Identification of *Mycobacterium* spp. isolates from suspected tuberculosis patients using molecular method in Zahedan, IRAN

Identification of *Mycobacterium* spp. isolates

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Abstract

Tuberculosis is one of the oldest zoonotic diseases, with a high prevalence in many low-income countries. Specific and sensitive tuberculosis diagnostic methods in the early stages play a significant role in the life-saving of patients. There is little data available in Zahedan on the prevalence of the Mycobacterium species, so this study aimed to identify the Mycobacterium species in patients with pulmonary tuberculosis in Zahedan. This study included 500 samples collected from sputum from Zahedan. The samples were cultured on LJ, and simultaneously stained with cold ZN. After growth, DNA was extracted and used for molecular identification of the Mycobacterium species from samples. RD typing was used to differentiate members of the *Mycobacterium tuberculosis* complex. Finally, the PCR-RFLP method was used as a comparison method. The typical 543 bp band was observed in all isolates via amplicon PCR-16S rRNA, emphasizing that all isolates belong to the genus Mycobacterium. Sixty isolates were identified as belonging to the MTBC and were classified as M. tuberculosis species. The PCR-RFLP analysis using Alu I on the oxyR gene confirmed that all 60 isolates were M. tuberculosis. Three samples (4.7%) were also positive for NTM. One isolate was categorized in the M. terrae complex group (MTC), and two isolates belonged to M. simiae group. Our results indicated that M. tuberculosis has a high prevalence in the human population of this city. Therefore, screening these individuals plays a significant role in reducing the disease prevalence in Zahedan. It is suggested further studies on the human population to find Mycobacterium strains in the future.

Keywords: *Mycobacterium tuberculosis*, NTM, PCR, PCR-RFLP, Zahedan

1. Introduction

Tuberculosis is a major health problem worldwide, affecting more than one million people every year. *Mycobacterium tuberculosis* (*M. tuberculosis*) is the causative agent of tuberculosis that is transmitted using airborne particles and develops as a latent infection in most cases. In some cases, the patients will experience reactivated disease and dissemination to other organs (1). Antibiotic resistance and co-infection with COVID-19 or HIV in immunocompromised patients are also considered one of the most challenging health issues (1, 2). According to the World Health Organization's (WHO) 2023 Global TB Report, there was a very small increase compared with 2022. Most of the cases were observed in 30 countries, which accounted for 87% of cases of the disease. Five countries accounted for 56% of cases of the disease, including India (26%), Indonesia (10%), China (6.8%), the Philippines (6.8%), and Pakistan (6.3%) (3). Sensitive, specific, and time-saving diagnostic tools are essential to controlling TB. However, the conventional tools for detecting tuberculosis are often inaccurate or time-consuming, particularly when distinguishing between active and latent TB infections. PCR-based methods allow for the accurate and rapid identification of *mycobacterium* species (4). Iran is located in a critical region in the world due to its vicinity to Afghanistan, Pakistan, Iraq, and countries in the north of Iran. The prevalence of TB is not identical throughout the country. In 2019, the highest prevalence of tuberculosis was reported in the provinces of Sistan and Baluchestan, and Golestan (5). Limited data is available on the prevalence of members of the *M. tuberculosis* complex in the human population of Zahedan. Shakiba et al. (2015) reported *M. bois* from suspected patients with tuberculosis in Zahedan (unpublished data). The lack of industrial and semi-industrial livestock increases the possibility of bovine tuberculosis spreading in this city. So, there is a possibility of bovine tuberculosis transmission to humans. Since *Mycobacterium bovis* is inherently resistant to pyrazinamide (the first-line drug for tuberculosis treatment), if *M. bovis* is not correctly identified, the patient may not receive appropriate and effective treatment. According to recent studies, infections caused by Non-Tubercolous Mycobacteria (NTM) are increasing worldwide, especially in poor and developing countries. The clinical symptoms caused by this group of mycobacteria are indistinguishable from *Mycobacterium tuberculosis* complex MTBC. NTM is also resistant to anti-tuberculosis drugs, and it has a different treatment protocol compared to MTBC. Therefore, the rapid and correct diagnosis of this group of mycobacteria is crucial. Thus, this study aimed to identify the species of disease.

2. Materials and Methods

2.1. Ethics approval

All humans involved in the study were handled by the ethics guidelines and protocols approved by the "Research Ethics Committees" of Razi Vaccine and Serum Research Institute, Iran (Approval ID: IR. RVSRI.REC.1403.006).

2.2. Patient population and sample collection

In this study, we included 500 tuberculosis-suspected patients with tuberculosis who were referred to the regional tuberculosis reference laboratory in Zahedan, the center of Sīstān and Baluchistan province, Iran, from 2024 to 2025. A total of 500 specimens (53.9% women and 46% men), between the ages of 22-99 years, were studied. The patient population exhibited symptoms of pulmonary tuberculosis, including cough, phlegm, hemoptysis, and dyspnea. They were referred to the regional tuberculosis reference laboratory in Zahedan from 2024 to 2025, where sputum samples were collected from suspected cases.

2.3. Microbiological process

The samples were cultured as below in Zahedan. Briefly, they were decontaminated with 3.5 M NaOH for 15 min. After that, the samples were centrifuged and neutralized with HCL (0.1 N). The sediments were cultured in two slope tubes of glycerinated and pyruvate Lowenstein–Jensen (LG) medium under Biosafety Level 3 (BSL3). They were then incubated at 37 °C for 8 weeks. Additionally, the sediment from each sample was stained using the cold Ziehl-Neelsen (ZN) technique. The culture tubes were monitored for bacterial growth. After growth, these samples were transferred to the laboratory of the Microbiology Department at Razi Vaccine & Serum Research Institute, Karaj, Iran.

2.4. DNA extraction

DNA was extracted according to van Soolingen's method as below (6). The samples were kept at -20°C for further analyses.

2.5. PCR amplification

Briefly, primers were synthesized (Metabion, Germany) and used to amplify a 543 bp fragment of the *16S rRNA* gene. Then, PCR IS*6110* was carried out for the amplification of the TB complex. The master mix without DNA template and *M. tuberculosis* strain C were used as negative and positive controls, respectively. Subsequently, region of difference (RD) typing (RD1/RD4/RD9/RD12) was performed to differentiate between members of the TB complex. *M. tuberculosis* strain C, *M. bovis* AN5, and *M. bovis* BCG strain were used as positive controls. Distilled water was also used as a negative control. *oxyR* PCR-RFLP was used for final confirmation. Primers and PCR conditions are listed in Table 1.

2.6. PCR-RFLP of *oxyR* gene

PCR-RFLP of a 548-bp region of the *oxyR* gene was carried out according to Sreevatsan et al. (7). The final volume was 12 µL, including PCR product (6 µL), *AluI* (2.5 µL) (Thermo Fisher Scientific, Lithuania), restriction enzyme buffer (2 µL), and distilled water (1.5 µL). The mixture was incubated at 37°C overnight. The products were electrophoresed on agarose gel for 100 min at 90 V. In this study, *M. tuberculosis* strain C, *M. bovis* AN5, and *M. bovis* BCG strain were used as the positive and water without DNA for the negative controls.

Table 1. Primers used in this study

Locus	PCR (Primer sequence)	Size (bp)	PCR condition	References
16S rRNA	ACGGTGGGTACTAGGTGTGGGT TTC TCTGCGATTACTAGCGACTCCG ACTTCA	543	Initial denaturation: 95°, 5 min; 35 cycles: 95°, 1 min, 62°, 1 min, 72°, 1 min Final extension 72°, 10 min	(8)

IS*6110*	CGTGAGGGCATCGAGGTGGC GCGTAGGCGTCGGTGACAAA	245		Initial denaturation: 94°, 3 min; 30 cycles: 94°, 30 sec, 65°, 33 sec, 72°, 40 sec; Final extension 72°, 10 min	(9)
RD1	AAGCGGTTGCCGCCGACCGACC CTGGCTATATTCCTGGGCCCGG GAGGCGATCTGGCGGTTTGGGG	146[1]	196[2]	Initial denaturation: 95°, 5 min; 35 cycles: 95°, 1 min, 62°, 1 min, 72°, 1 min Final extension 72°, 10 min	(10)
RD4	ATGTGCGAGCTGAGCGATG TGTACTATGCTGACCCATGCG AAAGGAGCACCATCGTCCAC	172	268	Initial denaturation: 95°, 5 min; 35 cycles: 95°, 1 min, 62°, 1 min, 72°, 1 min Final extension 72°, 10 min	(10)
RD9	CAAGTTGCCGTTTCGAGCC CAATGTTTGTTGCGCTG GCTACCCTCGACCAAGTGTT	235	108	Initial denaturation: 95°, 5 min; 35 cycles: 95°, 1 min, 62°, 1 min, 72°, 1 min Final extension 72°, 10 min	(10)
RD12	GGGAGCCCAGCATTTACCTC GTGTTGCGGGAATTACTCGG AGCAGGAGCGGTTGGATATTC	369	306	Initial denaturation: 95°, 5 min; 35 cycles: 95°, 1 min, 62°, 1 min, 72°, 1 min Final extension 72°, 10 min	(10)
PCR-RFLP on *oxyR* gene	GGTGATATATCACACCAT CTATGCGATCAGGCGTACTTG	230	79, 148, and 236	Initial denaturation: 94 °C 3 min; 30 cycles: 94 °C, 30 sec, 65 °C, 30 sec, 72 °C, 40 sec Final extension: 72 °C, 10 min	(7)
16S rRNA long	TAACACATGCAAGTCGAACGG AAA GG ACTTCGTCCCAATCGCCGATCCC A CC	1436		Initial denaturation: 95 °C 5 min; 35 cycles: 95 °C, 1 min, 60 °C, 45 sec, 72 °C, 40 sec Final extension: 72 °C, 10 min	(8)
hsp65	ACCAACGATGGTGTGTCCAT CTTGTCGAACCGCATACCCT	441		Initial denaturation: 95 °C 5 min; 35 cycles: 95 °C, 1 min, 60 °C, 45 sec, 72 °C, 40 sec Final extension: 72 °C, 10 min	(8)
rpoB	TCAAGGAGAAGCGCTACGA GGATGTTGATCAGGGTCTGC	359		Initial denaturation: 95 °C 5 min; 35 cycles: 95 °C, 1 min, 60 °C, 45 sec, 72 °C, 40 sec Final extension: 72 °C, 10 min	(8)

[1] *M. tuberculosis*

[2] *M. bovis* BCG

2.7. Identification of NTM species

Targeting *16S rRNA long*, *hsp65*, and *rpoB* genes using PCR and sequence analysis was performed to identify nontuberculous mycobacteria (NTM). *M. tuberculosis* strain C and master mix without DNA template were used as positive and negative controls, respectively. The outcomes of the nucleotide sequencing were analyzed using Chromas and Clustal X programs, and NCBI BLAST was used to align the analyzed sequences.

2.8. Phylogenetic analysis of the *16S rRNA* long, *hsp65,* and *rpoB* genes

Phylogenetic trees of NTM species were constructed using the Neighbor-Joining method via Molecular Evolutionary Genetics Analysis (MEGA) XI software.

3. Result

Sixty-three isolates grew in two LJG and LJP after 2 months. However, LJG was better than that one. Acid-fast bacilli were observed in all the samples. All purified DNAs had a concentration greater than 100 ng/µL; the 260/280 ratio of all samples was between 1.8 and 2.2. The electrophoresis results of the purified DNAs showed that all DNAs had high quality without any breaks in the electrophoresis gel. In this study, the molecular identification of 63 isolates of pulmonary tuberculosis patients was investigated using PCR-*16S rRNA*. The typical 543 bp amplicons were observed in all isolates, which showed all isolates belonged to the genus *Mycobacterium*. In the next stage, PCR IS*6110* was performed, and it was determined that 60 isolates out of 63 isolates (95/3%) were members of the MTBC. Based on the RD Typing result, it was determined that all the TB complex group were recognized as *M. tuberculosis* (**Fig 1**).

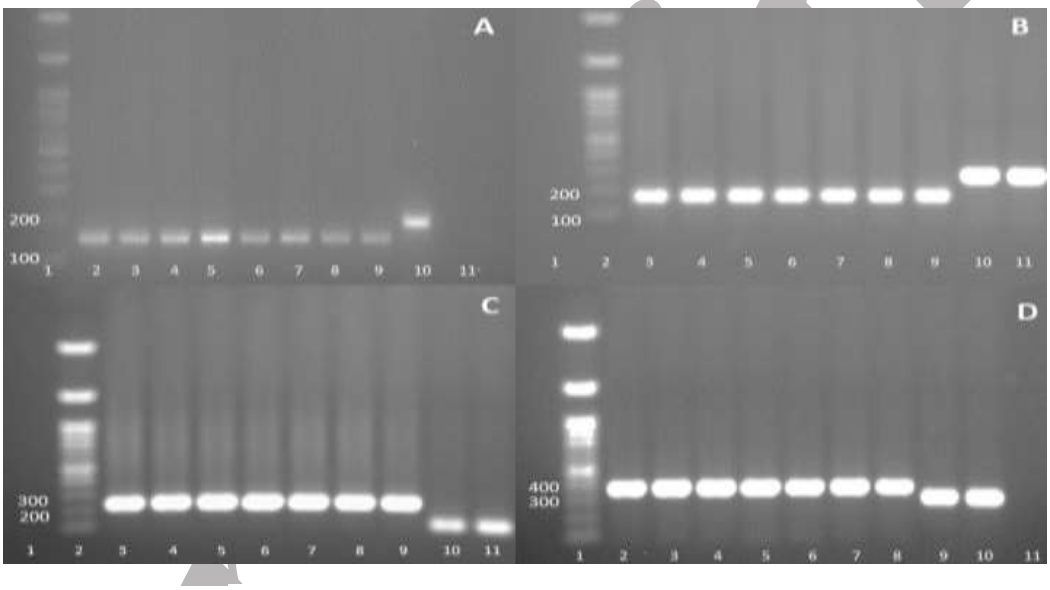


Fig. 1. RD typing. A: Samples were analyzed by primers specific for RD 1. Lane 1: Marker DNA (100-bp ladder), lane 2: Positive control of *M. tuberculosis* C strain, lanes 3–9: suspected samples, Lane 10: *M. bovis* BCG strain, Lane 11: Negative control (no DNA added). B: Samples were analyzed by primers specific for RD 4. Lane 1: Negative control (no DNA added). lane 2: Marker DNA (100-bp ladder), lane 3: Positive control of *M. tuberculosis* C strain, lanes 4–9: suspected samples, Lane 10: *M. bovis* AN5 strain, Lane 11: *M. bovis* BCG strain, C: Samples were analyzed by primers specific for RD 9. Lane 1: Negative control (no DNA added). lane 2: Marker DNA (100-bp ladder), lane 3: Positive control of *M. tuberculosis* C strain, lanes 4–9: suspected samples, Lane 10: *M. bovis* AN5 strain, Lane 11: *M. bovis* BCG strain. D: Samples were analyzed by primers specific for RD 12. Lane 1: Marker DNA (100-bp ladder), lane 2: Positive control of *M. tuberculosis* C strain, lanes 3–8: suspected samples, Lane 9: *M. bovis* AN5 strain, Lane 10: *M. bovis* BCG strain, Lane 11: Negative control (no DNA added).

Digestion with *AluI* yielded three fragments of 79, 146, and 236 bp for DNA samples from *M. bovis* AN5 and *M. bovis* BCG strain. However, only one band at 230 bp was observed for the DNA sample from *M. tuberculosis* strain C and all of the isolates. PCR-RFLP on *oxyR* demonstrated that all of them were recognized as *M. tuberculosis*, this was confirmed as the

result of PCR-RD typing. Three isolates were negative for PCR-*IS6110*. The nucleotide sequencing results based on *16S rRNA l*ong, *hsp65,* and *rpoB* genes showed that these three samples (4.7%) were positive for NTM. It should be noted that these 3 isolates were isolated from three patients with the following characteristics: Patient 1: was a 70-year-old male without underlying diseases. Patient 2: was a 57-year-old male with high blood pressure and diabetes. Patient 3: was a 49-year-old male with asthma. The outcomes of the nucleotide sequencing based on *16S rRNA* long showed the similarity to *M. kumamotonensis* in one isolate (99.5%) and *M. simiae* in two isolates (99.6% and 99.8%, respectively). Gene sequence alignment results based on *hsp65* showed the similarity to *M. senuense* in one isolate (97.8%) and *M. simiae* in two isolates (99.3% and 99.1%, respectively). Finally, the outcomes of the nucleotide sequencing based on *rpoB* showed the similarity to *M. terrae* in one isolate (93.3%) and *M. simiae* in two isolates (99.1% and 99.4%, respectively). So, the NTM isolates obtained from this study can be divided into two groups in the phylogenetic trees of the *16S rRNA* long, *hsp65,* and *rpoB* genes: One isolate was categorized in the *M. terrae* complex group (MTC), and two isolates belonged to *M. simiae* group. (**Fig 2-4**).

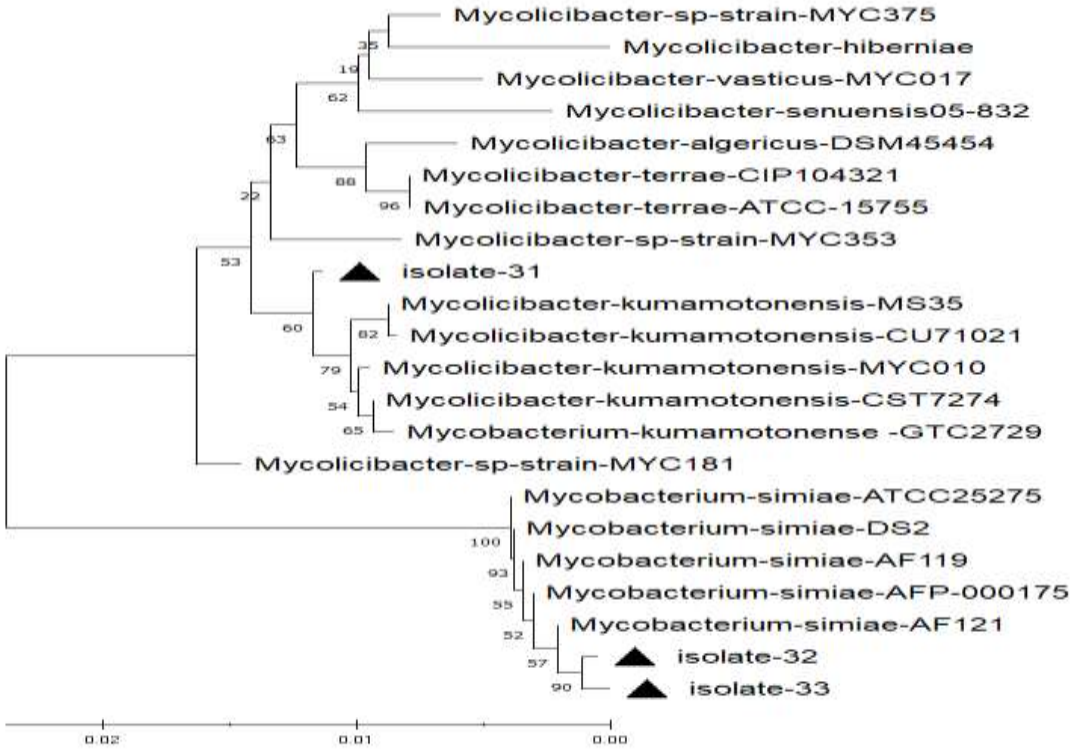
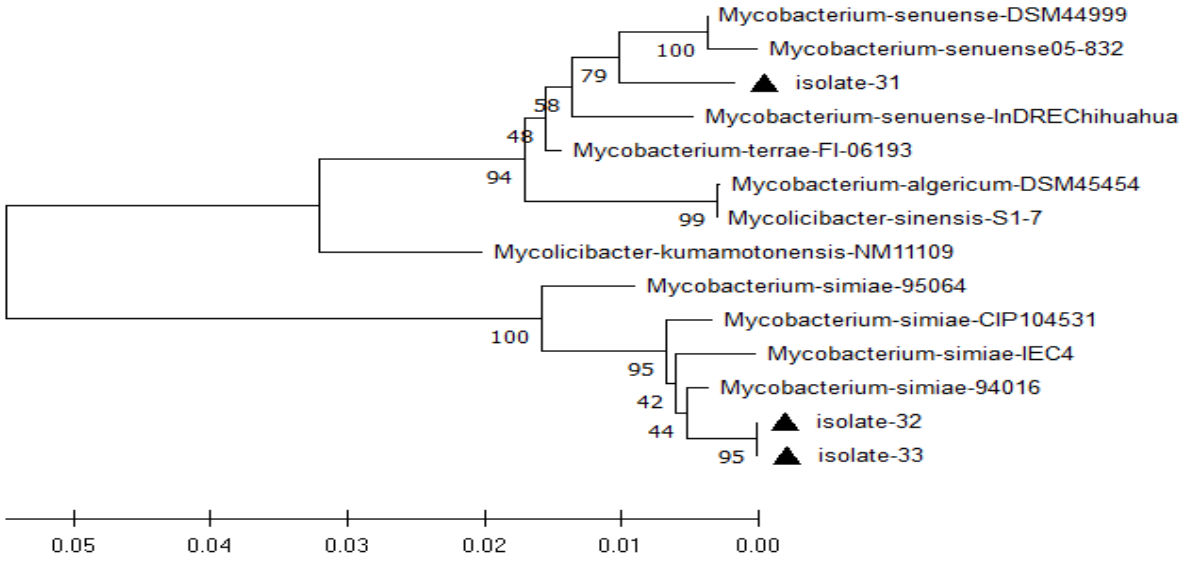
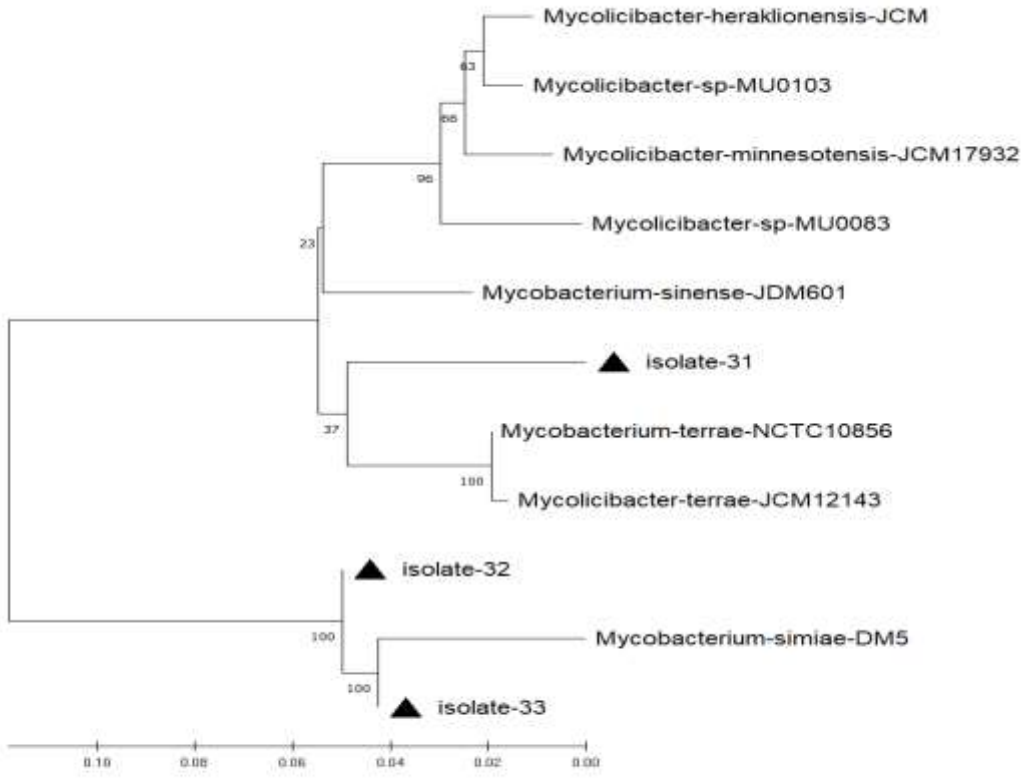


Fig. 2. Phylogenetic tree of different strain and isolates of *Mycobacterium* based on *16S rRNA* long



182

183 **Fig. 3**. Phylogenetic tree of different strain and isolates of *Mycobacterium* based on *hsp65*



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185 **Fig. 4.** Phylogenetic tree of different strain and isolates of *Mycobacterium* based on *rpoB*

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4. Discussion

Different genotyping methods were used to differentiate members of the *M. tuberculosis* complex, which showed a wide range of discrimination power. In this study, the molecular identification of 63 isolates of pulmonary tuberculosis patients in Zahedan was investigated. For this purpose, PCR-*16S rRNA* was performed, which showed that all isolates belonged to the genus *Mycobacterium*. In the next stage, PCR-IS*6110* was performed, and it was determined that 60 isolates out of 63 isolates (95/3%) were members of the MTBC. Based on the RD Typing result, it was determined that all 60 isolates studied were *M. tuberculosis,* similar to Tourabi,study (11). Warren reported that the multiplex PCR method based on the genomic regions of difference enabled the rapid and accurate differentiation of the *M. tuberculosis* complex (10). In this study, we used this method. Finally, the comparative genotyping technique of PCR-RFLP on the *oxyR* gene was performed. The result showed the belonging of 60 isolates to the *M. tuberculosis* species, and none of them were infected with *M .bovis*; it confirmed the result of RD typing. Sreevastan reported that *oxyR* PCR-RFLP is useful for differentiating *M. bovis* and *M. bovis* BCG from other members of MTBC (7). The result of this study showed the good ability of this method for confirmation of RD typing, similar to previous study (11)**.** In this city, 60 isolates were *M. tuberculosis,* and none of the 60 isolates were infected with *M. bovis,* similar to Tourabi study (11). However, in the Dehghanpour study, *M. bovis* was identified in approximately 7.4% of cases in Zanjan province (12). Shakiba Mehr also conducted a study on pulmonary tuberculosis and reported infection with *M. bovis* in Zahedan (unpublished data), contrary to this study. It is perhaps due to the improvement of the health level and the use of pasteurized milk and dairy products in this city. On the other hand, due to drought and the subsequent loss of traditional livestock farming, especially in rural areas, and the development of industrial livestock farming, the consumption of local milk and dairy products has decreased. This is very important because *M. bovis* is considered a health indicator of the community. Based on our results, the other three isolates (4.7%) were recognized as an NTM species. Nowadays, non-tuberculous mycobacteria are increasing worldwide, especially in developing countries. Karami-Zarandi (2019) reported 12% of positive cases as an NTM sample (13). Differentiation of non-tuberculous mycobacteria from the TB group is not possible by conventional methods; therefore, molecular-based methods play a very important role in the differentiating of non-tuberculous mycobacteria. For example, Leidsema reported that the sequencing of *16S rRNA*, *hsp65,* and *rpoB* genes is a reliable method for identifying these bacteria (14). In this study, we used the sequencing of *16S rRNA* long, *hsp65,* and *rpoB* genes for identifying non-tuberculous mycobacteria and phylogenetic study. Previous studies reported that the geographical distribution of the NTM group varies throughout (15). In this study, *M. simiae* was the most predominant among the NTM group, similar to the Shafipour study (16). Another study showed *M. avium* complex as the most predominant among NTM species in Asia including in South Korea, Japan, and Taiwan (17). Firoozeh et al. reported that the prevalence of non-tuberculous mycobacteria has increased in Iran. *M. simiae* was the most common slow-growing NTM group, similar to our study, while *M. fortuitum*, *M. terrae,* and *M. gastri* were the most common fast-growing NTM species, respectively (18). In this study, one NTM isolate showed heterogeneity resulting from the sequencing of all three genes: Based on our result, sequencing using the *16S rRNA* gene showed higher discriminatory power and percentage similarities (99.5%) than the *hsp65* gene (97.8%) and the *rpoB* gene (93.3%). *16S rRNA long* gene produces a 1436 bp fragment, which is the largest amplified fragment of the *16S rRNA* gene, so the results of the *16S rRNA* long gene are more valid than the two other genes similar to Hassansoltan Solaghani study (19). Lee et al. also reported that the sequencing of *16S rRNA* and *hsp65* genes is useful for the identification of the MTC, but the discriminatory power of the *hsp65* gene is lower compared

to *16S rRNA*,-similar to our study (20). In this study, one NTM isolate was identified as *M. kumamotonensis*. Also, the similarity of this isolate to *M. senuense* and *M. terrae* was identified through the *hsp65* and *rpoB* genes, respectively. According to studies, *M. kumamotonensis* and *M. senuense* are new species of the MTC (21). *M. kumamotonensis* has a great similarity to members of the MTC based on the sequencing of *16S rRNA, hsp65*, and *rpoB* genes (22). *M. senuense* is also classified in MTC based on phenotypic characterization (23). So, one of three NTM isolates belonged to MTC, and two of three NTM isolates were identified as *M. simiae* by sequencing of *16S rRNA, hsp65*, and *rpoB* genes. Two out of three NTM isolates were isolated from sputum samples of patients with underlying diseases. The risk of infection with NTM in underlying disease such as diabetic patients, and immunodeficiency diseases is greater than in patients without underlying disease (24), similar to our study. Wang reported a relationship between patients with diabetes mellitus and NTM. Although there were no reported significant differences in age and gender groups (24). According to the previous study and our study, *M. simiae* is the most common slow-growing nontuberculous mycobacterium among NTM species in Iran. Due to *M. simiae* is only niacin-positive NTM, similar to *M. tuberculosis*, and it can cause pulmonary infections in both immunocompromised and normal immune system individuals (25), so its differentiation from *M. tuberculosis* is very vital, and it will help in the rapid recovery of the patients. Another NTM species, *M. kumamotonensis* was isolated from the sputum sample of a 70-year-old male patient without underlying disease. As mentioned, this bacterium is a new species of the MTC. So far, no report has been announced of its isolation in Iran. Based on our results, *M. tuberculosis* has a high prevalence in this city, similar to the previous study, due to its proximity to the borders of Afghanistan and Pakistan. Khammarnia reported that there was observed a significant relationship between non-Iranian nationality with tuberculosis in Sistan and Balochistan province (26). In this study, 5% of disease cases belonged to the non-Iranian population, so, we should consider the possibility of disease transmission and circulating strains in non-Iranian populations as one of the important factors in this city. We should also take into account other factors such as malnutrition, smoking, tropical climate, population density, and poverty. Thus, the high prevalence of circulating *M. tuberculosis* in the human population of Zahedan suggests the screening of these patients for the reduction of the disease cases. So, further studies on the human population to find *Mycobacterium* strains are required in the future.

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Declarations

Author 's contribution

Acquisition of data, Analysis and interpretation of data, Drafting of the manuscript: **F.A.**

Study concept and design, Drafting of the manuscript, Critical revision of the manuscript for important intellectual content, Study supervision: **N.M**

Study concept and design, Drafting of the manuscript –Critical revision of the manuscript for important intellectual content, Study supervision: **S.M.B**

Drafting of the manuscript, Critical revision of the manuscript for important intellectual content, Study supervision: **A.R.S.K.**

Drafting of the manuscript, Critical revision of the manuscript for important intellectual content: **L.A.K**

Conflict of Interest

The authors declare no conflict of interest.

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Data availability

The data that support the findings of this study are available on request from the corresponding author.

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