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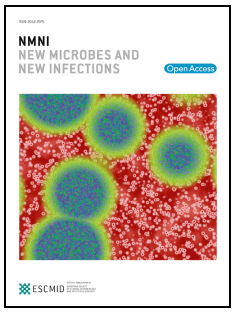

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High Rates of Non-Tuberculous Mycobacteria Isolation from Patients with Presumptive Tuberculosis in Iran

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Abstract

Introduction: Non-tuberculous mycobacteria (NTM) can cause disease which can be undistinguishable from tuberculosis (TB), posing a diagnostic and therapeutic challenge, particularly in low and middle income settings. This study was aimed to investigate the mycobacterial agents associated with presumptive clinical pulmonary TB in Iran.

Materials and Methods: A total of 410 mycobacterial isolates, obtained between March 2014 to January 2016, from 7.600 clinical samples, from consecutive cases with presumptive diagnosis of TB were identified. Phenotypic and molecular tests were used to identify the isolated organisms to species level. Single locus and multi locus sequence analysis (MLSA) based on *16S rRNA*, *rpoB*, *hsp65* and ITS locus were used to confirm the results.

Results: Out of 410 consecutive strains isolated from suspected TB subjects, 62 (15.1%) isolates 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 *M. fortuitum* (19.3%), *M. kansasii* (17.7%) and *M. avium* complex (8.0%) onward.

Conclusions: Isolation of NTM, including *M. simiae*, from suspected TB cases is a serious public health problem and merit further attention by health authorities, physicians, and microbiologists.

Keywords: Tuberculosis, mycobacterium, Nontuberculous, *M. simiae*, Iran

Introduction:

Non-tuberculous mycobacteria (NTM) are environmental bacteria that incidentally cause opportunistic infections in humans [1-3]. The frequency of pulmonary disease from NTM is reportedly on the rise in different parts of the world [4-8]. Iran is an intermediate tuberculosis- (TB-) burden country where TB remains a major public health problem. According to World Health Organization (WHO) the incidence rate of TB in Iran was 22 cases per 100,000 people in the year 2015 [9]. Although the epidemiology of TB is well described, the prevalence and epidemiology of NTM disease in Iran are 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 [10, 11]. The clinical and radiological manifestations of NTM infections are frequently overlap with pulmonary TB [12-16]. Furthermore, failure to characterize acid fast bacilli positive NTM infections has led to mistake in treatment for TB in Iran [12]. A recently published study showed that 30% of patients receiving treatment for pulmonary TB had NTM infections [17]. In Iran, some regional laboratories do not have proper facilities for patients admission. Consequently, TB cases have to come to the central laboratories in Tehran, the capital of Iran, for further identification of isolates, treatment and hospitalization. Therefore, the demonstrated measure of NTM infections can represent the statistical analysis from all around 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 el, indicated that *M. fortuitum* and *M. simiae* were the most prevalent mycobacteria among rapid growing mycobacteria (RGM) and slow growing mycobacteria (SGM) in clinical samples, respectively [18]. Unfortunately, these studies failed to capture a comprehensive extent of NTM. The majority of them confined to small metropolitan areas or to specific group of mycobacterial

species as well as specific groups of patients [10, 11]. This study was aimed to reports the species spectrum and the prevalence of NTM infections among pulmonary TB suspects in Iran.

Materials and methods:

Patients and samples

This cross-sectional study evaluated suspected TB patients referred to one of the main TB reference centers of Iran (Regional TB Reference laboratory; located in Tehran, capital of Iran), from Mar 2014 to Jan 2016. This center with drug susceptibility testing (DST) capability are among the main TB centers of Iran that regionally report the data on TB and acts as local center for 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 case of NTM infection. All investigated patients had clinical signs and symptom of TB and undergoing 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 the patients have signed informed consent.

Culture and isolation

Sputum specimens (2.5 to 10 mL) were processed using 2% NaOH method (Petroff's method) and concentrated at 4.000 × g for 15 minutes [19]. Sediments of each treated sample were used to prepare a Ziehl-Neelsen smear and were cultured in Lowenstein-Jensen medium [19]. Only one culture isolated per study subject was considered for further analysis.

Phenotypic identification

All mycobacterial isolates were grown on Lowenstein Jensen (LJ) medium and examined for growth rate, macroscopic and microscopic morphological features, growth at different temperatures and also 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 [20].

Molecular assignment of isolates to *M. 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 using QIAamp DNA Mini Kit (QIAGEN, USA) according to kit instruction. A 123-bp fragment of insertion element *IS6110* of the *M. tuberculosis* complex was used as a target and amplified using previously described PCR primers [21]. Genomic DNA of *M. tuberculosis* H37Rv (ATCC27294) and *M. fortuitum* (ATCC 49404) were used as positive and negative controls, respectively.

Molecular assignment to species level

PCR restriction analysis (PRA) was used to speciate mycobacteria. Single locus and multi locus sequence analysis (MLSA) based on 16S rRNA, *rpo*B, *hsp*65 and ITS locus were used to confirm the results.

hsp65-PRA

An approximately 441-bp fragment of *hsp*65 gene was amplified by PCR using two specific primers Tb11 (50-ACCAACGATGGTGTGTCCAT-30) and Tb12 (50-CTTGTCGAACCGCATACCCT-30). PCR products were digested with 5 U of restriction enzyme *Hae* III and *Bst* II for 24 hours at 37°C [22]. The pattern of digested products was analyzed using 8% polyacrylamide gel. *M. fortuitum* (ATCC 49404) and double distilled water were used as positive and negative

control in all PCR experiments respectively. Species identification was performed using algorithm proposed by Roth et al and Telenti et al [22, 23].

PCR and sequencing of 16S rRNA, rpoB, hsp65 and ITS

16S rRNA. Full lengths of the *16S rRNA* genes (1.500-bp) from isolates were amplified using primers pA (50-AGAGTTTGATCCTGGCTCAG-30) and pI (50-TGCACACAGGCCACAAGGGA-30) as described previously [24].

rpoB. A 750-bp fragment of the *rpo*B gene was amplified and sequenced using two specific primers MycoF (50-GGCAAGGTCACCCCGAAGGG-30) and MycoR (50-AGCGGCTGCTGGGTGATCATC-30) as previously described [25, 26].

hsp65. The amplified PCR products of *hsp*65 gene for each isolate were purified and the sequences were determined as described above using the specific primers Tb11 and Tb12 [22].

ITS. The universal primers 16S-1511f (50-AAGTCGTAACAAGGTARCCG-30) and 23S-23r (50-TCGCCAAGGCATCCACC-30) were used for amplification of the ITS region as previously described [27].

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 GenBank database at the NCBI website via the nucleotide BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Results:

Out 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*) (Figure 1). All of the patients with positive NTM cultures met ATS/IDSA diagnostic criteria for NTM disease. Based on the available data for DST, 6 of 62 isolates of NTM were from patients who were misdiagnosed as multi-drug resistant TB (MDR-TB) and 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 123-base-pair segment of a repetitive sequence of IS*6110*.

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%) onward. The remaining strains represented a variety of NTM species (Table 2).

***Identification by 16S rRNA, rpoB, hsp65,* 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 based on *hsp*65-PRA are summarized in Table 3. Clinical isolates were confidently identified by each of the 16S rRNA, *rpo*B, *hsp*65 and ITS. There was also a strong correlation between 16S rRNA, *rpoB, 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*, *rpoB*, *hsp*65 and ITS.

Discussion:

This study found that an unexpected number of cases 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 pulmonary TB and NTM based on symptoms [28-33]. We have recently reported that, 5% of mycobacterial species that were cultured from TB patients were NTM [34]. Likewise, a few other studies observed nearly the similar percentages: 4% to 10% of culture positive samples were diagnosed as NTM [11, 35]. In Iran, as the incidence of TB has declined, NTM have been increasingly recognized as human pathogens [11, 36]. This may be explained in part by increased recognition of NTM infections as a clinical entity and advances in laboratory methods [34]. Furthermore, increased susceptibility due to HIV, malignancy, pre-existing lung diseases, the relative immunodeficiency or occupational exposure to dusts may predispose an individual to NTM infection [37, 38]. The rising number of NTM infections in Iran may have several negative effects on public health statues. 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 settings and stigmatization of affected persons, with important social and economic consequences [17, 39]. Given the importance and increasing prevalence of NTM, rapid and reliable identification of NTM should be carried out as a means of effective patient managements [40-43].

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 an emergence of *M. simiae* as the most frequently isolated NTM in respiratory specimens [10, 36, 44]. *M. simiae* may present with clinical and radiological manifestations consistent with TB [10]. According to the ATS/ IDSA guideline, NTM lung disease can be diagnosed if *M. simiae* is isolated in two out of three sputum cultures, accompanying with pulmonary symptoms and abnormalities in the chest radiograph or high resolution computed tomography scan of chest, together with appropriate exclusion of other disorders [38]. In our study, *M. simiae* were isolated from patients that had either been previously diagnosed as being infected with MDR-TB, had received other types of TB treatments, or were new TB cases with pulmonary symptoms. These findings indicated that *M. simiae* is capable of colonization in previously damaged lungs and causing pulmonary disease [36]. 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 [38, 45]. Agents reported to have *in vitro* activity against *M. simiae* include clarithromycin, ethambutol, ethionamide, fluoroquinolones, amikacin, and cycloserine [10, 46].

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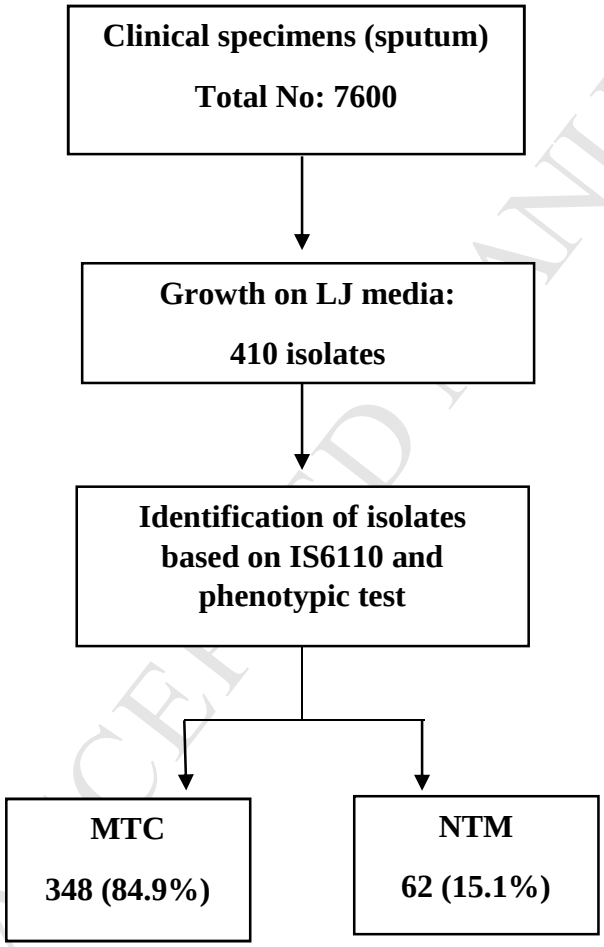

Figure 1. A flow chart of samples collection and isolation.
LJ: Lowenstein Jensen medium; MTC: *M. tuberculosis* complex; NTM: non-tuberculous mycobacteria.

Table1. The demographic and identification data of patients with NTM disease.

Variables	Cure N (%)	Poor outcome* N (%)
Number of subjects	56 (90.3)	6 (9.7)
Mean age	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)
Extra-pulmonary	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

*Poor outcome includes relapse, failure to treatment and death.

Table2. Results of NTM identification by phenotypic and genotypic tests

Numbers of isolates	Lab designation	Phenotypic tests	Patterns by hsp65-PRA		Identification by PRA
			Bst E II	*Hae* III	
24	12*	*M. simiae*	235/210	185/130	*M. simiae*
12	10*	*M. fortuitum*	235/120/85	145/120/60/55	*M. fortuitum*
11	14*	*M. kansasii*	235/210	130/105/80	*M. kansasii*
5	11*	*M. avium complex*	235/120/100	145/130/60	*M. intracellulare*
4	41*	*M. chelonae*	235/210	200/70/60/50	*M. abscessus*
2	35*	*Mycobacterium sp.*	235/210	130/115	*M. gordonae*
1	47*	*Mycobacterium sp.*	235/120/85	160/105/60	*M. xenopi*
1	48*	*Mycobacterium sp.*	320/115	145/65/60	*M. phocaicum*
1	9*	*Mycobacterium sp.*	235/210	180/135/70/50	*M. thermoresistibile*
1	40*	*Mycobacterium sp.*	235/210	140/125/60/50	*M. senegalense* or *M. conceptionense*

*The isolates randomly selected from each cluster of hsp65-PRA patterns for Multi locus sequence analysis (MLSA)

Table 3. Details of identification of NTM by sequence analysis 437

Lab designation*	16S rRNA (1500 bp)	rpoB (750 bp)	hsp65 (450 bp)	ITS (230–350 bp)	MLSA*
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.cheimera 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*

*The isolates randomly selected from each cluster of hsp65-PRA patterns for Multi locus sequence analysis (MLSA). 438 439

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