. At this time, the mass was 4 × 3 cm, cystic and not lobulated, and located in the right parotid tail overlying the ramus and mandible anterior to the tragus. On March 9, aspiration of the abscess yielded 4 mL of yellow pus; the smear was positive for acid-fast bacilli and was identified by the Provincial Mycobacteriology Laboratory on April 18 as M. avium complex (with unusual biochemical characteristics). On August 30, the surgical site drained a few drops of a yellow-green discharge. On September 13, the swelling had subsided and no further drainage had been noted so it was decided that further treatment was not indicated. The present study identified the organism as closest to M. avium/Mycobacterium silvaticum/Mycobacterium paratuberculosis, with a 99.1% identity by partial 16S rDNA analysis.

**Case 98**

A 41-year-old woman with a history of tuberculosis infection sought care on March 26, 1982, because of a 1-month history of a left-sided cervical mass located in the lower posterior triangle. The initial mass was estimated to be 1 cm in diameter and was associated with a sore throat lasting about 2 weeks and production of approximately a tablespoon of green sputum for approximately 1 month. The patient also had a fever for 3 days and lost 4 lb (1.8 kg) during that month. Meanwhile, the mass gradually enlarged and became tender, and the skin became red.

On March 26, the mass was 4 × 3 cm, tender, tense, firm, mobile, warm, and located in the left lower posterior triangle of the neck. The physical examination revealed 2 × 3-cm central lymph nodes in each axilla that were nontender and mobile, with old scars in the right middle cervical area. The medical history included the initial diagnosis of pulmonary tuberculosis in 1956, with discontinued medications owing to adverse effects, followed by relapses in 1966 and 1972. About 2 mL of pus was aspirated from the mass, and a few acid-fast bacilli were revealed by microscopy; the culture was positive 10 weeks later. Findings of a chest radiograph, showing calcification in the left upper lobe and a raised right hilum with scarring in the right upper lobe, had not changed since 1979.

In August 1983, small tissue calcifications in the neck and submandibular region, presumed to be calcified lymph nodes, were seen on the radiographs, signaling a change in findings from those of a cervical radiograph performed in 1973. The patient was given a diagnosis of tuberculous adenitis and treated with isoniazid, rifampin, and pyrazinamide. In August 1982, culture reports identified the organism as M. terrae complex that was resistant to all drugs except capreomycin, cycloserine, and ethambutol. Because the mass was not caused by a Mycobacterium tuberculosis infection, the antituberculous medications were discontinued and the lesion was observed. The present study identified the organism as MCRO 16, for which the closest known relative is M. terrae with a 98.2% sequence identity by partial 16S rDNA analysis.
Case 69
A 71-year-old man, a 75-pack-per-year smoker and a retired farmer, was examined in April 1981 because of hemoptysis and a lung lesion. The patient described the production of brown sputum in the morning with blood specks for 1 month and night sweats for the previous 2 years. Aspiration biopsy showed poorly differentiated large cell carcinoma, and a chest radiograph revealed multiple lesions, with the largest lesion in the right lower lobe of the lung. Destruction of the 9th and 10th ribs posteriorly also was noted. The diagnosis of bronchogenic carcinoma was made. A sputum sample was obtained, and the laboratory isolated an organism identified as *M. terrae* complex. This study also identified the organism as MCRO 16.

Case 81
This case also involved MCRO 16 and was from a 29-year-old woman with asthma who was in acute respiratory distress. The organism was isolated from a sputum culture. The isolate was likely a contaminant or a colonizer, and the acute illness causing the respiratory distress was probably due to allergic aspergillosis.

Case 1852
This case involved an 18-month-old girl who was examined because of a 4-cm mass in the left submandibular area. The mass was excised and was shown to have granulomatous inflammation with caseation. Microscopy revealed acid-fast bacilli. The isolate from this case of lymphadenitis was identified as *M. avium* complex by the laboratory and was deemed unique by sequencing of the 16S rRNA gene, having a 99.1% identity with both *Mycobacterium intracellulare* and *M. silvaticum/M. paratuberculosis/M. avium* complex. The isolate was likely a contaminant or a colonizer, and the acute illness causing the respiratory distress was probably due to allergic aspergillosis.

Case 273
This case involved a 77-year-old man with a constant cough, occasional mucoid sputum associated with chest discomfort, and exertional dyspnea. An investigation led to the diagnosis of squamous cell carcinoma in the right main bronchus. Chest radiographs showed calcified granulomas in the upper lobes. The laboratory identified *Mycobacterium scrofulaceum* from the sputum culture using biochemical techniques, but in the present study using molecular techniques, the isolate was determined to be MCRO 33, for which the closest established relative is *M. simiae* with a 98.4% sequence identity. From analysis of the medical history, it could not be determined whether the disease was caused by the MCRO 33 or was related to the underlying lung cancer.

**Discussion**
Phenotypic characterization, including pigmentation, growth rate, and biochemical test algorithms, has been used in the identification of *Mycobacterium* species since the earliest isolation of NTM in clinical specimens. However, in keeping with previous findings,9,24-27 the present study reveals the advantages of molecular techniques over conventional testing for identifying mycobacterial species and detecting novel species. It generally can be assumed that a new species has been detected if base pair discrepancies are found in the variable regions of the 16S rRNA gene and the organism has distinct phenotypic properties.28

Of the 52 isolates identified as a known species by sequencing of the 16S rRNA gene (groups 1 and 2), the Provincial Mycobacteriology Laboratory, using the best available methods available at the time, accurately identified only 29 specimens to the species level. Biochemical testing can be misleading for various reasons, including difficulty in test reproducibility, interspecies phenotypic variability, and the algorithms of phenotypic characteristics that are limited to only common species.5,16 Thus, when faced with unusual biochemical test results, it remains uncertain whether the results are due to intraspecies variation or whether the organism is a new species. Most often, there is a bias toward identifying an isolate as the closest matching established species. Alternatively, owing to the limited number of biochemical tests routinely performed, a genotypically unique species may show a profile similar to an established species, thereby resulting in inaccurate identification.

Of the 23 misidentified strains, some had a sequence described in previously published articles as subspecies of *M. gordonae*,21 *Mycobacterium interjectum*,23 or *M. kansasi*22 and may still be considered as such. However, it also is possible that these isolates showed phenotypic characteristics distinct from the type strain of the species, which may have misled identification. It also is possible that, in the future, these might be described as new species.

The unique NTM isolates (group 3) consisted of single isolates from various sample sites from both sterile and respiratory sites. Six patient charts were reviewed fully, and the laboratory requisitions for the 24 remaining cases were analyzed. Of these 24 cases, it was deemed likely that several cases were clinically significant for NTM disease, with isolates obtained from a uterine cervix mass, brain biopsy, lung biopsies, pleural fluid, cervical lymph node, and peritoneal fluid. The pathogenic role in the 17 cases from respiratory specimens or urine was difficult to determine. However, in cases such as case 970, multiple isolations of what is believed to be the same organism might suggest disease.

We identified 3 main species making up 20 of the 30 unique isolates. These were MCRO 6, MCRO 16, and MCRO 33. Both MCRO 6 and MCRO 16 were identified originally in 14 of 15 cases as *M. terrae* complex, consisting of *M. terrae*, *Mycobacterium nonchromogenicum*, and *Mycobacterium triviale*.29-31 while 1 MCRO 16 isolate was...
identified initially as *M. avium* complex in 1982, before the use of commercial probes. The MCRO 6 and 16 strains are described as unique isolates related to *M. terrae* and *M. nonchromogenicum*. Differentiation to the species level may be considered insignificant because these organisms are considered nonpathogenic; however, cases have been described in which these organisms have caused debilitating disease, usually tenosynovitis of the hand, that is relatively resistant to antibiotic therapy. Smith et al. reviewed 54 reports from the literature describing *M. terrae* complex infections and found that 62% of patients were immunocompetent with no underlying medical conditions. 59% of the cases involved the upper extremities (arm, wrist, hand, fingers), 26% involved the lungs, 7% involved the lower extremities (hip, knee, ankle), and 6% involved the gastrointestinal or genitourinary tract. In the majority of cases, the diagnosis of *M. terrae* complex infection took longer than 6 months to make, with the initial diagnosis often believed to be a noninfectious inflammatory condition.

Of the 3 MCRO species, it seems from the analysis of the specimen isolation site that MCRO 6 is the most clinically significant, with 4 of 7 isolates obtained from sterile sites (brain, 2 lung biopsies, and peritoneal fluid). Of the MCRO 16 strains, we were able to analyze 3 clinical cases. Case 98 shows that this species is capable of causing disease (lymphadenitis) based on analysis of the clinical history and isolation of the organism from a sterile site. Two other MCRO 16 cases (69 and 81) are believed to be examples of colonization or a contaminant. These cases did not meet the American Thoracic Society criteria, and the symptoms more likely were due to the underlying illnesses. In addition, 2 cases of MCRO 16 infection that were not reviewed were isolated from sterile sites (pleural fluid and another cervical lymph node), which are likely to be significant for disease. Owing to the lack of differentiation in the *M. terrae* complex when identifying these organisms, it might be possible that a few members are more pathogenic than others, such as *M. nonchromogenicum*, which has been reported to cause disease, and possibly MCRO 6 and MCRO 16. Future work and case reports determining which members of the *M. terrae* complex are the most pathogenic are warranted.

The third major species isolated in this study, MCRO 33, has been identified by the Provincial Mycobacteriology Laboratory on every occasion as *M. scrofulaceum*, an organism that is associated with cervical lymphadenitis in children and that has been known to cause ulcerative and nodular skin lesions, disseminated disease, and pulmonary infection. We observed that the biochemical profile of these isolates was identical to that known for *M. scrofulaceum*. However, the 16S rDNA sequences of both species are different (95% identity). Springer et al. described MCRO 33 as a slow-growing species related to *M. simiae*, with a 98.4% identity. Of 5 MCRO 33 isolates, 1 was from a cervix biopsy and 4 were isolated from sputum. In 1 patient, the organism was isolated from 2 sputum samples. Case 273 underscores the difficulty in diagnosing NTM disease, particularly when only 1 sputum sample is obtained. When reviewing medical records from the early 1980s, such as in this case, it may be anticipated that repeated cultures might not have been done as follow-up to a positive NTM culture result, because the role of NTM in causing disease may not have been documented as it is today.

Two other examples of novel NTM species causing disease are illustrated by cases 1658 and 1852. Both of these species, initially identified by the Provincial Mycobacteriology Laboratory as *M. avium* complex, were found to have 16S rRNA sequences not belonging to an established species or a sequence on a public database. Case 1658, an abscess in the parotid area, and case 1852, lymphadenitis in the left submandibular area, were deemed to be cases of clinical disease due to the organism isolated from a sterile site. These species sequences have been deposited in GenBank with the following accession numbers: AY184225 and AY184226. Future encounters with these organisms will provide clinicians with examples of disease caused by these organisms. Eight other isolates (GenBank accession numbers AY306200-AY306207), each with unique 16S rRNA sequences, were found in the present study. Five of these isolates were identified originally as *M. terrae* complex, 1 as *M. simiae*, 1 as *M. kansasi*, and 1 as *M. gordonae*. Five of these isolates were isolated from sputum, urine, a mouth ulcer, and a bronchial wash. Without the ability to review the medical records for any of these cases, determination of clinical significance cannot be made.

We have characterized 30 strains encompassing 13 novel NTM. We have provided a valuable clinical history for 6 of these isolates, for which the cases have not been described, permitting a reference point for clinicians faced with these NTM. It is our intention to complete their characterization to include susceptibility testing in addition to molecular and phenotypic data for their establishment as a new species, for ease of recognition by other laboratories, and to encourage other mycobacteriologists to do so as well. Along with other case studies, we hope that this information will permit a better appreciation of the clinical disease or colonization caused by NTM organisms. Future reports on NTM disease will provide further insight into the treatment of the diseases caused by these organisms and help decrease the morbidity associated with NTM.
References


