Original article

An 8-year study on the prevalence and drug resistance of mycobacteria in clinical specimens (2011–2018)

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ABSTRACT

Non-tuberculous mycobacteria cause a wide range of clinical disorders. Isoniazid (INH) and rifampin (RIF) are the most effective first-line antibiotics against for *Mycobacterium tuberculosis*. The aim of this study was to investigate the prevalence of mycobacteria in clinical samples collected during 8 years (2011–2018) in the National Research Institute of Tuberculosis and Lung Disease, Tehran, Iran and to determine the tuberculosis drug resistance to first-line antibiotics, INH and RIF. In this study, 15829 different clinical specimens were collected at the NRITLD National Tuberculosis Center. A multiplex allele specific polymerase chain reaction (MAS-PCR) was used to identify mutations related to RIF and INH resistance. The genes involved were *katG315*, *inhA* for INH and *rpoB516*, *rpoB526* and *rpoB531* for RIF. In total (7528/15829, 47.56%), mycobacterial isolates including 6937 MTBC (43.82%) and 591 NTM (3.73%) were obtained. The frequency of MTBC isolates decreased from 65.17% (2015/3092) in 2011 to 47.06% (1224/2601) in 2018. Among NTM isolates, *M. simiae* was the most prevalent with 55.33% (327/591). The average INH resistance ratio between 7528 MTB and NTM isolates was 21%, from 15.98% in 2011 to 18.76% in 2018. In the case of RIF, the same resistance trend has been gradually increasing from 12.45% in 2011 to 14.55% in 2018. The prevalence of MDR TB has increased during the study period, from 6.49% (134/2065) in 2011 to 12.58% (174/1354) in 2018. The results of this study indicate that early detection of mycobacterial strains and determination of their drug resistance are necessary.

1. Introduction

Mycobacterium species, including more than 150 species, many of them cause disease.^{1,2} The majority of these species do not cause disease but may, under certain conditions, become opportunistic pathogens and cause infection.³ Non-tuberculous mycobacteria (NTMs) are opportunistic pathogens that cause skin and chronic lung infections. These species are widely present in the environment.⁴

Because of the similar clinical pulmonary and X-ray symptoms of NTM infection with MTBC, NTM is not as prominent as TB. As a matter of fact, NTM infection is generally ignored. Significantly, there is a reciprocal trend between the prevalence of MTBC and NTM, while the rate of NTM isolates has doubled.⁴

Reports of non-tuberculosis mycobacterial infections worldwide have increased over the past two decades.⁵ This type is also isolated from clinical specimens, but cannot always be considered a disease because NTMs are capable of colonization in various parts of the body

such as the respiratory tract, digestive tract and skin.^{6,7} Treatment of patients infected with different strains of NTM is very difficult and complex because of the effective antibiotic strains that should be used. One of the reasons for the different percentages of resistance of isolated strains to different drugs is the variety of the species isolated.^{7–12}

Patients with NTM require frequent and intensive health care resources such as hospitals and administrative visits, as well as complex treatment. These challenges also exist when associated with multiple infections, which are common in this population. The emergence of drug resistance has gradually become an obstacle to TB control, especially multidrug resistance (MDR).^{4,13–15}

Clinically, rapid diagnosis and drug-susceptibility testing (DST) are crucial for infection control and drug treatment in *Mycobacterium tuberculosis* and non-tuberculosis mycobacteria.¹⁶ The increasing isolation and detection of non-tuberculous mycobacteria from patients with mycobacterial infections in Iranian laboratories reflects the fact that most patients are treated with inappropriate and unnecessary

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medication.^{15,17–21}

Non-tuberculous mycobacteria are one of the most important causes of death, which are often a form of progressive pulmonary disease.^{22,23} Diagnosing NTM infection can be quite challenging. First, NTM culture can be misleading because these bacteria are present in any environment, so clinical specimens can be contaminated with unrealistic factors. Contamination may occur before, during, and even after sampling. For example, sputum samples collected may be contaminated with fresh water mouthwash.²² In order to distinguish between contamination and infection, pulmonary NTM disease diagnosis must be considered a combination of clinical, radiological, bacteriological, and histological criteria.^{24–27}

The use of simple, fast, and efficient tools for the differentiation of mycobacteria such as the World Health Organization (WHO), DST and line probe assay (LPA) is highly needed, especially in developing countries with high TB and Non-tuberculosis mycobacteria burden.^{28,29}

In countries with endemic tuberculosis, isolation of non-tuberculous mycobacteria from clinical specimens, especially respiratory specimens, is not uncommon.^{20,24,29,30} However, in clinical conditions, in patients with suspected TB, physicians rely on local epidemiological knowledge to assess whether positive culture is related to *Mycobacterium tuberculosis* or NTM. Therefore, the prevalence of *M. tuberculosis* and NTM isolation should be studied to better understand the epidemiological features of these diseases.^{19,31}

Non-tuberculous mycobacteria are one of the most important human pathogens in non-endemic and endemic areas of TB such as Iran. It is well known that the determination of the drug sensitivity of non-tuberculosis mycobacterial clinical isolates is one of the key points in the treatment of these diseases.²⁴ In Iran (where endemic tuberculosis is present) all patients with mycobacterial infections are treated with first-line anti-TB drugs, while most non-TB isolates have been found to be resistant to first-line anti-TB drugs (24). Therefore, accurate identification of these clinical specimens can be a great step in early diagnosis and treatment of the disease.

In this study, bacterial infection and drug resistance data are reviewed to provide an appropriate solution for the control of mycobacterial infections. It is recommended that Identification, detection and sensitivity tests for NTMs should be standardized and implemented as standard operating protocols in clinical and laboratory settings to counter the increased NTM infections. The aim of the present study was to evaluate the frequency and prevalence of clinical isolates of Mycobacteria and their drug resistance pattern from 2011 to 2018, so as to be a strategy for their control process.

2. Method and material

The clinical samples studied in this manuscript were collected from the National Research Institute of Tuberculosis and Lung Disease, Tehran, Iran (NRITLD) between 2011 and 2018.

Samples were collected from patients suspected of Mycobacteria and hospitalized in the hospital and clinic and transferred to the Mycobacteriology Research Center (MRC) Laboratory for bacterial culture, identification and antibiotic susceptibility testing.

A total of 15829 specimens were collected, including 12830 sputum (81.05%), 152 pleural effusion (0.96%), 1087 bronchial specimens (6.87%), 169 Biopsy specimens (1.07%) and others.

Initial isolation of Mycobacterium strains was performed by Petrov method using Leven-Stein Johnson medium.^{32,33} Subsequently, mycobacterial DNA was extracted from patient samples. To identify Mycobacterium species, first a 190 bp fragment of IS6110 gene was used by primers Tb 1: 5'- ATC CTG CGA GCG TAG GCG TCGG -3' and Tb2: 5'- CAG GAC CAC GAT CGC TGA TCC GG -3'.^{33,34}

PCR mix were used with a final volume of 50 µl containing 8 picomol of Tb1 and Tb2 primer. 1.5 mM magnesium chloride, 1 unit Taq polymerase enzyme (Synagen Corporation), 0.2 mM dNTP, 1.5 mM buffer 10 X and 2% DMSO. The PCR reaction in Thermal Cycler (Astec

Table 1

Positive isolation rate of mycobacterial from year 2011–2018.

year	No. of specimen	No. of isolates (%) ^a		
		M.TB	NTM	total
2011	3092	2015(65.17)	50(1.62)	2065(66.79)
2012	399	171(42.86)	10(2.51)	181(45.36)
2013	1131	526(46.51)	25(2.21)	551(48.72)
2014	2843	237(8.34)	31(1.09)	268(9.43)
2015	1120	511(45.63)	94(8.39)	605(54.02)
2016	1927	884(45.87)	114(5.92)	998(51.79)
2017	2716	1369(50.41)	137(5.04)	1506(55.45)
2018	2601	1224(47.06)	130(5)	1354(52.06)
total	15829	6937(43.82)	591(3.73)	7528(47.56)

M.TB = mycobacterium tuberculosis group, NTM = nontuberculous mycobacteria.

^a Numbers in the parenthesis indicated the corresponding positive isolation rate to the number of specimens each year.

Japan) is as follows: The first cycle was 95 °C for 10 min to an initial denaturation followed by 35 cycles of 93 °C for 20 s, 65 °C for 1 min and 72 °C for 20 s, and the last cycle for 5 min at 72 °C was performed for final elongation. PCR reaction products were loaded onto 1.5% agarose gel containing ethidium bromide.^{33,35}

Specimens positive for IS6110 were considered as *Mycobacterium tuberculosis* complex. Samples with negative PCR result based on IS6110 were analyzed for non-tuberculous mycobacteria using PCR-RFLP method.

This gene was amplified using a Nested-PCR with primer pair Tb15: 5'-CGT AYG ACG AAG AGG CCC GT 3' and Tb17: 5'-WAS GGR TCC TCS AGG ACS GC -3'(33).

PCR mixture with a final volume of 50 µl containing 4 Pico moles of Tb15 and Tb17 primer. 1.5 mM magnesium chloride, One unit of Taq polymerase (Synagen Corporation), 0.2 mM dNTP, 1.5 mM 10 X buffer and 1% DMSO were used. The PCR reaction in Thermal cycler consisted of 30 cycles with annealing temperature of 60 °C.

The second step was performed using primer pairs Tb11: 5'ACCAA CGATGGTGTGTCCA 3' and Tb12: 5'CTTGTCGAACCGCATACCCT.³⁶

PCR reaction with 50 µl volume containing 8 Pico moles of Tb11 and Tb12 primer, 1.5 mM magnesium chloride, 1 unit of Taq polymerase enzyme (Synagen Co.), 0.2 mM dNTP, 1.5 mM 10 X buffer and 2% DMSO was used.

The conditions of the reaction temperature as follows: the first cycle of 95 °C for 5 min denaturation primary by 30 cycles of 94 °C 30 s, 56 °C 1 min and 72 °C in 40 s and the last cycle 72 °C for 10 min.

Finally, amplified fragments obtained by PCR on 5.1% agarose gel were electrophoresed and 439 bp band was absorbed. To analyze the data, SPSS statistical software was used and the frequency and percentages were calculated. MAS-PCR was used to identify mutations in the katG codon 315 and rpoB gene codons 526, 516, 513 and inhA15.^{37,38} In this method, at each allele-specific primer, the 3' end is positioned for pairing with the associated codons, where most point mutations are found to compare with the wild-type sequence ip37Rv. For each MAS-PCR reaction, a standard 35 µl reaction was used.

3. Results and discussion

In order to monitor and develop appropriate treatment strategies for mycobacterial infections, the rates of mycobacterial infections (MTBC and NTM clinical isolation) and their drug resistance were investigated during 2011–2018. To investigate drug resistance, resistance profiles of clinical isolates of MTBC and NTMs to two first-line antibiotics, namely INH (isoniazid) and RIF (rifampin) were evaluated during the study.

Table 2

The number of clinical samples prepared by type of study during the study period 2011–2018.

Year Specimen type	2011	2012	2013	2014	2015	2016	2017	2018	total
	MTB	NTM							
CSF	16(0.52)	–	90(7.96)	93(3.27)	1(0.09)	4(0.21)	3(0.11)	5(0.19)	212(1.34)
Sputum	3013 (97.45)	362 (90.73)	893 (78.96)	660 (23.21)	1032 (92.14)	1809 (93.88)	2571 (94.66)	2490 (95.73)	12830 (81.05)
BAL	3(0.1)	–	2(0.18)	52(1.83)	2(0.18)	10(0.52)	7(0.26)	9(0.35)	85(0.54)
Pleural	4(0.13)	4(1)	6(0.53)	120(4.22)	1(0.09)	2(0.1)	10(0.37)	5(0.19)	152(0.96)
bronchia	4(0.13)	4(1)	5(0.44)	1062(37.35)	2(0.18)	3(0.16)	5(0.18)	2(0.08)	1087(6.87)
Urine	4(0.13)	–	3(0.27)	52(1.83)	2(0.18)	5(0.26)	5(0.18)	6(0.23)	77(0.49)
g/w	7(0.23)	1(0.25)	6(0.53)	381(13.4)	5(0.45)	2(0.1)	4(0.15)	18(0.69)	424(2.68)
Biopsy	3(0.1)	1(0.25)	8(0.71)	134(4.71)	8(0.71)	4(0.21)	5(0.18)	6(0.23)	169(1.07)
Ascites	–	1(0.25)	45(3.98)	19(0.67)	–	–	2(0.07)	–	67(0.42)
lavage	1(0.03)	–	–	5(0.18)	1(0.09)	2(0.1)	1(0.04)	–	10(0.06)
other	37(1.2)	26(6.52)	73(6.45)	265(9.32)	66(5.89)	86(4.46)	103(3.79)	60(2.31)	716(4.52)
total	3092	399	1131	2843	1120	1927	2716	2601	15829

Fig. 1. Reciprocal alteration in the relative isolation rates (%) in y-axis) of M.TB and NTM strains from 2011 to 2018 (X-axis).**Table 3**

The number and type of nontuberculous mycobacteria during the study period 2011–2018.

Year strain	2011	2012	2013	2014	2015	2016	2017	2018	Total
M.abscessus	–	–	2	1	2	11	14	36	66
M.atypical	–	–	1	–	–	–	–	–	1
M.avium	–	–	–	3	2	2	5	1	13
M.BCG	1	–	–	–	3	–	9	4	17
M.bovis.BCG	–	–	–	11	–	–	–	–	11
M.brendly	1	–	–	–	–	–	–	–	1
M.chelonae	13	2	2	7	7	4	4	3	42
M.chelonae sub abscessus	–	2	–	–	–	–	–	–	2
M.fortuitum	3	–	–	–	–	–	1	–	4
M.genavense	–	–	–	–	–	–	1	–	1
M.gordonae	–	–	–	–	1	2	–	–	3
M.intracellular	1	–	–	1	12	17	23	14	68
M.kansasii	1	–	–	1	3	7	9	2	23
M.parafortuitum	–	–	–	–	–	–	1	–	1
M.parascrofulaceum	–	–	–	–	–	2	–	2	4
M.simiae	30	6	20	6	58	69	70	68	327
M.szulgai	–	–	–	1	–	–	–	–	1
Other NTM	–	–	–	–	6	–	–	–	6
Total NTM isolated	50	10	25	31	94	114	137	130	591

4. Positive isolation rate by microscopic smear, mycobacterial culture and PCR method

During the 8-year study, 15829 samples were collected. Among these samples (7528/15829, 47.56%) mycobacterial isolates including

6937 MTBC (43.82%) and 591 NTM (3.73%) were obtained. The number of positive clinical specimens for MTBC and NTM isolates are also listed in Table 1 and Table 2, respectively. MTBC isolation rates were 47.06% (1224/2601, year 2018) and 65.17% (2015/3092, year 2011) during the study. It is noteworthy that the NTM positive isolation

Table 4

Resistance rate of MTB clinical isolates against first-line antituberculosis drug from year 2011–2018.

Year Antibiotic	2011	2012	2013	2014	2015	2016	2017	2018
No. + PCR specimen	2065	181	551	268	605	998	1506	1354
INH (R)	330(15.98)	53(29.28)	153(27.77)	31(11.57)	221(36.53)	242(24.25)	333(22.11)	254(18.76)
INH (S)	1740(84.26)	103(56.91)	373(67.7)	237(88.43)	380(62.81)	753(75.45)	1172(77.82)	1098(81.09)
RIF (R)	257(12.45)	66(36.46)	151(27.4)	31(11.57)	189(31.24)	196(19.64)	239(15.87)	197(14.55)
RIF (S)	1813(87.8)	120(66.3)	379(68.78)	237(88.43)	412(68.1)	799(80.06)	1266(84.06)	1156(85.38)
MDR TB	134(6.49)	40(22.1)	68(12.34)	4(1.49)	165(27.27)	171(17.13)	200(13.28)	174(12.85)

MDR TB = multidrug resistance tuberculosis, INH = isoniazid, RIF = rifampicin.

*No (%), (R) = resistant (S) = sensitive.

Fig. 2. Resistance rates (%) in y-axis) of 7528 MTB and NTM clinical isolates against the 2 first-line antituberculous drugs, namely INH, RIF during study years (X-axis). M.TB = mycobacterium tuberculosis group, NTM = nontuberculous mycobacteria, MDR TB = multidrug resistance tuberculosis, INH = isoniazid, RIF = rifampicin.

rate increased from 1.62% (30/5092, year 2011) to 5% (266/13001, year 2018).

5. Distribution of MTB and NTM isolates

The distribution of MTB and NTM among mycobacterial clinical isolates was evaluated and illustrated in Fig. 1. Among 7528 clinical isolates of Mycobacterium isolates, the frequency of MTBC isolates showed a decreasing trend as ratios decreased from 65.17% (2015/3092) in 2011 to 47.06% (1224/2601) in 2018. In contrast, there is a significant upward trend in NTM isolates, increasing from 1.62% (50/3092) in 2011 to 5% (130/2601) in 2018. In particular, the frequency of MTB and NTM isolates during these eight years showed a reciprocal change that approached each other during 2014.

In addition, the number and types of NTM isolates during the years studied are shown in Table 3.

M. simiae with 55.33% (327/591) had the highest number followed by *M. intracellulare*, *M. abscessus* and *M. chelonae* with 11.51%, 11.17% and 7.11%, respectively.

Our results show that there is a significant interaction change in MTB and NTM isolation rates at the NRITLD center during the study period despite a concomitant decrease in MTB incidence and a parallel increase in NTM incidence. These observations are supported by our previous findings as well as conclusions from other reports, namely that an increase in the isolation of NTM clinical specimens is associated with a decrease in the prevalence of TB. Possible factors leading to this trend toward growth in NTM strains isolation may include increased susceptibility to these strains, increased recognition of NTM infections by physicians and microbiologists, implementation of public health strategies, TB targeting, and it will also enhance the diagnostic methods used in medical laboratories.

Also, the current findings support our earlier suggestion that improving the availability of new techniques that enable accurate identification of NTM strains may lead to further enhancement of NTM detection in the future.³⁹ The clinical symptoms of MTB infections often

overlap, which is a major problem that needs to be addressed especially when diagnosing NTM infections.^{40,41}

In addition, the continued increase in NTM infections makes it difficult to implement a direct TB treatment program. The short-term strategy (DOTS) indicates that tuberculosis only be treated according to the microscopic results of acid fast sputum smear. As a result of the DOTS program, most patients with NTM are likely to be treated with anti-TB drugs when acid fast bacilli are in their sputum smear before their NTM outcome is available. Unfortunately, this can lead to treatment failure or relapse because of differences between bacterial components, drug resistance patterns, clinical manifestations and treatment outcome between NTM and MTB infection despite some similarities.^{4,14,42,43} The aforementioned problems, ranging from initial diagnosis to treatment outcome, are probably all contributing factors to the increase and spread of NTM strains that appear to be reflected in the upregulation pattern identified in the present study. In this context, early and accurate diagnosis of patients with NTM infection is a prerequisite for effective intervention. Standard operating protocols for the diagnosis and identification of NTM susceptibility need to be established in medical laboratories to improve the early detection of NTM infections. This is all the more necessary as the frequency of NTM isolates is constantly and significantly increasing. In addition, potential causes of this increase should be explored to enable public health strategies that can effectively control these emerging NTM infections.

6. MTBC and NTM clinical isolation antibiotic resistance profiles

Among the 7528 MTB and NTM isolates, the average resistance to INH ratio was 21%, from 15.98% in 2011 to 18.76% in 2018 (Table 4 and Fig. 2). In the case of RIF, the same resistance trend has been increasing and gradually increasing from 12.45% in 2011 to 14.55% in 2018. Concerning the average susceptibility of mycobacterial isolates to INH and RIF, it was almost constant and did not show such a change, with 63% for INH and 78% for RIF, respectively. Finally, the prevalence of MDR TB during the study period increased steadily from 6.49%

(134/2065) in 2011 to 12.58% (174/1354) in 2018.

The subject of the continued expansion of MDR TB, which causes intolerable tuberculosis and leads to patient death, is a worldwide study (21, 44). About 5% of global tuberculosis cases have MDR tuberculosis with about 3.5% incidence of new MDR tuberculosis.^{14,15,44,45} The average prevalence of MDR TB in our study, approximately 14.18%, which is much higher than the global average of 3.5% of MDR TB, and this is very alarming. The MDR tuberculosis rate has remained stable due to the high level of resistance to INH and RIF. Despite global efforts to reduce the number of TB cases, the spread of MDR TB is still a threat, leading to the spread of TB and a significant number of deaths. MDR TB treatment is expensive and time-consuming for both patients and physicians, and is often associated with a high incidence of mismatches and side effects (21, 44). Therefore, more effort and more effective strategies are needed to control TB in different areas. Knowledge and information on the prevalence of mycobacteria, the trend of antibiotic resistance, advanced identification techniques and drug susceptibility testing are important for the effective control of mycobacterial infections. Based on the findings of the present study on mycobacterial infections and drug allergies that have been documented for 8 years, we conclude and recommend that the diagnosis, identification and sensitivity testing for NTM should be standardized and integrated in clinical systems.

Declaration of competing interest

There are no conflicts of interest.

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