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ORIGINAL ARTICLE

Occurrence and risk factors of nontuberculous mycobacteria in tuberculosis-suspected patients in the north of Iran

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ABSTRACT

Background and Objectives: Some Nontuberculous Mycobacteria (NTM) can occasionally infect the human population and cause infections having symptoms similar to tuberculosis (TB). This study tried to provide updated data about the frequency and diversity of NTM species.

Materials and Methods: Suspicious samples of *Mycobacterium tuberculosis* (MTB) with both positive results in Ziehl-Neelsen (ZN) staining and Löwenstein-Jensen medium culturing were evaluated during January 2016 and December 2018 in Gorgan, Iran. After determination of MTB isolates by the growth rate, pigmentation status, the niacin test, and the insertion sequence 6110 (IS6110) PCR assay, other unknown isolates (presumably NTM) were detected by the *16S* rDNA sequencing method and drawing the phylogenetic tree. Based on the patients' demographic information, their risk factors were also assessed.

Results: Among 226 culture-positive samples, obtained from 2994 individuals with suspected symptoms of TB, the analyses found 12 (5.3%) NTM and three *Mycobacterium caprae* isolates. *Mycobacterium simiae* (6/12) was the most prevalent NTM species. The average nucleotide similarity value was 98.2% ± 3.7. In comparison to patients with MTB (211 confirmed cases), other mycobacterium infections were more common in patients over 65 years old (Odd ratio (95% convenience interval): 2.96 (0.69 - 12.59), P = 0.14).

Conclusion: Although the NTM species has a small portion in TB suspected patients, their prevalence has increased, mainly in elderly patients. Moreover, *M. simiae* was the most prevalent NTM species in our region. Therefore, identification of common species in each region is recommended and clinicians should pay more attention to them in each region.

Keywords: Nontuberculous mycobacteria; Tuberculosis; *16S* rDNA; Sequence alignment; Sequence homology; *Mycobacterium simiae*; *Mycobacterium virginiense*

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INTRODUCTION

There are more than 200 known species and subspecies in the genus of *Mycobacterium*. Most of these species are saprophytes and widespread in environmental sources, whereas some are opportunistic or even highly probable pathogens. Among

them, *Mycobacterium tuberculosis* (MTB) and *Mycobacterium leprae* are the most important species and obligate pathogens (1, 2). MTB with some other species can cause tuberculosis in humans or other animals, which are known as the *Mycobacterium tuberculosis* complex (MTBC) group. The most famous species of MTBC are *Mycobacterium africanum (M. africanum), Mycobacterium caprae (M. caprae),* and *Mycobacterium bovis.* The other species are Nontuberculous Mycobacteria (NTM) and can cause pulmonary, skin and soft tissue infections, lymphadenitis, and disseminated infections in both immunocompromised and immunocompetent populations (3, 4).

Governments and clinicians had underestimated the real incidence of diseases caused by NTM for years, because of poor available practical diagnostic techniques. But nowadays, several national studies suggested an increase in the incidence of NTM infections in Asia, the Middle East, Europe, and the United States, indicating the importance of attention to the infections (5, 6). This increase may be due to improvement in the molecular detection methods for NTM identification or even to the changing behavior of these environmental bacteria (7). This becomes even more important when we know that the prevalence of TB is decreasing in most countries, according to the World Health Organization (WHO) report (8). However, there are some gaps in the knowledge of NTM infections and their clinical manifestation. Moreover, in some studies, researchers suggested that following the increase in the rate of NTM infections, the rate of mortality can also rise worldwide due to inappropriate therapy (9).

Most of the clinical signs of NTM infections overlap with TB. This similarity leads clinicians to use inappropriate empirical anti-TB treatment for patients with NTM infections (10, 11). One study showed that around 30% of the patients who had NTM infections, had wrongly received TB treatment (12). NTM species can have different treatment strategies. Identification of the dominant of them in each region can help develop the treatment protocols (13). There is another important point in treating and controlling the disease. The risk factors can be similar to TB and patients with immunodeficiency or blood cancers are at the top of the risk and more attention should be paid to them (14).

The distribution of the bacterial species varies across geographic regions. Some species are unique in the specific areas, but some others are worldwide and are predominant species for the majority of the NTM infections. *Mycobacterium avium* complex (MAC), *M. fortuitum, M. kansasii, M. abscessus* and *M. simiae* were worldwide predominant species in the most previous studies (15, 16). In these regards, the epidemiology of NTM infections needs updating in the developing countries including Iran to cover all gaps in this field. Therefore, this study has attempted to determine the prevalence and diversity of NTM species among TB-suspected patients, according to their gender and age.

MATERIALS AND METHODS

Patient population and study setting. We worked on all samples that had TB-suspected positive cultures, obtained from January 2016 to December 2018 (Fig. 1). The samples belonged to the TB-suspected patients (extrapulmonary or pulmonary tuberculosis) who were referred to the regional tuberculosis reference laboratory in Gorgan, the center of Golestan province, Iran. The province is located in the north of Iran and is the second province with the highest prevalence of TB in Iran. Several urban and rural health centers surveyed the suspected patients and took sputum samples from patients with symptoms of TB after the examinations or referred the patients to the hospitals for collecting the clinical specimens. Reference laboratory staff routinely stained the samples for bacilli by Ziehl-Neelsen (ZN) stain and then conducted for culture on Löwenstein-Jensen medium according to the WHO guideline (17).

The ethics committee of the Golestan University of Medical Science reviewed and approved the study. All the required information was anonymous and the names of patients were de-identified. Samples were obtained with the consent of the patient to identify and treat TB.

Definitions. The American Thoracic Society standard was the reference for the definition of both NTM and TB infections. The most characteristics in these definitions were clinical symptoms of pulmonary TB along with abnormal chest radiograph, positive ZN test, and the growth of colonies similar to MTB morphology (3).

Laboratory examinations. The work focused only

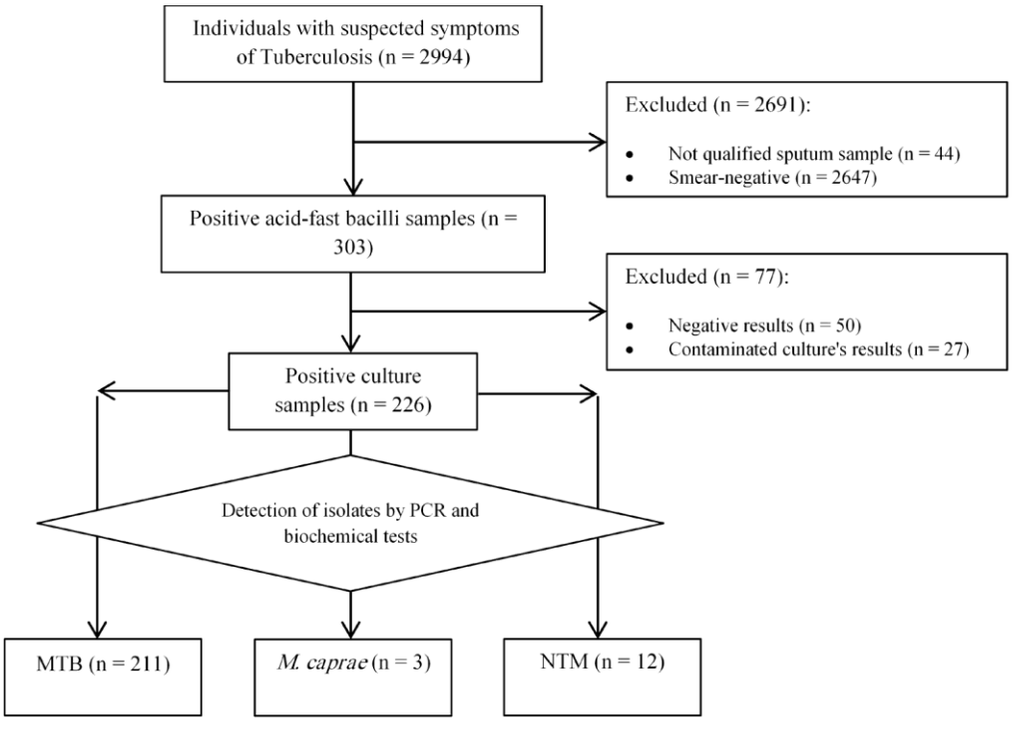

Fig. 1. Flowchart of sample collection and *Mycobacterium* species isolation; MTB: *Mycobacterium tuberculosis*, *M. caprae*: *Mycobacterium caprae*, NTM: Nontuberculous mycobacteria.

on the positive culture samples. Colonies obtained from each sample were evaluated morphologically for *Mycobacterium* genus and used for DNA extraction to identify the species using molecular techniques. DNA of each sample extracted using High Yield DNA Purification Kit (DNPTM Kit, CinnaGen Company, Iran). Each DNA sample was solved in 50 µL of Milli-Q water and stored at - 70ºC. NanoDrop (ND-1000, Thermo Scientific, DE, USA) checked and measured the quality and quantity of each extracted DNA. MTB isolates were checked and differentiated from the other species using the growth rate, pigmentation status, the niacin test, and the insertion sequence *6110* (IS*6110*) PCR assay. This PCR assay can differentiate MTBC from NTM species. MTB species was identified and differentiate using the niacin test. To identify non-MTB species (including other members of MTBC and NTM), all samples with a negative result for the niacin test and a negative result for the IS*6110*-PCR assay were applied for further evaluation. Based on the Eisenach protocol, the PCR assay was used to amplify a 123-bp IS*6110* fragment (18). MTB H37Rv (ATCC 27294) and *M. fortuitum* (ATCC 49404) were the positive and negative controls in this assay, respectively. After excluding the MTB isolates, an amplification of the *16S* rRNA gene with 1500-bp in length was sequenced in each sample using a set of primer (forward: AGAGTTTGATCCTGG CTCAG, and reverse: TGCACACAGGCCACAAGGGA) (19). Briefly, each PCR microtube was filled with the following materials and reached a volume of 50 µl: 25 µL of 2× PCR Master Mix (DNA amplification mixture (PR8251C) containing *Taq* DNA Polymerase, reaction buffer, dNTPs, and $MgCl_2$; CinnaGen Company, Iran), 0.3 µM of each primer and 4 µL DNA template. The PCR program included the initial denaturation at 95°C for 4 min, followed by 35 cycles of the denaturation step at 94°C for 30 s, the annealing step at 55°C for 30 s, and the extension step at 72°C for 2 min, and the final extension step at 72°C for 10 min. An electrophoresis machine (BioRad, USA) separated each PCR product on 1.5% agarose gel (CinnaGen Company, Iran) using a 100V power supply for 45 min. Moreover, the products were purified by AccuPrep PCR purification kit (Bioneer, Seoul, South Korea), according to the manufacturer's instructions. Macrogen Company (South Korea) sequenced the PCR products by an ABI Automated Sequencer system (Applied Biosystems, Foster City, CA).

Chromas software, version 2, analyzed the raw results of the sequencing (http://technelysium.com.au/wp/chromas/). Moreover, the GenBank™ database

was used to analyze and blast the data at the National Center for Biotechnology Information (NCBI) website (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Each sequence result of NTM samples was aligned and compared with several existing sequences of mycobacteria species in this database.

Molecular Evolutionary Genetics Analysis (MEGA) software, version 6, paired the samples and constructed a phylogenetic tree (Fig. 2) using the neighbor-joining algorithm (20). Finally, the sequences of samples of this study were submitted to the GenBank™ database.

Statistical analysis. Demographic and clinical characteristics of these positive culture samples were retrieved from the TB registry system, including age, sex, ethnicity, place of residence, as well as the status of resistance against TB drugs. The information was carefully entered into SPSS version 16 (SPSS Inc., Chicago, USA) and checked by two independent persons. All variables were tested for any statistical association between NTM species and TB confirmed cases using Chi-square or Fisher's exact tests. The association was expressed using odds ratios (ORs) with a 95% confidence interval (95% CI). A *p*-value of less than 0.5 was considered statistically differences.

RESULTS

Patient's characteristics and enrollment. During the study, the health centers received and evaluated 2994 individuals with suspected symptoms of TB. Among them, 303 (10.1%) samples were acid-fast bacilli positive (including suspicion (34, 11.2%), 1+ (73, 24.1%), 2+ (159, 52.5%), and 3+ (37, 12.2%)) in ZN smear test. The origin of 71 (23.4%) of these samples were extrapulmonary sites (details are not shown). Extrapulmonary positive cultures were 34 (15%) out of 226 samples. Out of these 303 samples, 226 (74.6%) were culture-positive and were enrolled in this study (Fig. 1). All 226 culture-positive samples

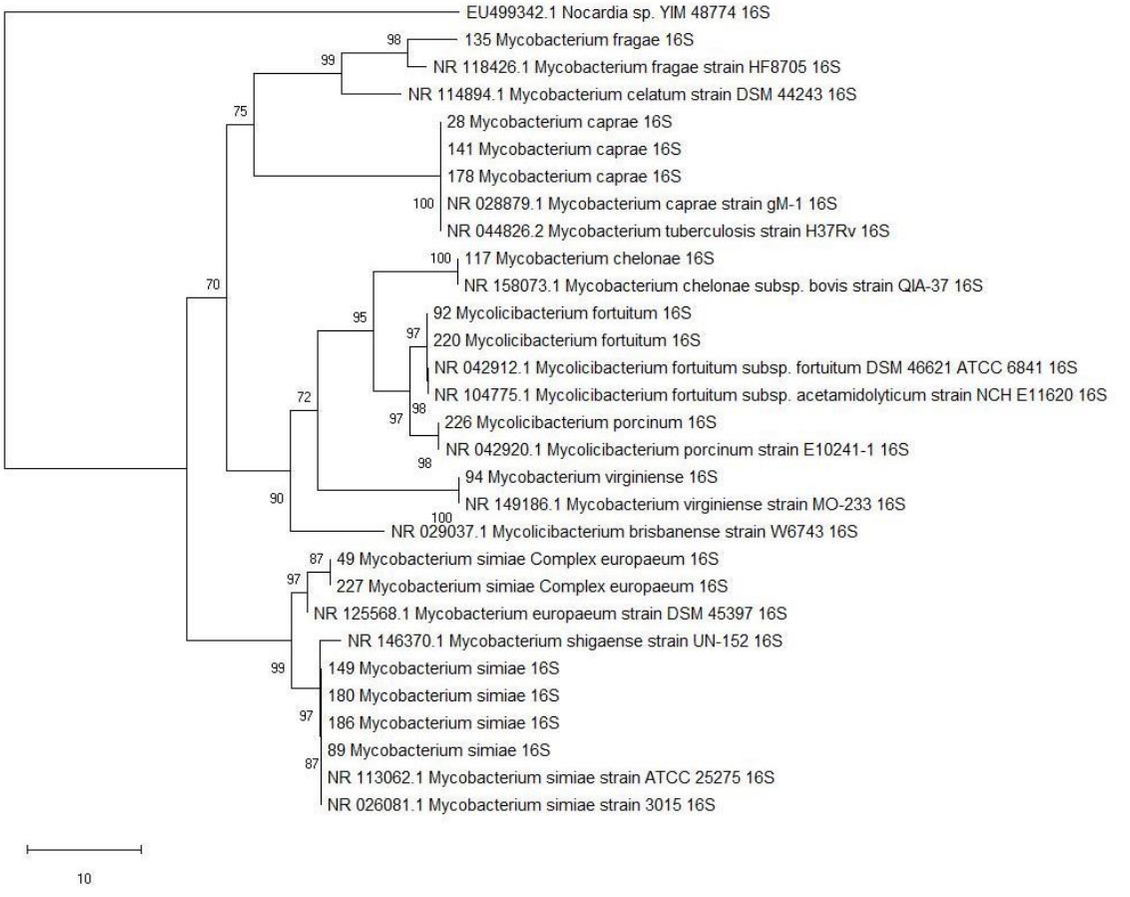

Fig. 2. The phylogenetic tree using the neighbor-joining algorithm based on *16S* rRNA gene sequences.

were a new case. Other samples were culture-negative (50, 16.5%) or had some fungal contamination in culture medium (27, 8.9%) and excluded from further analysis.

Among positive cultures, 211 (93.4%) sample were MTB, which 33 (15.6%) of them had an extrapulmonary origin. In other positive cultures, 3 (1.3%) *M. caprae* (one of the MTCB members), and 12 (5.3%) NTM isolates were detected. Only one NTM isolate (1/12 = 8.3%) had extrapulmonary origin. No samples had simultaneous presence of both MTB and NTM species. The mean age of the patients infected with non-MTB (positive for NTM or *M. caprae* isolates) was 59.9 ± 16.9 years and among them nine patients were men.

NTM species. Table 1 and 2 show the characteristics of the cases, as well as their NTM species. From eight types of detected non-MTB species (including *M. caprae* and seven types of NTM species), five types of species (including *M. caprae*) were slowly growing mycobacteria, whereas three other species were rapidly growing mycobacteria (Table 2). The source of one NTM species, *Mycobacterium virginiense (M. virginiense)*, was extrapulmonary sites (CNS), whereas other NTM and *M. caprae* species had pulmonary sources. The method of *16S* rDNA sequencing could identify all non-MTB isolates.

Among NTM species, *M. simiae* had the highest rate including four *M. simiae* isolates and two *M. simiae* complex European isolates. Two isolates were *M. fortuitum*, a new genus that was previously in *Mycobacterium* genus classification. Furthermore, one isolate was also identified from each of the following NTM species: *M. virginiense, M. chelonae, M. fragae*, and *M. porcinum*. The sequence results of most samples (80%) were completely similar to the reference type strains; however, the average nucleotide similarity value was 98.2% ± 1.8. As an alarming result, seven isolates of these non-MTB isolates were XDR and had a resistance to streptomycin, rifampin, isoniazid, and ethambutol drugs (Table 1). Fig. 2 shows the phylogenetic tree of the mycobacterial species, using the program MEGA.

Risk factors assessment in the non-MTB cases. Because *M. caprae* was detected in three samples and could not be statistically comparable, we put this species in a group with NTM species for statistical analysis called the non-MTB group and compared

Table 1. Demographic, clinical, and laboratory information of the evaluated patients

ID number	Demographic characteristics			Clinical characteristics		Identified species	Laboratory results			Accession Number
	Age	Sex	Place of residence	Isolated location	Previous treatment history		Smear	Nucleotide similarity[1] (%)	Number of Gap / Antibiogram total[2] (% of gap)	results[3]	
28	71	Male	Urban	Pulmonary	New case	*Mycobacterium caprae* (MTB complex)	2+	674/674 (100)	0/674 (0)	S[4]	MK514284
49	58	Male	Urban	Pulmonary	New case	*Mycobacterium simiae* Complex *europaeum*	3+	618/674 (92)	10/674 (1)	S	MK514285
89	60	Female	Rural	Pulmonary	New case	*Mycobacterium simiae Mycolicibacterium fortuitum Mycobacterium virginiense*	2+	643/643 (100)	0/643 (0)	XDR[5]	MK416189
92	62	Female	Urban	Pulmonary	New case		1+	660/660 (100)	0/660 (0)	XDR	MK514286
94	56	Male	Urban	CNS	New case	*Mycobacterium chelonae Mycobacterium fragae*	2+	677/677 (100)	0/667 (0)	S	MK514287
117	47	Female	Urban	Pulmonary	New case		2+	660/660 (100)	0/660 (0)	XDR	MK514288
135	33	Male	Urban	Pulmonary	New case	*M. caprae* (MTB complex)	1+	599/659 (91)	12/659 (1)	XDR	MK514289
141	35	Male	Rural	Pulmonary	New case	*M. simiae*	3+	654/654 (100)	0/654 (0)	S	MK514290
149	84	Male	Urban	Pulmonary	New case	*M. caprae* (MTB complex)	2+	643/643 (100)	0/643 (0)	XDR	MK514291
178	52	Female	Urban	Pulmonary	New case	*M. simiae*	2+	674/674 (100)	0/674 (0)	S	MK514292
180	71	Female	Rural	Pulmonary	New case	*M. simiae M. fortuitum*	2+	643/643 (100)	0/643 (0)	S	MK514293
186	55	Male	Urban	Pulmonary	New case	*Mycolicibacterium porcinum*	2+	643/643 (100)	0/643 (0)	S	MK514294
220	49	Female	Urban	Pulmonary	New case	*M. simiae* Complex *europaeum*	1+	660/660 (100)	0/660 (0)	S	MK514295
226	74	Male	Rural	Pulmonary	New case		2+	660/660 (100)	0/660 (0)	XDR	MK514296
227	78	Male	Rural	Pulmonary	New case		3+	587/651 (90)	8/651 (1)	XDR	MK514297

1: nucleotide similarity based on the reference type strains in NCBI; 2: based on the reference type strains in NCBI; 3: based on the antibiogram for anti-TB drugs; 4: sensitive; 5: resistance to streptomycin, rifampin, isoniazid, and ethambutol drugs.

Table 2. Distribution of mycobacteria species isolated from non-MTB patients

Classification	Species	Number of isolates, N = 15 (%)	Age average of patients
Slowly growing mycobacteria (n = 11)	*Mycobacterium simiae*	4 (26.7)	67.5
	Mycobacterium caprae (MTB complex)	3 (20)	52.7
	Mycobacterium simiae Complex *europaeum*	2 (13.3)	68
	Mycobacterium Virginiense	1 (6.7)	55.5
	Mycobacterium fragae	1 (6.7)	47
Rapidly growing mycobacteria (n = 4)	*Mycolicibacterium fortuitum*	2 (13.3)	56
	Mycobacterium chelonae	1 (6.7)	33
	Mycolicibacterium porcinum	1 (6.7)	74

them with MTB cases. Table 3 shows the details of associations between demographic characteristics of patients with MTB and non-MTB infections. The non-MTB infections were more common in males than females. Moreover, using the age group analysis, more non-MTB cases were in the age group of older than 65 years in comparison to the TB patients, but the difference was not statistical (OR (95% CI): 2.96 (0.69 - 12.59), P = 0.14). Surprisingly, the analysis also showed that living in the rural areas did not increase the chance of non-MTB infection compared to the urban areas (OR (95% CI): 0.45 (0.14 - 1.39), P = 0.16).

DISCUSSION

TB is still the leading cause of infectious death worldwide. In this regard, NTM infections are one of the hidden and underestimated causes of death due to the treatment failure of tuberculosis. Moreover, the classification of these NTM bacteria is rapidly updating and changing. NTM infections represent a growing problem and clinicians should update their information about them every year. Therefore, further annual research in each region can help to increase the understanding of healthcare providers and health system managers and reduce the incorrect treatment rate (21, 22).

The present data showed that around 6% of positive MTB cultures were NTM species. When our results were compared to the other research results in the same geographic region, an increased incidence of NTM infections was observed from 5% in 2015 to 7% in 2019 (2). Although the incidence of NTM infections in our result was lower than the average rate in Iran (23), similar to the most previous studies from Iran and other parts of the world, it showed an increase in the incidence rate (2, 6, 22). The reason behind this may be the advancement of technology

Table 3. Demographic and clinical characteristics of positive *Mycobacterium* culture samples

Characteristics	No. of cases (%)		Statistical results	
	TB (n = 211)	non-MTB (n = 15)	OR (95% CI)	P value
Gender				
Female	106 (50.20)	6 (40.00)	1	-
Male	105 (49.80)	9 (60.00)	1.79 (0.58, 5.50)	0.31
Age group (years)				
< 25	22 (100.00)	0 (0.00)	0	0.99
25-44	51 (29.30)	3 (20.00)	1	-
45-64	53 (30.50)	5 (33.30)	1.52 (0.34, 6.81)	0.58
⩾65	48 (27.60)	7 (46.70)	2.96 (0.69, 12.59)	0.14
Residency				
Urban	91 (48.90)	10 (66.70)	1	-
Rural	95 (51.10)	5 (33.30)	0.45 (0.14, 1.39)	0.16

and laboratory detection methods and/or changing the behavior of NTM bacteria due to changing environmental conditions (23, 24). Furthermore, in recent years, chronic lung diseases caused by air pollution, cancers, and AIDS has increased, which could also lead to an increase in NTM infections (3, 25).

The prevalence rates of NTM are highly variable in the previous studies; however, in most of them, it is below 15%. In a systematic review in china, the average prevalence rate was 6.3% (26). Moreover, a comprehensive report from Europe showed the existence of 6% NTM species in TB-suspected patients (27). In addition, the diversity of the bacterial species varied in the world based on previous studies. But, in each geographic area more similar bacterial species have been observed. Several studies highlighted that *M. simiae* was the highest NTM species in Iran (16, 28). The results of this study also implied the same. Instead, *Mycobacterium intracellulare* was the most isolated species in Zhejiang and Shanghai of China in two studies (2, 29). In two other studies from the United States and Taiwan, MAC was the most common species (3, 30).

The results of this study also confirmed the previous conclusions about the impact of geographical and environmental NTM diversity on the human disease (2, 24). However, some species are worldwide and have been reported in most studies, including *M. simiae*, MAC, and *M. fortuitum* (3, 24). *M. simiae* and *M. fortuitum* were also observed in our study.

The clinical manifestations of NTM infections are similar to TB. Unfortunately, most NTM species are intrinsically resistant to the first-line of the anti-TB drugs and cause treatment failure. Therefore, it is necessary to improve the laboratory capacity for the detection of NTM species in smear- and culture-positive patients, especially for the most well-known emerging NTM species in each geographic area (2).

Based on the statistical analysis in this study, elderly patients can be one of the risk factors for non-MTB infections and can increase the chance of the disease. Therefore, in areas with fewer laboratory facilities and knowledge of the prevalence of NTM and/or non-MTB infections, more attention should be paid to these cases. Xu et al. in 2019 and López-Roa et al. in 2020 also reported the same results. They considered reducing the power of the immune system in elderly patients as a possible reason (2, 4).

Some limitations of this study should be stated here. First, this study only evaluated the samples from one province of Iran. Analysis of full national data can help to get a comprehensive view of the prevalence of NTM. Second, some clinical information on the cases was missed including HIV and bronchiectasis status. Therefore, the statistical evaluation between them and the NTM infections could not be performed. Furthermore, data from suspected TB patients who had negative cultures were not recorded and evaluated in this study. It is better to evaluate data including the source of sampling and the status of being a new case / old case in future studies. Besides, based on the guideline, the samples are routinely cultured on egg-based media, in the laboratories. Some of the species may be lost as they cannot grow perfectly on these media. Therefore, the real prevalence rate could be even higher.

In conclusion, the results showed an increase in the prevalence of NTM infections, chiefly in elderly patients. Moreover, *M. simiae* was the most prevalent NTM species in our region. These bacterial isolates were also highly resistant to TB drugs that are alarming and are important in terms of public health and treatment, as it can lead to the failure of TB treatment. In this regard, the use of rapid molecular detection techniques at the species level and the identification of common species in each region with specific antibiogram patterns are recommended in each region with a high incidence of treatment failure of TB.

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