Original Article

Species Identification of Neglected Nontuberculous Mycobacteria in a Developing Country

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SUMMARY: In developing countries where tuberculosis is still a health challenge, the prevalence of nontuberculous mycobacterial diseases is expected to rise as medical conditions that compromise the immune system become more widespread. In the current study, we aimed to determine the presence and diversity of nontuberculous mycobacteria (NTM) causing infections in Iranian patients. Sixty-seven clinical NTM isolates were identified using conventional and molecular methods, including PCR-restriction fragment length polymorphism analysis (PRA) and 16S rRNA sequencing. Out of 67 patients with confirmed mycobacterial infection, 29 had an associated immunosuppressive syndrome, including 9 who were HIV-infected. Forty-nine NTM isolates were identified using PRA, and the remaining 18 isolates were identified using 16S rRNA sequencing. We obtained the following results:

- *Mycobacterium fortuitum*, 30 isolates;
- *M. kansasii*, 12 isolates;
- *M. gordonae*, 8 isolates;
- *M. porcinum*, 3 isolates;
- *M. conceptionense*, 3 isolates;
- *M. phlei*, 2 isolates; and
- *M. austroafricanum*, *M. intracellulare*, *M. lentiflavum*, *M. monacense*, *M. parascrofulaceum*, and *M. thermoresistibile*, 1 isolate each; and 1 potentially novel mycobacterial species. With regard to the complexity of identification, it is recommended that laboratory diagnosis of NTM diseases be centralized by strengthening or setting up quality national and regional infrastructure.

INTRODUCTION

Nontuberculous mycobacteria (NTM), also known as atypical mycobacteria and mycobacteria other than tuberculosis, are environmental organisms that are normally found in soil and water. They have been recognized since late in the 19th century, when avian tuberculosis was first described in 1868 (1). However, they were not recognized as a cause of human disease until the 1950s (2). At that time, NTMs were rare, and were found almost exclusively in patients with underlying diseases. However, NTM disease patterns and epidemiology have changed since the 1980s and gradually emerged in previously unrecognized populations (3,4). Given that the clinical presentation of NTM is often difficult to differentiate from that of *Mycobacterium tuberculosis* complex (MTBC), it is important to accurately identify NTM so that the correct epidemiological controls and specific treatments can be implemented (5).

To date, the genus *Mycobacterium* comprises over 150 species. Though *M. tuberculosis* is one of the most common, several other species are being increasingly recognized as significant human pathogens (http://www.bacterio.cict.fr/).

In countries like Iran, where tuberculosis is still a major health challenge that causes the suffering and death of many people each year, the prevalence of diseases caused by NTM is expected to rise as both the AIDS epidemic and medical conditions that compromise the immune system become more widespread. Unavoidable health consequences of ongoing and unending natural and human disasters, including social unrest, ethnic conflict, sectarian bloodshed, war, earthquake, and floods in the turbulent region of the Middle East, seem to be considerably worsening the situation.

In the current study, we aimed to assess the extent of NTM infection and the diversity of microorganisms found in an Iranian population using a combinatorial approach comprising conventional and molecular techniques.

MATERIALS AND METHODS

Isolates: From 2003 to 2009, a total of 67 clinical NTM isolates were collected by our molecular microbiology laboratory at the Infectious Diseases and Tropical Medicine Research Center (IDRC), Isfahan, Iran. The sources and clinical details of the isolates are summarized (see Table 1). They were isolated in or referred to our laboratory. The majority of cases (i.e., 53 strains, including M01, M02, M04 to M25, M27 to M34, M40 to M51, M61, M66, M70, M71, M79, M120, M137, M138, and M143) were received from peripheral laboratories in the region or from other laboratories across the country for the identification and characterization of the bacteria. These isolates had been grown in pure culture on Löwenstein-Jensen (LJ) slants, and were verified to be
acid-fast bacteria (AFB) by Ziehl-Neelsen (ZN) staining. All isolates displayed growth characteristics and colony morphology consistent with those of Mycobacterium spp. The remaining isolates (i.e., M201, M202, M206, M210, M213, M214, M216, M219, M220 to M223, M226, and M228) were recovered from clinical specimens of the patients referred to our laboratory by their practicing physicians. Species characterization of these isolates was then analyzed using a battery of conventional and molecular tests as described below.

**Conventional identification:** On specimens collected from nonsterile body sites, digestion and decontamination procedures were performed by Petroff’s method in order to minimize the interference of contaminating bacteria in the recovery of mycobacteria (6). However, sterile bodily fluids such as blood, soft tissues, and lymph node biopsies were processed without decontamination. Examination and reporting of smears stained by auramino O dye and ZN stain were performed within 24 h of specimen receipt. LJ slants were loosely capped and incubated at 37°C in an atmosphere of 5% CO₂. All culture tubes were examined daily for 30 days and twice weekly for 4 weeks thereafter.

Slants containing pure cultures of AFB were subjected to a series of conventional phenotypic tests and standard biochemical assays, including assessments of growth rate, niacin accumulation, pigment production, growth in LJ medium (at 37°C, 45°C, and 52°C), growth on MacConkey agar without crystal violet, semi-quantitative and heat-stable (68°C) catalase production, nitrate reduction, arylsulfatase activity, tolerance to 5% NaCl, iron uptake, urease activity, and tellurite reduction, according to standard procedures (6).

**Molecular identification:** Strains that were initially identified as mycobacteria and were classified into Runyon groups (7) on the basis of phenotypic features (see Table 1), were subjected to more definitive identification by molecular approaches using the standard procedures described below.

**DNA extraction:** Chromosomal DNA was extracted using the method described by Pitcher et al., with slight modifications to improve the susceptibility of cells to the standard digestion (8). Briefly, after thermal inactivation, pretreatment of biomass with lipase (Type VII; Sigma, Poole, UK) and additional treatment with proteinase K (100 pg/ml) and sodium dodecyl sulfate (final concentration, 0.5% NaCl, iron uptake, urease activity, and tellurite reduction, according to standard procedures (6).

**RESULTS**

Of the 67 patients with confirmed mycobacterial infections, 38 (56%) were female, 17 (25%) were between the ages of 50 and 60 years, 14 (21%) were between the ages of 30 and 40 years, 12 (18%) were between the ages of 40 and 50 years, 12 (18%) were over the age of 70 years, 10 (15%) were between the ages of 60 and 70 years, and 2 (3%) were between the ages of 20 and 30 years. Twenty-nine patients were found to have a history of various immunosuppressive syndromes, including cystic fibrosis (5 cases), previously treated tuberculosis (1 case), malignancy (3 cases), chronic obstructive pulmonary disease (6 cases), diabetes mellitus (1 case), bronchitis (3 cases), chronic pelvic pain (1 case), or HIV infection (9 cases), while the remainder of the patients had no apparent history of such disorders (Table 1).

**Conventional identification:** On the basis of growth characteristics and pigmentation (Table 1), the isolates studied were categorized into the four Runyon groups (7): 43 clinical isolates were categorized as rapidly growing (Runyon IV), and 24 isolates were categorized slowly growing mycobacteria, with 10 classified as scotochromogenic (Runyon II), 12 classified as photochromogenic (Runyon I), and 2 classified as nonchromogenic (Runyon III).

On the basis of the biochemical properties, identification was made for all 67 isolates with variable levels of confidence. The M. fortuitum-like group was the most frequently encountered (36 isolates), followed by M. kansasi-like strains (12 isolates), and M. gordonae-like strains (8 isolates). The remaining strains were mostly rare mycobacteria found in only 1 to 3 isolates.

**Molecular identification:** Genus-specific PCR amplification characteristically yielded a 228-bp fragment of the hsp65, confirming that all strains belonged to the genus Mycobacterium.

Using PRA, we were able to definitively identify 49
<table>
<thead>
<tr>
<th>No.</th>
<th>Designation</th>
<th>Origin(1)</th>
<th>Year of isolation</th>
<th>Immune defect(1)</th>
<th>Runyon group</th>
<th>Identification by conventional test</th>
<th>Species assignment by</th>
<th>16S rRNA sequencing</th>
</tr>
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<tr>
<td>30</td>
<td>M01, M02, M04, M06–M10, M12, M13, M15–M25, M27–M34, M41</td>
<td>BAL (13), sputum (10), soft tissue (4), abscess (2), urine (1)</td>
<td>2003–9</td>
<td>HIV (4), COPD (4), cancer (3), CF (2)</td>
<td>IV</td>
<td>M. fortuitum complex</td>
<td>M. fortuitum</td>
<td>M. fortuitum(1)</td>
</tr>
<tr>
<td>12</td>
<td>M42-M51, M216, M219</td>
<td>BAL (6), lymphadenitis (1), sputum (5)</td>
<td>2003-9</td>
<td>HIV (1), COPD (1), bronchitis (3), diabetes mellitus (1), CF (1)</td>
<td>I</td>
<td>M. kansasii</td>
<td>M. kansasii type I</td>
<td>M. kansasii</td>
</tr>
<tr>
<td>1</td>
<td>M214</td>
<td>BAL</td>
<td>2007</td>
<td>HIV</td>
<td>III</td>
<td>M. avium complex</td>
<td>M. avium</td>
<td></td>
</tr>
<tr>
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<td>M220</td>
<td>Sputum</td>
<td>2006</td>
<td>HIV</td>
<td>III</td>
<td>M. avium complex</td>
<td>M. intracellular</td>
<td></td>
</tr>
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<td>1</td>
<td>M206</td>
<td>BAL</td>
<td>2004</td>
<td>Healthy</td>
<td>IV</td>
<td>Mycobacterium sp.</td>
<td>M. phlei</td>
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<tr>
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<td>BAL</td>
<td>2004</td>
<td>Healthy</td>
<td>IV</td>
<td>Mycobacterium sp.</td>
<td>M. phlei</td>
<td></td>
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<tr>
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<td>M210</td>
<td>BAL</td>
<td>2003</td>
<td>Healthy</td>
<td>IV</td>
<td>Mycobacterium sp.</td>
<td>M. phlei</td>
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</tr>
<tr>
<td>1</td>
<td>M201</td>
<td>Sputum</td>
<td>2003</td>
<td>Healthy</td>
<td>IV</td>
<td>Mycobacterium sp.</td>
<td>M. phlei</td>
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<tr>
<td>3</td>
<td>M71, M213, M222</td>
<td>BAL (1), gastric washing (1), sputum (1)</td>
<td>2004-8</td>
<td>CF (1)</td>
<td>IV</td>
<td>M. fortuitum complex</td>
<td>M. porcinum</td>
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<td>2</td>
<td>M137, M70</td>
<td>Urine (1), blood (1)</td>
<td>2009</td>
<td>HIV, COPD</td>
<td>IV</td>
<td>M. fortuitum complex</td>
<td>M. conceptionense</td>
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<tr>
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<td>Urine (1)</td>
<td>2003</td>
<td>HIV</td>
<td>IV</td>
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<tr>
<td>1</td>
<td>M222, M223</td>
<td>Sputum, BAL</td>
<td>2003</td>
<td>Healthy</td>
<td>II</td>
<td>Mycobacterium sp.</td>
<td>M. gordonae</td>
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<tr>
<td>5</td>
<td>M14, M40, M61, M120, M143</td>
<td>BAL (3), urine (1), Lymph node (1)</td>
<td>2004-7</td>
<td>HIV (1), CF (1), Healthy (3)</td>
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<td>Mycobacterium sp.</td>
<td>M. gordonae</td>
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<td>1</td>
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<td>BAL</td>
<td>2003</td>
<td>Healthy</td>
<td>IV</td>
<td>M. flavescens</td>
<td>M. lentiflavum</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>M79</td>
<td>Vaginal discharge and urine</td>
<td>2008</td>
<td>Chronic pelvic pain</td>
<td>II</td>
<td>M. scrofulaceum</td>
<td>M. simiae</td>
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<tr>
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<td>M11</td>
<td>Sputum and BAL</td>
<td>2008</td>
<td>Healthy</td>
<td>IV</td>
<td>Mycobacterium sp.</td>
<td>M. monacense</td>
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<td>BAL</td>
<td>2003</td>
<td>Healthy</td>
<td>II</td>
<td>Mycobacterium sp.</td>
<td>M. lentiflavum</td>
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<td>M05</td>
<td>BAL</td>
<td>2008</td>
<td>Healthy</td>
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<td>Mycobacterium sp.</td>
<td>M. fortuitum-like</td>
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<tr>
<td>1</td>
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<td>BAL</td>
<td>2007</td>
<td>Healthy</td>
<td>IV</td>
<td>M. fortuitum complex</td>
<td>M. fortuitum-like</td>
<td></td>
</tr>
</tbody>
</table>

1: The digits in the parentheses indicate the number of cases.
2: Representative isolates from each cluster were subjected to 16S rRNA gene sequencing.
3: Unknown patterns were designated based on algorithm described by Kim et al. (10).

NTM, nontuberculous mycobacterium; PRA, PCR-restriction fragment length polymorphism analysis; BAL, bronchoalveolar lavage; COPD, chronic obstructive pulmonary disease; CF, cystic fibrosis.

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Table 1. Clinical details and identification information of Iranian isolates of NTM
and 4 single isolates of \textit{tiflavum} (1 isolate), and \textit{vum}, \textit{M. phlei}, and known PRA identities, including of the 16S rRNA gene (Table 1 and Fig. 1). Further analyzed by determining the nucleotide sequence to those of the reference strains (10). These isolates were presented a restriction pattern that was not comparable to the reference type species of closely related mycobacteria. However, 18 of the isolates, namely, M05, M11, M14, (1 isolate), \textit{M. phlei} (1 isolate), \textit{M. fortuitum} (1 isolate), \textit{M. porcinum} (3 isolates), \textit{M. intracellulare} (1 isolate), \textit{M. phlei} (1 isolate), \textit{M. lentiflavum} (1 isolate), and \textit{M. thermoresistibile} (1 isolate), were reevaluated by 16S rRNA sequencing to investigate the possibility of intraspecies variation when compared to the reference type species of closely related mycobacteria.

All the test strains analyzed by 16S rRNA sequencing showed mycobacterial nucleotide signatures; specifically, they shared sequences at positions 70–98 (A–T), 293–304 (G–T), 307 (C), 328 (T), 614–626 (A–T), 631 (G), 661–744 (G–C), 824–876 (T–A), 825–875 (A–T), 843 (C), and 1122–1151 (A–T) (14,15). The 16S rRNA sequences of all the rapidly growing mycobacteria test strains contained a characteristic short helix at the 451–482 position, while 16S rRNA sequences of all the slowly growing test strains had an extended helix at the 451–482 position, characteristic of slowly growing mycobacteria. However, among the slowly growing mycobacteria, two strains, M79 and M221, presented with sequences that were somewhat deviant. These isolates were characterized by a short helix 18 (12 nucleotides shorter than that in \textit{M. tuberculosis}) in hypervariable region B, placing them in an intermediate position between rapid and slow growers in the \textit{Mycobacterium} phylogenetic tree (Figs. 2 and 3).

The nucleotide signature sequences hypervariable A (positions 125–270) and hypervariable B (positions 408–503) for the test strains, together with those of closely related rapidly and slowly growing mycobacteria, are shown in Fig. 2.

Analysis of the 16S rRNA sequences of 18 isolates showing unknown PRA patterns revealed that six isolates, M11, M66, M70, M79, M137, and M202, belong to the type species of a recently described mycobacterial species that is not included in the algorithm described by Kim et al. (10) (Table 1).

The remaining isolates that showed unique PRA patterns (i.e., M14, M40, M61, M120, M138, M143, M210, M214, M222, M223, and M226) were found to be members of previously established species since their sequence similarity values fell within the cut-off range of relevant established species (Table 1). \textit{M. gordonea} strains produced three different patterns: “pattern i” for strains M222 and M223, “pattern ii” for strain M138, and “pattern iii” for the remaining strains M14, M40, M61, M120, and M143.

The nucleotide sequence of isolate M05 showed substantial differences from the corresponding sequence of its nearest neighbor, \textit{M. fortuitum}, within the hypervariable regions A and B (Fig. 2). Isolate M05 formed a distinct line of descent in the neighbor-joining based phylogenetic tree (Fig. 3).

**DISCUSSION**

The clinical presentation of NTM is often hard to differentiate from that of MTBC; therefore, it is important to accurately identify NTM, since the management and treatment of infected patients, as well as the epidemiological control methods implemented, must reflect the specific mycobacterial species encountered (16). In low-income countries like Iran, where the microbiological diagnosis of tuberculosis relies mostly on microscopy and where access to culture and drug susceptibility testing is virtually non-existent, physicians may neglect to consider the possibility that infections found in patients with smear-positive specimens may be due to NTM (16,17). Isolation and identification of NTM species from clinical specimens can often require specific preparatory
Fig. 2. Alignment of selected stretches of 16S rRNA sequencing of Iranian NTM isolates with those of type strains of closely related mycobacteria. 16S rRNA positions are according to *Escherichia coli* numbering system. **'-'** indicates that the base pair was identical to that of type strain of *M. tuberculosis*.

procedures, such as decontamination steps or selective incubation methods (e.g., the use of a CO₂-enriched atmosphere or a specific temperature for optimal growth). Since most clinical laboratories do not use these techniques, infections caused by these organisms are likely to be underdiagnosed. On the other hand, extensive identification schemes comprising a minimum of 10 phenotypic tests have been used to identify the entire spectrum of mycobacteria, which generally inconclusive results (17). These tests are difficult to interpret and are limited by subjectivity and low specificity (18). Consequently, attempts to identify mycobacteria by conventional phenotypic tests alone may result in erroneous or incomplete identification if a sufficient number of discriminative tests are not available. However, in response to the need for a more rapid and accurate identification of clinically relevant NTM, appropriate use of molecular testing, in conjunction with the key phenotypic tests, has shown significant promise (16,18).

The importance of molecular data in the accurate identification of NTM cannot be overstressed, since in this study a simple PRA provided accurate identification for 73% of isolates (49 out of 67). The identification of the 17 remaining anonymous mycobacterial isolates was not accomplished by preliminary PRA due to unique or non-identical PRA patterns. Instead, conclusive identification of each of these remaining isolates was achieved by 16S rRNA gene sequencing, demonstrating the significance of this sequencing approach in the diagnosis of NTM infections.

The substantial differences between the nucleotide signature sequences of the isolate MO5 and that of its nearest neighbor, *M. fortuitum*, as well as the formation of a recognizable phylogenetic subline, suggests that this organism exhibits features of a potentially novel species. Further studies, including *hsp65*, *rpoB*, and 16S-23S internal transcribed spacer (ITS) gene sequencing, are being conducted in order to reach an unambiguous identification for this isolate.

Among the 67 NTM isolates, *M. fortuitum* (n = 30, 45%) was the most prevalent species, followed by *M. kansasii* (n = 12, 18%), and *M. gordonae* (n = 8, 12%). These findings are in accordance with those of a multi-country retrospective survey conducted in 1996 by the Working Group of the Bacteriology and Immunology Section of the International Union Against Tuberculosis and Lung Disease, which contacted 50 laboratories in many different countries, including Iran (19). According to this report, which included evaluation of 63 Iranian NTM isolates, *M. fortuitum* was found to be predominant in Iran and Turkey. In fact, with the exception of these two countries and countries like Belgium and the Czech Republic, the *M. avium* complex (MAC) has been the species most frequently isolated from clinical specimens. The authors have suggested that the increase in identification of MAC is most likely due to the rising incidence of HIV infection (19). This might explain the low frequency of MAC in our study,
especially since, according to UNAIDS and the World Health Organization’s Report on the global AIDS epidemic, the prevalence of HIV infection in Iran has remained rather low (0.2% of the population aged 15–49 years) during the past 4 years (20).

Unfortunately, our data does not permit us to estimate the true prevalence of unknown mycobacteria species in the laboratory isolates because the majority of the strains examined in our study were collected from other laboratories, and therefore, a selection bias might exist. However, the high number of inconclusively identified NTM strains among a collection of isolates from...
patients with initial presumptive tuberculosis diagnosis is disturbing.

The clinical relevance of the strains that were isolated from patients with pulmonary infection (54 out of 67 cases, 81%) was demonstrated on the basis of the fact that they were all obtained from pure cultures from at least two independent clinical samples with a preliminary positive smear of AFB. The etiologic role of the remaining extrapulmonary or rare mycobacterial isolates (7 cases, namely, M05, M11, M66, M70, M79, M137, and M202) might be inferred from the fact that AFB were microscopically observed in all clinical specimens. Furthermore, all isolates were recovered from the pure culture.

In conclusion, the high number of NTM found in a collection of unidentified isolates and the diversity of species involved in the various clinical manifestations of healthy and immunocompromised patients merit special attention by health authorities, doctors, and microbiologists in developing countries. In health centers that deal with tuberculosis, action should be taken to increase awareness of appropriate diagnostic criteria and management guidelines for NTM diseases. Moreover, expanding the number and quality of national and regional reference laboratories may facilitate more accurate laboratory diagnosis of NTM diseases.

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Conflict of interest None to declare.

REFERENCES