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Prevalence and Molecular Typing of Non-Tuberculous Mycobacteria in Hospital Water Sources of Tehran, Iran

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ABSTRACT

Background: Non-tuberculous mycobacteria (NTM) pose a growing public health concern, especially in regions with high prevalence rates. Iran, situated near high-burden countries like Afghanistan and Pakistan, is particularly vulnerable. This study aimed to investigate the molecular epidemiology of NTM in water samples from Farhikhtegan Hospital, Tehran.

Methods: A total of 70 water samples were collected from various hospital departments. After culture and phenotypic identification, 33 mycobacterial isolates were subjected to polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis of the rpoB gene.

Results: *Mycobacterium fortuitum* type I was the most prevalent NTM species, accounting for 81% of the isolates. *Mycobacterium kansasii* type I and *Mycobacterium gordonae* type I followed, each comprising 6% of the isolates. Less common species included M. gordonae type II and *Mycobacterium intracellulare*. The PCR-RFLP method proved to be a sensitive and accurate tool for identifying NTM species.

Conclusion: This study provides valuable insights into the NTM profile in Farhikhtegan Hospital and underscores the importance of effective surveillance and control measures to mitigate the risk of NTM infections.

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Introduction

The epidemiology of NTM infections varies greatly across geographical regions. The Middle East, with its high burden of tuberculosis, faces unique challenges in managing NTM due to several factors. Firstly, proximity to high-burden countries like Afghanistan and Pakistan increases the risk of NTM transmission and creates confusion during diagnosis, potentially leading to delays in identifying and treating NTM infections (1-3). Secondly, warm climates in this region favor the persistence of NTM in environmental sources like soil and water. Contaminated water systems in hospitals and inadequately treated recreational facilities can act as reservoirs for NTM, posing a threat to immunocompromised individuals and those undergoing procedures using contaminated water (4).

Furthermore, limited data on the specific NTM species prevalent within healthcare settings in the Middle East hinders the development of effective preventive and treatment strategies. This lack of comprehensive data makes it difficult to assess the true burden of NTM infections and implement targeted control measures to prevent transmission and optimize patient care (5).

Recent studies conducted after 2015 in Iran have begun to shed light on the NTM landscape within the country. In a 2017 study by Mortazavi et al., researchers investigated the prevalence of nontuberculous mycobacteria in respiratory samples from Iran. They identified *Mycobacterium fortuitum* as the most frequent isolate, followed by *Mycobacterium simiae*, *Mycobacterium kansasii*, *Mycobacterium gordonae*, and *Mycobacterium conceptionense* (6). This highlights the diverse range of NTM species present in Iran and their potential role in respiratory illnesses. Similarly, another study explored the clinical significance of NTM isolated from respiratory samples. Their findings suggest that NTM infections in endemic settings may contribute to the dissemination of various diseases and impact tuberculosis control. However, further research is needed to fully evaluate the clinical significance of NTM isolates (7). These studies underscore the growing recognition of NTM as a public health concern in Iran and emphasize the need for continued research to understand the specific distribution and clinical impact of different NTM species (8-9).

Our present study investigates the prevalence and diversity of Non-tuberculous Mycobacteria (NTM) in Farhikhtegan Hospital, Tehran, Iran. By employing PCR-RFLP targeting the rpoB gene, we aimed to contribute to a more comprehensive understanding of NTM epidemiology in the region (10). This research provides crucial information about the diversity of NTM species present within the hospital environment. By evaluating the performance of PCR-RFLP, we demonstrate its reliability for accurate NTM detection. Our findings underscore the significance of molecular methods like PCR-RFLP for timely and precise NTM identification, which is essential for initiating appropriate treatment regimens. By shedding light on the molecular characteristics of NTM in Farhikhtegan Hospital, this study can inform the development of targeted prevention strategies, improve diagnostic accuracy, and optimize resource allocation for effective NTM management within healthcare settings.

Materials and Methods

Sampling

Between January 2021 and January 2023, researchers conducted a 24-month study to collect 70 water samples from diverse locations within Farhikhtegan Hospital in Tehran, Iran. The sampled water sources comprised tap water and drinking water tanks. Approximately one liter of water was collected per sample in sterile glass bottles and transported to the laboratory on ice for immediate analysis. Chlorinated drinking water was utilized to supply both the water tanks and dental unit water systems. Sterile water served as

the water source for ventilator systems, while hemodialysis machines employed a standardized procedure to generate water (11). Physical and chemical characteristics of the collected samples were determined using standard methods (11-13).

Mycobacteria isolation

Water samples were processed and decontaminated according to standard laboratory protocols, ensuring the removal of any potential contaminants that could interfere with NTM isolation (13). Briefly, this involved filtering 500 ml of each sample through a specialized membrane filter with pores measuring 0.45 micrometers (Millipore, USA). The filtered samples then underwent decontamination using a 0.05% cetylpyridinium chloride solution for 30 minutes at room temperature (14). Next, 200 microliters of the remaining sediment were inoculated onto two separate Lowenstein-Jensen media plates (Thermo Scientific, USA). These plates were incubated at two different temperatures: 37°C and 25 °C, for a period of two months. The cultures were examined twice weekly for signs of growth, including rate of growth, colony appearance, and any pigmentation. Any colonies that grew were then stained using the Ziehl-Neelsen method to identify the presence of acid-fast bacilli (AFB). If a colony stained positive for AFB, standard phenotypic tests were performed to further identify the bacteria. These tests included niacin accumulation, nitrate reduction, catalase activity, iron uptake, and arylsulfatase activity. The specific protocols for these tests followed established guidelines (11, 15). Finally, for confirmed acid-fast positive isolates, molecular identification methods were employed.

Molecular identification of species: PCR-RFLP

The PCR-RFLP technique was utilized to ascertain the species of the bacterial isolates by targeting the rpoB gene (16). The DNA of these bacteria was isolated using the HiPurA® Mycobacterium tuberculosis DNA Purification Kit (HiMedia, India), following the guidelines provided by the manufacturer. Post-extraction, the DNA underwent purification and was then conserved for subsequent analysis. The amplification of the rpoB gene was conducted with the RPOB1 forward primer (5'-TCAAGGAGAAGCGCTACG-3') and the RPOB2 reverse primer (5'- GGATGTTGATC AGGGTCTGC-3'). The PCR mixture, with a total volume of 25µl, included 2U of Taq polymerase, 200 mM of dNTPs,10 mM of Tris-HCl (at pH 8.3), 1.5 mM of MgCl2, 20 pmol of each primer, and 2ng of the DNA template. The PCR cycling conditions were set to an initial denaturation at 95 °C for 5 minutes, followed by 45 cycles of amplification (each cycle consisting of 60 seconds at 95 °C, 60 seconds at 56 °C, and 60 seconds at 72 °C, respectively), and concluding with a final extension at 72 °C for 7 minutes, all performed in an Eppendorf X40 thermocycler. The resultant PCR products were then electrophoresed on a 1.5% agarose gel, with the DNA bands being made visible through erythrogel staining and subsequently photographed. For further analysis, the amplified *rpoB* gene segments were enzymatically cleaved using the HaeIII and MspI (HpaII) restriction enzymes (Thermo Scientific, USA), adhering to the manufacturer's protocols. The amplified fragments were then separated using 2% polyacrylamide gel electrophoresis, and the RFLP patterns were examined based on the size of the fragments.

Results

Sample Analysis

The initial analysis focused on the physical properties of the samples. The pH was measured to be between 7 and 7.9, indicating a neutral to slightly alkaline range. Temperature readings ranged from 15 to 60 °C. Next, filtration was

employed to separate desired components from the samples. A specialized filtration device, the Pall-Aqua Safe Water Filter AQ14F1SA (USA) with a 0.2 µm pore size, was used. This device is capable of withstanding high pressure (-400 bar) and is equipped with a 0.2-micron filter for efficient particle removal.

Phenotypic analysis

Phenotypic analysis of 70 water samples identified 33 isolates as NTM. Pigmentation profiling further differentiated these isolates, with three exhibiting scotochromogenic characteristics suggestive of *M. gordonae*, one displaying non-chromogenic features consistent with *M. intracellulare*, and two demonstrating photochromogenic properties indicative of *M. kansasii*. Growth rate analysis revealed a predominance of fast-growing NTM (27 isolates), potentially belonging to the *M. fortuitum* group. The remaining 6 isolates exhibited slow growth, suggesting they may represent other NTM species.

Genotypic Characterization of NTM Isolates

PCR-RFLP analysis targeting the rpoB gene definitively identified previously isolated NTM. Amplification yielded a 360 bp fragment, subsequently digested with HaeIII and MspI (HpaII) restriction enzymes. Polyacrylamide gel electrophoresis separated the digested fragments, revealing distinct patterns for species identification (Table 1). Notably, 27 isolates were confirmed as *M. fortuitum* subtype I based on the characteristic banding pattern (70/105/175 bp for MspI and 120/90/80 bp for HaeIII). Similarly, slow-growing isolates were identified: one *M. intracellulare* type I (175/105/80 bp for MspI and 180/90 bp for HaeIII), two *M. kansasii* subtype I (175/60/40/30 bp for MspI and 205/90 bp for HaeIII), and three *M. gordonae* (including two subtype I with 145/95/40/30 bp for MspI and 210/98/90 bp for HaeIII, and one subtype II with 145/100/40 bp for MspI and 330 bp for HaeIII). The patters of digested ropB PCR products are shown in Figures 1 and 2.

Discussion

This study investigated the presence and characteristics of non-tuberculous mycobacteria (NTM) in water samples. Our findings demonstrate the presence of a diverse NTM population, highlighting the potential public health concern associated with these environmental mycobacteria. Initial phenotypic analysis identified 33 NTM isolates from 70 water samples. Pigmentation characteristics provided preliminary differentiation, suggesting the presence of *M. gordonae*, *M. intracellulare* , and *M. kansasii*.

Table 1. Restriction Fragment Length Polymorphism profile of isolated strains.

Isolates	Pattern	
	HaeIII	MspI
M. *fortuitum* subtype 1	120/90/80	175/105/70
M. *gordonae* subtype 1	210/98/90	145/95/40/30
M. *gordonae* subtype 2	330	145/100/40
M. *kansasii* subtype 1	205/90	175/60/40/30
M. intracellulare type I	180/90	175/105/80

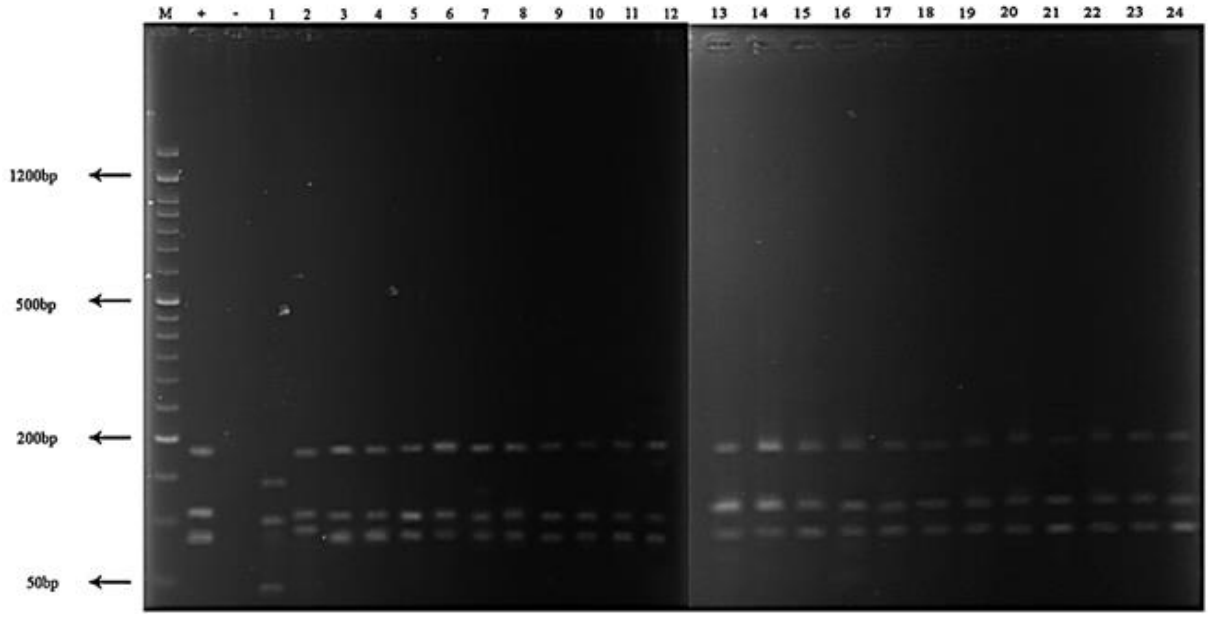

Fig 1. Differential identification of non-tuberculosis mycobacteria by digestion of rpoB 360-bp PCR amplicons by MspI. M:100 bp ladder; +: control,-:blank, 1-24 samples. Sample number 1 is considered as *M. gordonae* subtype 2 and samples number 2 to 24 are *M. fortuitum* subtype 1.

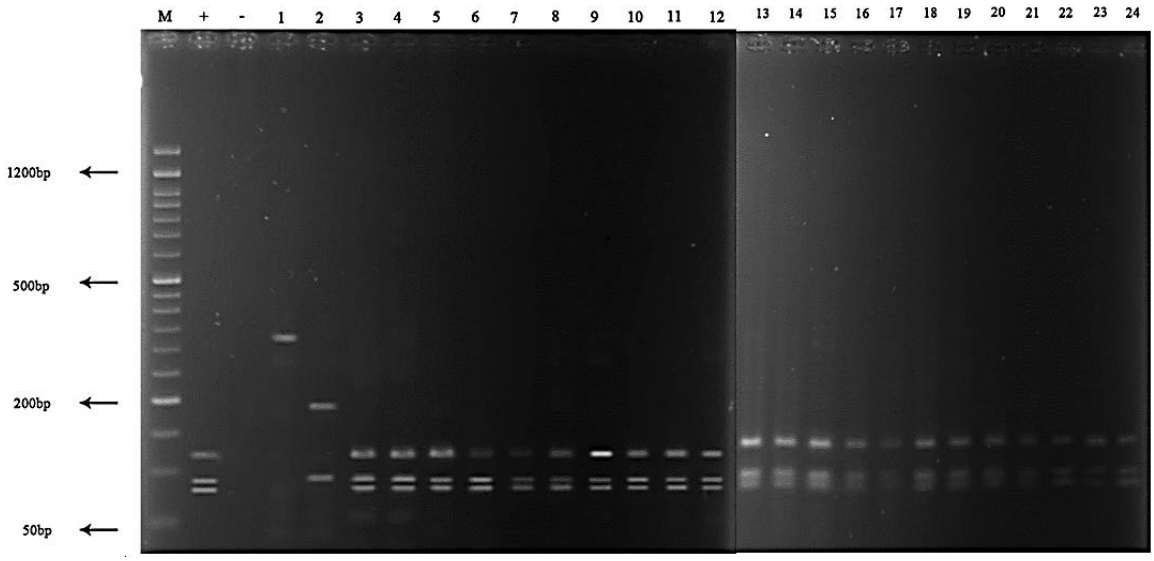

Fig 2. Differential identification of non-tuberculosis mycobacteria by digestion of rpoB 360-bp PCR amplicons by HaeIII. M:100 bp ladder; +: control,-:blank, 1-24 samples. Sample number 1 is considered as *M. gordonae* subtype 2 and, sample number 2 is considered as *M. kansasii* subtype 1, and samples number 3 to 24 are *M. fortuitum* subtype 1.

Growth rate analysis further categorized the isolates, with a predominance of fast-growing NTM (n=27), potentially belonging to the *M. fortuitum* group. These findings are consistent with previous studies reporting *M. fortuitum* as a common NTM found in water environments (17-19).

Interestingly, no significant correlation was observed between the presence of mycobacteria in the water samples and the total chlorine concentrations. However, a link was identified between the presence of NTM and the water temperature ($p<0.05$). These findings suggest that factors beyond chlorine disinfection may influence NTM presence in water.

Our investigation of pigmentation revealed a diverse range of NTM isolates within the water samples. Three isolates were classified as scotochromogenic (*M. gordonae*), one as non-chromogenic (*M. intracellulare*), and two as photochromogenic (*M. kansasii*). These findings suggest that scotochromogenic NTM species, particularly *M. gordonae*, may exhibit greater resistance to the water treatment conditions prevalent in Farhikhtegan Hospital, as well as various physical and chemical factors, aligning with previous observations by previous studies (20, 6, 21).

While phenotypic methods remain valuable for initial NTM identification at the species level, they have limitations in terms of accuracy and specificity. Alternative methods, such as high-performance liquid chromatography (HPLC), can provide more precise identification but often require specialized equipment and a significant amount of microbial material (21).

This observation aligns with previous research in this background (16). Studies in different locations have all documented the presence of NTM in various water sources, including ice, public drinking water, and even hospital tap water (22-24). The NTM prevalence in our study falls within the range reported by these prior investigations (16), suggesting a widespread occurrence of these environmental mycobacteria.

Chlorination, while a widely used and effective method for disinfecting water against many microorganisms, exhibits limitations in its ability to eradicate mycobacteria. These resilient bacteria possess complex cell walls that confer resistance to chlorine-based disinfectants (25). This resistance stems from several factors, including the thick layer of mycolic acids in their cell walls, which are highly hydrophobic and resistant to penetration by disinfectants (26). Additionally, specialized pores in the mycobacterial cell walls can limit the entry of hydrophilic molecules like chlorine. Furthermore, mycobacteria can form biofilms, which provide a protective barrier against disinfectants and other environmental stresses (27). These factors contribute to the persistence of mycobacteria in chlorinated water environments, emphasizing the need for complementary or alternative disinfection strategies to effectively control their growth and transmission (28).

Studies investigating NTM prevalence in water sources in Iran hospitals, for example, revealed higher resistance in M. chelonae and M. fortuitum. While *M. aurum* appeared somewhat more susceptible to chlorine, it's important to note that both *M. aurum* and *M. gordonae* still exhibit significantly higher chlorine resistance compared to a common indicator bacteria, *E. coli*. They were, in fact, 100 and 330 times more resistant, respectively (18). Studies have shown a higher prevalence of NTM colonization in older compared to newly built drinking water systems, highlighting the potential risk associated with aging infrastructure. Similarly, hospitals, dialysis wards, and dental offices are at increased risk due to NTM colonization, with reported prevalence rates ranging from 60% to 100% (29). To address this concern, implementing disinfection methods like high chlorine concentrations, UV irradiation, hot water, or copper-silver ion generation systems in potable water supplies could significantly

reduce the risk of NTM diseases in hospitalized patients (30).

Subsequent genotypic characterization using PCR-RFLP analysis of the rpoB gene definitively identified the NTM isolates. This approach confirmed the dominance of *M. fortuitum* subtype I (n=27) among the fast-growing isolates. Additionally, slow-growing isolates were identified as *M. intracellulare* type I (n=1), *M. kansasii* subtype I (n=2), and *M. gordonae* (n=3, including two subtype I and one subtype II). The distinct banding patterns observed after restriction enzyme digestion with HaeIII and MspI (HpaII) provided clear differentiation between the identified NTM species.

Our findings align with existing research demonstrating that individuals with compromised immune systems, such as those with HIV/AIDS or organ transplant recipients taking immunosuppressants, are more susceptible to opportunistic infections including non-tuberculous mycobacteria. The alarming rise in NTM prevalence in recent years poses a significant challenge for global healthcare systems. This highlights the need for further investigation, as numerous studies have documented increasing rates of diverse mycobacterial infections (31, 32). Notably, water sources – encompassing rivers, streams, watersheds, and urban distribution systems – have been consistently identified as a key reservoir and transmission route for these microorganisms to humans (33, 34).

The presence of these NTM in water samples, particularly *M. kansasii* and *M. intracellulare*, warrants further investigation due to their potential pathogenic nature. These species have been linked to pulmonary infections in immunocompromised individuals (35-37). Future studies employing more comprehensive genotypic methods, such as whole-genome sequencing, could provide deeper insights into the specific strains present and their potential virulence. Additionally, investigating the environmental factors influencing NTM presence and distribution in water sources could be crucial for developing effective control strategies.

Conclusion

This study identified a diverse population of NTM species in water samples, with *Mycobacterium fortuitum* being the most frequently isolated species. The presence of potentially pathogenic species, such as *M. kansasii* and *M. intracellulare*, highlights the importance of further investigating their distribution and potential health risks. By utilizing more comprehensive genotypic methods and exploring environmental factors, future studies can contribute to the development of effective strategies for managing NTM in water environments.

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Ethics approval and consent to participate

It is declared that all ethical considerations were taken into account in the preparation of the submitted manuscript..

Conflict of interest

The authors declare that there are no conflicts of interest.

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