ORIGINAL ARTICLE

High rates of nontuberculous mycobacteria isolation from patients with presumptive tuberculosis in Iran

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Abstract

Nontuberculous mycobacteria (NTM) can cause disease which can be indistinguishable from tuberculosis (TB), posing a diagnostic and therapeutic challenge, particularly in low- and middle-income settings. We aimed to investigate the mycobacterial agents associated with presumptive clinical pulmonary TB in Iran. A total of 410 mycobacterial isolates, obtained between March 2014 and January 2016, from 7600 clinical samples taken from consecutive cases of presumptive diagnosis of TB were identified. Phenotypic and molecular tests were used to identify the isolated organisms to the species level. Single-locus and multilocus sequence analysis based on *16S rRNA, rpo*B, *hsp65* and ITS locus were used to confirm the results. Of 410 consecutive strains isolated from suspected TB subjects, 62 isolates (15.1%) were identified as NTM. Patients with positive NTM cultures met American Thoracic Society diagnostic criteria for NTM disease. *Mycobacterium simiae* was the most frequently encountered (38.7%), followed by *Mycobacterium fortuitum* (19.3%), *M. kansasii* (17.7%) and *M. avium* complex (8.0%). Isolation of NTM, including *M. simiae,* from suspected TB cases is a serious public health problem and merits further attention by health authorities, physicians and microbiologists
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Introduction

Nontuberculous mycobacteria (NTM) are environmental bacteria that incidentally cause opportunistic infections in humans (M. Mirsaeidi *et al.*, 'Geographic diversity of non-tuberculous mycobacteria species among NTM patients in the USA,' paper presented at the American Thoracic Society International Conference, Washington, DC, 19–24 May 2017, abstract A7807) [1,2]. The frequency of pulmonary disease from NTM is reportedly on the rise in different parts of the world [3–7]. Iran is an intermediate tuberculosis (TB) burden country where TB remains a major public health problem. According to the World Health Organization, the incidence rate of TB in Iran was 22 per 100,000 people in 2015 [8]. Although the epidemiology of TB is well described, the prevalence and epidemiology of NTM disease in Iran remain largely unknown. However, recent studies have reported the isolation of NTM from both TB patients and the general public in some regions of the country [9,10].

The clinical and radiologic manifestations of NTM infection frequently overlap with pulmonary TB [11–15]. Furthermore, failure to characterize acid-fast bacilli–positive NTM infection has led to mistaken treatment for TB in Iran [11]. One study showed that 30% of patients receiving treatment for pulmonary TB had NTM infections [16]. In Iran, some regional laboratories do not have proper facilities for patient admission. Consequently, TB patients have to come to the central laboratories in Tehran, the capital of Iran, for further identification of isolates,

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treatment and hospitalization. Therefore, the demonstrated measure of NTM infections can statistically represent all of Iran.

Given the fact that TB is still a major public health problem in Iran, there is a growing concern that NTM infections could be misdiagnosed as TB. In recent years, some researchers attempted to determine the prevalence of NTM and its importance in Iran. For example, Velayati *et al.* [17] indicated that *Mycobacterium fortuitum* and *Mycobacterium simiae* were the most prevalent mycobacteria among rapid growing mycobacteria and slow-growing mycobacteria in clinical samples, respectively. Unfortunately, these studies failed to capture a comprehensive extent of NTM. Most were confined to small metropolitan areas or to a specific group of mycobacterial species and/or to specific groups of patients [9,10].

We therefore aimed to report the species spectrum and prevalence of NTM infections among cases of suspected pulmonary TB in Iran.

Materials and methods

Patients and samples

This cross-sectional study evaluated patients suspected to have TB who were referred to one of the main TB reference centres in Iran, the Regional TB Reference laboratory, in Tehran, the capital of Iran, from March 2014 to January 2016. This centre, which has drug susceptibility testing capability, is among the main TB centres of Iran that regionally report the data on TB and acts as local centre for the diagnosis and treatment of infectious diseases. Moreover, regional TB laboratories from different provinces of Iran (e.g. Qom, Golestan, Markazi, Ghazvin, Kerman and Guilan) transfer TB samples to this laboratory for further identification of isolates and in cases of NTM infection.

All investigated patients had clinical signs and symptom of TB and underwent examination for possible active TB. If the patient had multiple longitudinal sampling, only the first set of samples was included into the study. In total, 7600 sputum specimens were tested. The ethics committee of Shahid Beheshti University of Medical Sciences approved the study, and all patients provided written informed consent.

Culture and isolation

Sputum specimens (2.5 to 10 mL) were processed using 2% NaOH method (Petroff method) and were concentrated at 4000 × *g* for 15 minutes [18]. Sediments of each treated sample were used to prepare a Ziehl-Neelsen smear and were cultured in Löwenstein-Jensen medium [18]. Only one culture isolated per study subject was considered for further analysis.

Phenotypic identification

All mycobacterial isolates were grown on Löwenstein-Jensen medium and examined for growth rate, macroscopic and microscopic morphologic features, and growth at different temperatures; they also underwent a set of biochemical tests, including Tween 80 hydrolysis, nitrate reduction, niacin production, arylsulfatase, urease production, tellurite reduction, salt tolerance and catalase production according to standard procedures [19].

Molecular assignment of isolates to *Mycobacterium tuberculosis* complex (MTC)

For the identification of MTC organisms and the differentiation of MTC and NTM from positive cultures, *IS6110*-based PCR assay was used.

Genomic DNA for *IS6110*-based PCR assay was extracted with the QIAamp DNA Mini Kit (Qiagen, Germantown, MD, USA) according to the manufacturer's instruction. A 123 bp fragment of insertion element *IS6110* of the MTC was used as a target and was amplified using previously described PCR primers [20]. Genomic DNA of *M. tuberculosis* H37Rv (ATCC 27294) and *M. fortuitum* (ATCC 49404) was used as positive and negative controls, respectively.

Molecular assignment to species level

PCR restriction analysis (PRA) was used to speciate mycobacteria. Single-locus and multilocus sequence analysis based on *16S rRNA*, *rpo*B, *hsp65* and ITS locus were used to confirm the results.

PRA of *hsp65* gene (HSP65-PRA)

An approximately 441 bp fragment of the *hsp*65 gene was amplified by PCR using two specific primers, Tb11 (5′-ACCAACGATGGTGTGTCCAT-3′) and Tb12 (5′-CTTGTCGAACCGCATACCCT-3′). PCR products were digested with 5 U of restriction enzymes *Hae*III and *Bst*II for 24 hours at 37°C [21]. The pattern of digested products was analysed using an 8% polyacrylamide gel. *M. fortuitum* (ATCC 49404) and double-distilled water were used as positive and negative controls respectively in all PCR experiments. Species identification was performed using algorithms previously proposed by others [21,22].

PCR and sequencing of *16S rRNA*, *rpo*B, *hsp65* and ITS

16S rRNA. Full length of the *16S rRNA* gene (1500 bp) from isolates were amplified using primers pA (5′-AGAGTTTGATCCTGGCTCAG-3′) and pI (5′-TGCACACAGGCCACAAGGGA-3′) as described previously [23].

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rpo*B*. A 750 bp fragment of the *rpo*B gene was amplified and sequenced using two specific primers, MycoF (5′-GGCAAGGTCACCCCGAAGGG-3′) and MycoR (5′-AGCGGCTGCTGGGTGATCATC-3′), as previously described [24,25].

hsp65. The amplified PCR products of the *hsp65* gene for each isolate were purified, and the sequences were determined as described above using the specific primers Tb11 and Tb12 [21].

ITS. The universal primers 16S–1511f (5′-AAGTCGTAACAAGGTARCCG-3′) and 23S–23r (5′-TCGCCAAGGCATCCACC-3′) were used for amplification of the ITS region as previously described [26].

Analysis of sequence data

The obtained sequences for each isolate from different loci were aligned separately and compared with all existing relevant sequences of mycobacteria retrieved from the GenBank database at the National Center for Biotechnology Information (NCBI) website via nucleotide Basic Local Alignment Search Tool (BLAST) search (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Results

Of 410 consecutive strains isolated from suspected TB subjects, 62 isolates (15.1%) were identified as NTM using conventional and molecular methods (all NTM isolates were negative for *IS6110*) (Fig. 1). All of the patients with positive NTM cultures met American Thoracic Society/Infectious Diseases Society of America (ATS/IDSA) diagnostic criteria for NTM disease. On the basis of the available data for drug susceptibility testing, six of 62 isolates of NTM were from patients who were misdiagnosed as having multidrug-resistant TB and whose disease failed to respond to first-line treatment (Table 1).

Assignment of isolates to TB group

Of 410 confirmed cases of mycobacterial isolates, 348 were confirmed as MTC using conventional tests along with the presence of a 123 bp segment of a repetitive sequence of *IS6110*.

Molecular assignment of NTM to species level

HSP65-PRA-based identification. According to HSP65-PRA results, an identical pattern was detected for the isolated microorganisms from every patient. Using HSP65-PRA, *M. simiae* was the most frequently encountered (38.7%), followed by *M. fortuitum* (19.3%), *M. kansasii* (17.7%) and *M. avium* complex (8.0%). The remaining strains represented a variety of NTM species (Table 2).

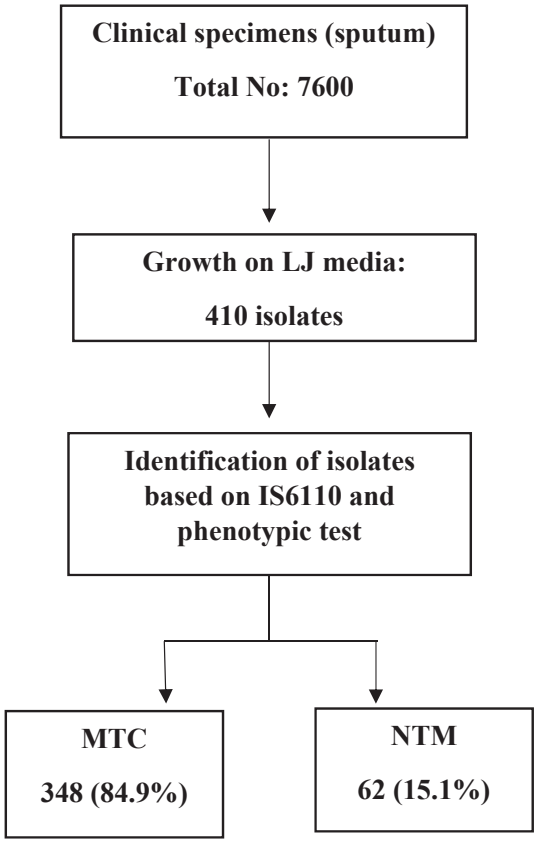

FIG. 1. Flowchart of sample collection and isolation. LJ, Löwenstein-Jensen medium; MTC, *Mycobacterium tuberculosis* complex; NTM, non-tuberculous mycobacteria.

Identification by 16S rRNA, rpo*B*, hsp65 and ITS. The percentage similarities of almost full 16S rRNA and partial sequences of *rpo*B, *hsp*65 and ITS of representative clinical isolates of each group of NTM, which was clustered on the basis of HSP65-PRA, are summarized in Table 3. Clinical isolates were confidently identified by each of the *16S rRNA*, *rpo*B, *hsp*65 and ITS assessment. There was also a strong correlation between *16S rRNA*, *rpo*B, *hsp65* and ITS gene sequencing results. Clinical isolates including *M. simiae, M. fortuitum, M. kansasii, M. intracellulare, M. thermoresistibile, M. abscessus, M. gordonae, M. senegalense, M. xenopi* and *M. phocaicum* can be confidently identified by each of the *16S rRNA*, *rpo*B, *hsp*65 and ITS analyses.

Discussion

We found that an unexpected number of patients who sought diagnosis and treatment for TB in Iran were infected by mycobacteria other than TB (15.1%), in particular *M. simiae* (38.7%). This result is consistent with prior reports of an increased prevalence of NTM and the difficulty in distinguishing

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TABLE 1. Demographic and identification data of patients with NTM disease

Variable	Cure	Poor outcome[a]
No. of subjects	56 (90.3)	6 (9.7)
Mean age, years	51.4	42.2
Sex		
Female	26 (46.4)	3 (50)
Male	30 (53.6)	3 (50)
NTM location		
Pulmonary	53 (94.6)	6 (100)
Extrapulmonary	3 (5.4)	0
Mycobacteriology		
M. simiae	21 (37.5)	3 (50)
M. fortuitum	10 (17.8)	2 (33.3)
M. kansasii	11 (19.6)	0
M. intracellulare	5 (9)	0
M. abscessus	3 (5.3)	1 (16.7)
M. thermoresistibile	1 (1.7)	0
M. xenopi	1 (1.7)	0
M. phocaicum	1 (1.7)	0
M. gordonae	2 (3.5)	0
M. senegalense	1 (1.7)	0

Data are presented as *n* (%) unless otherwise indicated.
NTM, nontuberculous mycobacteria.
[a]Poor outcome includes relapse, failure to respond to treatment and death.

pulmonary TB and NTM according to symptoms [27–32]. We previously reported that 10% of mycobacterial species that were cultured from TB patients were NTM [33]. Likewise, a few other studies observed similar percentages: 4% to 10% of culture-positive samples were diagnosed as NTM [10,34]. In Iran, as the incidence of TB has declined, NTM have been increasingly recognized as human pathogens [10,35]. This may be explained in part by increased recognition of NTM infections as a clinical entity and advances in laboratory methods [33]. Furthermore, increased susceptibility due to human immunodeficiency virus (HIV), malignancy, preexisting lung diseases, the relative immunodeficiency or occupational exposure to dust may predispose an individual to NTM infection [36,37]. The rising number of NTM infections in Iran may have several negative effects on public health. Importantly, most TB laboratories in Iran are not equipped to perform mycobacterial culture and species identification; consequently, NTM infections are frequently misdiagnosed as TB. Missing NTM disease results in unnecessary anti-TB treatment, inappropriate use of high-cost care and stigmatization of affected persons, with important social and economic consequences [16,38]. Given the importance and increasing prevalence of NTM, rapid and reliable identification of NTM should be carried out as a means of effective patient management [39–42].

In the current study, *M. simiae* was the most frequently encountered species of NTM in clinical samples. In Iran, *M. simiae* is an endemic NTM. Recent studies in Iran have reported the emergence of *M. simiae* as the most frequently isolated NTM in respiratory specimens [9,35,43]. *M. simiae* may present with clinical and radiologic manifestations consistent with TB [9]. According to the ATS/IDSA guideline, NTM lung disease can be diagnosed if *M. simiae* is isolated in two of three sputum cultures, accompanied by pulmonary symptoms and abnormalities on chest radiograph or high-resolution computed tomographic scan of chest, together with appropriate exclusion of other disorders [37]. In our study, *M. simiae* was isolated from patients who had either been previously diagnosed as being infected with multidrug-resistant TB, who had received other types of TB treatments or who comprised new TB cases with pulmonary symptoms. These findings indicate that *M. simiae* is capable of colonization in previously damaged lungs, causing pulmonary disease [35]. Therapy of *M. simiae* pulmonary infection also remains an important issue. There are no published clinical trials for the treatment of infection caused by *M. simiae*. This bacterium usually shows poor *in vivo* response to therapy, and most isolates are resistant to first-line anti-TB drugs [37,44]. Antimicrobial drugs reported to have *in vitro* activity against *M. simiae* include clarithromycin, ethambutol, ethionamide, fluoroquinolones, amikacin and cycloserine [9,45].

In conclusion, isolation of NTM, including *M. simiae,* from suspected TB cases is a serious public health problem in Iran and merits further attention by health authorities, physicians and microbiologists. *M. simiae* may present with clinical and radiologic manifestations consistent with TB, and it may be

TABLE 2. Results of nontuberculous mycobacteria identification by phenotypic and genotypic tests

No. of isolates	Lab designation	Phenotypic test result	Pattern by HSP65-PRA		Identification by HSP65-PRA
			*Bst*EII	*Hae*III	
24	12[a]	*M. simiae*	235/210	185/130	*M. simiae*
12	10[a]	*M. fortuitum*	235/120/85	145/120/60/55	*M. fortuitum*
11	14[a]	*M. kansasii*	235/210	130/105/80	*M. kansasii*
5	11[a]	*M. avium complex*	235/120/100	145/130/60	*M. intracellulare*
4	41[a]	*M. chelonae*	235/210	200/70/60/50	*M. abscessus*
2	35[a]	*Mycobacterium* sp.	235/210	130/115	*M. gordonae*
1	47[a]	*Mycobacterium* sp.	235/120/85	160/105/60	*M. xenopi*
1	48[a]	*Mycobacterium* sp.	320/115	145/65/60	*M. phocaicum*
1	9[a]	*Mycobacterium* sp.	235/210	180/135/70/50	*M. thermoresistibile*
1	40[a]	*Mycobacterium* sp.	235/210	140/125/60/50	*M. senegalense* or *M. conceptionense*

HSP65-PRA, PCR restriction analysis (PRA) of *hsp65* gene.
[a]Isolates randomly selected from each cluster of HSP65-PRA patterns for multilocus sequence analysis.

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TABLE 3. Details of identification of nontuberculous mycobacteria by sequence analysis

Lab designation[a]	*16S rRNA* (1500 bp)	*rpoB* (750 bp)	*hsp65* (450 bp)	ITS (230–350 bp)	MLSA[a]
10	100% *M. fortuitum* 98% *M. farcinogenes* 98% *M. senegalense*	100% *M. fortuitum* 98% *M. senegalense*	100% *M. fortuitum* 99% *M. farcinogenes* 99% *M. houstonese* 98% *M. senegalense*	100% *M. fortuitum*	*M. fortuitum*
11	100% *M. intracellulare* 99% *M. avium*	100% *M. intracellulare* 99% *M. chimera* 99% *M. avium* 99% *M. yongonense*	99% *M. intracellulare* 98% *M. avium* 98% *M. yongonense*	100% *M. intracellulare* 96% *M. avium*	*M. intracellulare*
12	100% *M. simiae*	100% *M. simiae* 95% *M. sherrisii* 94% *M. genavense*	99% *M. simiae* 97% *M. genavense*	100% *M. simiae* 95% *M. genavense*	*M. simiae*
14	100% *M. kansasii*	100% *M. kansasii* 97% *M. gastri*	100% *M. kansasii* 98% *M. gastri*	100% *M. kansasii*	*M. kansasii*
9	100% *M. thermoresistibile*	100% *M. thermoresistibile*	99% *M. thermoresistibile*	100% *M. thermoresistibile*	*M. thermoresistibile*
47	99% *M. xenopi*	99% *M. xenopi*	99% *M. xenopi*	99% *M. xenopi*	*M. xenopi*
48	99% *M. phocaicum*	99% *M. phocaicum*	99% *M. phocaicum*	99% *M. phocaicum*	*M. phocaicum*
41	99% *M. abscessus*	98% *M. abscessus*	99% *M. abscessus*	99% *M. abscessus*	*M. abscessus*
40	99% *M. senegalense*	100% *M. senegalense*	99% *M. senegalense*	99% *M. senegalense*	*M. senegalense*
35	99% *M. gordonae*	99% *M. gordonae*	99% *M. gordonae*	99% *M. gordonae*	*M. gordonae*

HSP65-PRA, PCR restriction analysis (PRA) of *hsp65* gene; MLSA, multilocus sequence analysis.
[a]Isolates randomly selected from each cluster of HSP65-PRA patterns for MLSA.

resistant to anti-TB agents. Finally, establishment of rapid and reliable methods for identification of NTM infections, selection of an appropriate treatment regimen for NTMs such as *M. simiae* and expanding the number of the facilitated laboratories are strongly recommended.

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Conflict of Interest

None declared.

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