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Isolation and Identification of Nontuberculous Mycobacteria from Environmental Water Samples in the Northeast of Iran

İran'ın Kuzeydoğusundaki Çevre Suları Örneklerinden Nontüberküloz Mikobakterilerin İzolasyonu ve Tanımlanması

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Abstract

Introduction: The importance of nontuberculous mycobacteria (NTM), a group including over 150 species, has recently received increased attention as an environmental source of human infection. This study aimed to isolate and identify NTM species in the environment in the northeast of Iran.
Materials and Methods: A total of 344 water samples were obtained from water resources and drinking water distribution networks in 73 cities in the Khorasan Razavi province and were examined using culture. Nontuberculous mycobacteria identification was also accomplished by polymerase chain reaction amplification of the *hsp65* gene, sequencing and data analysis. Statistical analysis was performed by Pearson's correlation coefficient test and two-sample T-test using SPSS 16.0. Statistical significance was determined at a significance level of 0.05.
Results: Nontuberculous mycobacteria were identified in 10.46% of the water samples (36/344) by culture. The most frequent NTM species was *Mycobacterium gordonae* (13/36). Other *Mycobacterium* species detected included *M. mucogenicum*, *M. senegalense*, *M. gadium*, *M. genavense*, *M. simiae*, *M. frederiksbergense*, *M. flouranthenivorans*, *M. neoaurum*, and *M. pallens*. Statistically significant correlations were observed between the presence of NTM in water and nitrate level, turbidity, pH, and age of water resource (p<0.001). The relation between NTM presence and depth of the water resource was not statistically significant (p>0.05).
Conclusion: It was concluded that NTM water contamination was not very high in this area, but further studies are needed to investigate the sources of contamination and origins of these species.
Keywords: Nontuberculous mycobacteria, environment, water resources, Iran

Öz

Giriş: Çevresel insan enfeksiyonu kaynağı olarak son zamanlarda giderek artan düzeyde dikkat çeken ve toplamda 150'den fazla türü olan nontüberküloz mikobakterilere (NTM) verilen önem artmaktadır. Bu çalışmanın amacı, İran'ın kuzeydoğusunda çevresel NTM türlerini izole etmek ve tanımlamaktı.
Gereç ve Yöntem: Razavi Horasan eyaletinin 73 şehrindeki içme suyu dağıtım ağları ve su kaynaklarından toplanan 344 su örneği kültür yoluyla incelendi. Nontüberküloz mikobakteriler tanımlaması ayrıca *hsp65* geninin polimeraz zincir reaksiyonu amplifikasyonu, sekanslama ve veri analizi yoluyla da yapıldı. İstatistiksel analiz, SPSS 16.0 kullanılarak Pearson korelasyon katsayısı testi ve iki örnek t-testi ile yapıldı. istatistiksel anlamlılık seviyesi 0,05 olarak belirlendi.
Bulgular: Nontüberküloz mikobakteriler, kültürde su örneklerinin %10,46'sında (36/244) tespit edilmiştir. En yaygın NTM türü *Mycobacterium gordonae* (13/36) olarak belirlenmiştir. Diğer saptanan türler *M. mucogenicum*, *M. senegalense*, *M. gadium*, *M. genavense*, *M. simiae*, *M. frederiksbergense*, *M. flouranthenivorans*, *M. neoaurum*, ve *M. pallens*'di. Ayrıca, suda NTM varlığı ile nitrat seviyesi, türbidite, pH ve su kaynağının

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yaşı arasında istatistiksel olarak anlamlı bir korelasyon gözlendi (p<0,001). Nontüberküloz mikobakteriler varlığı ile su kaynağının derinliği arasındaki ilişki istatistiksel olarak anlamlı bulunmadı (p>0,05).

Sonuç: Bu bölgede çevre sularında NTM kontaminasyonunun çok yüksek olmadığı sonucuna ulaşılmıştır, ancak suların kontaminasyon kaynaklarını ve bu türlerin kökenlerini araştırmak için ek çalışmalar yapılması gereklidir.

Anahtar Kelimeler: Nontüberküloz mikobakteri, çevre, su kaynakları, İran

Introduction

Due to an increase in immunodeficiency diseases in recent decades, nontuberculous mycobacteria (NTM) that previously were known as environmental pathogens have emerged as important pathogens. Nontuberculous mycobacteria, which are also known as environmental or atypical mycobacteria, are acid-fast bacteria that may be found in surface waters, soil, dust, and vegetables[1]. These bacteria are opportunistic pathogens in people with weakened immune systems such as the elderly or those with underlying diseases such as AIDS (acquired immune deficiency syndrome), diabetes mellitus and cancer[2-6]. Environmental exposure to contaminated water is the cause of several infections due to NTM. Since exposure to these bacteria is common, the incidence of water-borne respiratory, skin, and soft tissue infections in immunocompromised patients is increasing[2,7].

The importance of mycobacterial studies can be considered from multiple angles. The rising prevalence of infection with these bacteria is one of the severe problems facing the health systems worldwide and increases the importance of studying and tracing them in resource extractions and water distribution networks[7,8]. Unfortunately, due to lack of access to advanced diagnostic facilities and the use of traditional methods, many of these mycobacterial infections are not recognized, or are treated as tuberculosis because of their similarity with *Mycobacterium tuberculosis*. The geographical distribution of NTM in Iran has not been well studied[9]. Evidence suggests that there are cases of infections caused by NTM. The prevalence of NTM infections has been reported 10.2% (95% confidence interval: 6.3-15.9) among culture-positive cases of tuberculosis, 38.3% in environmental samples, and 1.3% in clinical specimens in different studies from Iran[10-12]. Thus, there is a need to assess the presence of NTM and to identify and find solutions to control these infections[13].

In this study, we aimed to investigate the presence of NTM in drinking water resources and distribution networks and to identify their species.

Methods

This study was performed between December 2015 and November 2016 on resource extractions and drinking water distribution systems of 73 cities in the Khorasan Razavi province. The ethical approval for performing this study was obtained from the Ethics Committee of the Mashhad University of Medical Sciences (921330). Sampling was rational-classified. Sample size (344 samples) and sampling locations were determined based on the total number of resource extractions and population covered in each city. The resource extractions in each city which had the highest share of water resources (more than 30% of the total volume of water production in each city) were selected for sampling (126 samples).

In addition, 218 samples were taken from the water distribution network. In each city, three points (based on the highest number of users determined by the water and wastewater company) were used for sampling. In all cases, disposable sterilized (gamma-irradiated) 1.5-L polyethylene packaging was used for sampling from the taps. To prevent possible elimination of bacteria due to the free residual chlorine in the water during sampling, sterile 3% sodium thiosulfate was added to neutralize the free residual chlorine[14].

The environmental mycobacteria were separated using 0.2-µm polystyrene filters, which were then washed in 5 ml of DNase-free distilled water. To grow NTM after decontamination of the supernatant, 2 ml of this solution was inoculated on 7H10 Middlebrook medium (HiMedia, Mumbai, India). Fungal growth was inhibited using cycloheximide added to a concentration of 100 µl/ml[15].

After three weeks of incubation at two different temperatures (30 °C and 37 °C) with examination every 48 hours to detect rapidly growing mycobacteria, smears and Ziehl-Neelsen staining were performed to identify the NTM from the resulting colonies. The colonies were transferred to the Löwenstein-Jensen medium (bioMérieux, Marcy l'Etoile, France) and incubated at 30 °C for three weeks. Pigment production in the presence and absence of light was examined using *Mycobacterium smegmatis* as a positive control. The genus and species of the identified mycobacteria were verified by isolating and sequencing *hsp65* gene fragments[16]. DNA was extracted using the boiling method, based on standard protocols[17]. DNA concentration and purity were determined in all samples before polymerase chain reaction (PCR) amplification.

A pair of primers for the target gene was used based on previous studies[18]. The primers (forward 5'-ACC AAC GAT GGT GTG TCC AT-3', reverse 5'-CTT GTC GAA CCG CAT ACC CT-3') were used to amplify a 420-bp sequence of *hsp65*. Gene amplification was performed by a Touchgene Gradient thermal cycler (Eppendorf, Hamburg, Germany). Cycling consisted of initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 35 s, annealing at 61 °C for 40 s and extension at 72 °C for 30 s, and a final extension at 72 °C for 5 min[18]. Amplicons were analyzed using electrophoresis in 1.5% agarose gel (Sigma, Deissenhofen, Germany), followed by ultraviolet fluorescence visualization after staining with ethidium bromide (Figure 1). Precautions to avoid cross-contamination and false-positive results were taken in every assay.

The PCR products were sequenced and the NTM species were determined through the phylogenic diagram. Using Mega6 software, the FASTA format of each pair of F & R chains were aligned separately, and then the R chain was modified by use of the F chain (consensus sequence). Each of the F chains was then individually aligned with original mycobacteria using the Nucleotide BLAST (basic local alignment search tool) section of the NCBI (National Center for Biotechnology Information) site. The FASTA format of the target gene of suggested mycobacteria was then stored in the Mega6 software. Along with the above steps, all environmental water-borne mycobacteria that were detectable by *hsp65* gene were searched from the NCBI site, and the FASTA format of their target gene was obtained from the site and stored in the Mega6 program.

Using the Mega6 program, the FASTA format of all isolated bacteria, blasted bacteria, and F chains of samples were aligned together. In this way, the phylogenic tree was outlined for 1873 NTM along with 36 samples from this study. Species was predicted based on the location of the samples with regard to phylogenic similarity percentage.

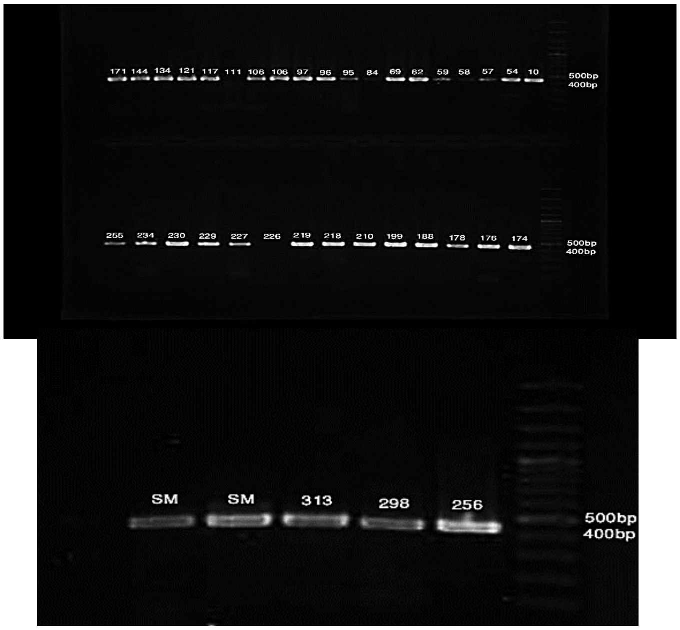


Figure 1. Results for *hsp65* polymerase chain reaction of water samples showing specific 420 bp bands on 1.5% gel agarose. Thirty-six samples were positive for nontuberculous mycobacteria

Statistical Analysis

Pearson's correlation coefficient test and two-sample T-test were used to investigate the significance of the relationship between the presence of NTM and parameters including pH, turbidity, nitrate, free residual chlorine, age and depth of water resources. Statistical analysis was performed using SPSS 16.0 for Windows (IBM Corporation, New York, USA) and statistical significance was determined at a significance level of 0.05.

Results

The average age of the resource extractions (number of years between the digging of the water source and the time of sampling) was 9.47±4.95 years, and their average depth was 108.6±78.6 m. In the distribution network, the average level of free residual chlorine was 0.67±0.17 ppm. Values of pH, turbidity, and nitrate and their mean values in both resource extractions and water distribution networks are presented in Table 1.

Of 126 samples from water resource extractions, 12 (9.5%) yielded NTM. The most common species were related to *M. gordonae* (5 samples, 41.6%), followed by *M. mucogenicum* (3 samples, 25%), *M. senegalense* (2 samples, 16.6%), *M. neoaurum* (1 sample, 8.3%), and *M. pallens* (1 sample, 8.3%).

Of 218 samples from the distribution network, NTM were isolated in 24 (11%). The most common species was again *M. gordonae* (13 cases, 54.1%). Other isolated species included *M. mucogenicum* (4 samples, 16.6%), *M. gadium* (2 samples, 8.3%), *M. senegalense* (1 sample, 4.1%), *M. genavense* (1 sample, 4.1%), *M. simiae* (1 sample, 4.1%), *M. frederiksbergense* (1 sample, 4.1%), and *M. flouranthenivorans* (1 sample, 4.1%).

Out of the 36 NTM-positive samples, two samples had higher than optimum levels of chlorine and turbidity and four had higher pH. None of the samples had higher than acceptable levels of nitrate.

Most NTM were found in the southern part of the Khorasan Razavi province, with fewer in the northern region. In the

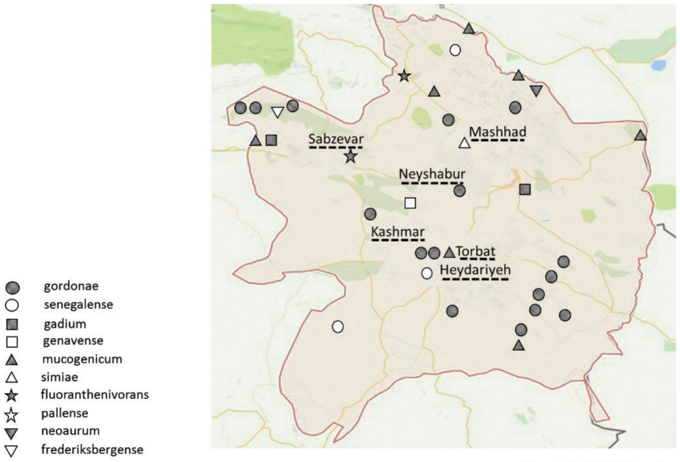
Table 1. The average of the three quantitative parameters in the separation of resource extractions and water distribution networks

Parameter	Resource extractions	Distribution networks
pH	7.26±0.28	7.27±0.29
Turbidity (NTU)	0.47±0.37	0.48±0.39
Nitrate (ppm)	15.57±9.89	15.94±9.43

NTM: Nontuberculous mycobacteria, NTU: Nephelometric turbidity units, PPM: Parts per million eastern and western geographical regions, the scattering was at zero or within a minimal range. Dispersion was seen on the central strip from the northern to southern parts of the Khorasan Razavi province (schematic map is shown in Figure 2).

According to our findings, *M. pallens* and *M. neoaurum* were found only in resource extractions and *M. gadium, M. frederiksbergense*, and *M. flouranthenivorans* were found only in the distribution networks. This study is the first report of these five species in the drinking water of Iran.

Most of the pipes in the distribution networks were made of polyethylene (121, 55.5%), followed by asbestos pipes (69, 31.7%) and galvanic pipes (28, 12.8%). Although the highest number of NTM was found in polyethylene pipes, there was no statistical significance between the presence of NTM and the type of pipe (p=0.715).



Map data ©2017 Google

Figure 2. The schematic map of *Mycobacterium* species distribution in the Khorasan Razavi province

Most of the taps in the distribution network were made of brass (146, 67%), steel (61, 28%), and aluminum (25, 11.5%). Although the highest number of NTM was found in brass valves, there was no statistical significance between the presence of NTM and the type of tap (p=0.502).

There was a significant relationship between NTM presence and the amounts of quantitative indicators including free residual chlorine, pH, turbidity, and nitrate in the distribution networks (Table 2).

In the distribution network, there were significant direct correlations between turbidity and the parameters of chlorine (r=0.254, p<0.001), pH (r=0.308, p<0.001), and nitrate (r=0.275, p<0.001).

Presence of NTM was significantly associated with the age of water resources (but not depth) and other quantitative water indicators (pH, turbidity, and nitrate) (Table 3).

In water resources as well as distribution networks, there was a significant direct correlation between turbidity and the parameters of pH (r=0.290, p<0.001) and nitrate (r=0.408, p<0.001). Furthermore, age of water resource was directly related to turbidity (r=0.253, p=0.004), pH (r=0.263, p=0.003), and nitrate level (r=0.378, p<0.001).

Table 2. Comparison of quantitative indicators (mean±standard deviation) of water distribution network based on the presence or absence of nontuberculous mycobacteria

	Absence of NTM	Presence of NTM	p value
Free residual chlorine (ppm)	0.62±0.1	1.09±0.08	p<0.001
pH	7.15±0.53	8.0±0.94	p<0.001
Turbidity (NTU)	0.37±0.2	1.44±0.23	p<0.001
Nitrate (ppm)	13.47±6.33	36.94±2.88	p<0.001

NTM: Nontuberculous mycobacteria, NTU: Nephelometric turbidity units, PPM: Parts per million

Table 3. Comparison of quantitative indicators (mean±standard deviation) in water resources based on the presence or absence of nontuberculous mycobacteria

	Absence of NTM	Presence of NTM	p value
Age (years)	8.15±2.84	21.91±2.64	p<0.001
Depth (meters)	78.69±7.37	81.42±23.5	0.86*
pH	7.18±0.10	8.08±0.10	p<0.001
Turbidity (NTU)	0.37±0.21	1.40±0.27	p<0.001
Nitrate (ppm)	6.98±0.65	1.38±0.39	p<0.001

NTM: Nontuberculous mycobacteria, NTU: Nephelometric turbidity units

*There was no statistically significant relationship between NTM-positive and NTM-negative samples with regards to depth of water resources (p=0.86)

Discussion

Nontuberculous mycobacteria are causative agents of opportunistic infections in humans. In recent years, contaminated water systems have been responsible for some of these infections[19]. In this study, we determined the geographical distribution of NTM in drinking water resources and distribution networks in the northeast of Iran. Our results showed that some water supplies and distribution networks are colonized with NTM that are often not pathogenic, although a small percentage of them could potentially be pathogenic for immunecompromised hosts systems.

As revealed in this study, *M. gordonae* was the most prevalent NTM species in both water resources and distribution networks that cause clinical infections. *M. mucogenicum, M. senegalense, M. genavense*, and *M. simiae* are also opportunistic pathogens that are capable of causing disease in those with impaired immune systems[20]. *M. mucogenicum* was also detected in a similar study by Nasr Esfahani et al.[21] in central Iran, where the dominant isolates were *M. fortuitum, M. chelonae*-like organism, and *M. mucogenicum.* A similar study by Mohajeri et al.[22] evaluating the frequency of NTM in drinking water supplies revealed NTM in 32% of 110 samples, with *M. gastri* as the most common species.

Five species of NTM detected in Iranian drinking water for the first time in the present study (*M. pallense* and *M. neoaurum* in water resources and *M. gadium, M. frederiksbergense*, and *M. flouranthenivorans* in water distribution networks) are known to be nonpathogenic to humans and cause opportunistic infections mostly in animals and plants[9].

Novel detection of these rare species in our study compared to other studies of drinking water may be attributed to our relatively large sampling area (73 cities in the Khorasan Razavi province) with a high number of samples (344), as well as having an accurate filtration technique and focusing on molecular diagnosis and sequencing instead of biochemical methods.

In this study, the presence of NTM was associated with the amount of free residual chlorine in drinking water. Chlorine and its derivatives are the oldest, most common, and cheapest chemical disinfectant for drinking water and are added to the vast majority of water distribution networks in Iran. However, this can sometimes have serious consequences in terms of water quality and health[23]. The allowable level for free residual chlorine in drinking water, according to the authoritative internal reference (standard 1053 of Iran) and the United Nations, including the standard method [published by World Health Organization (WHO)] is 0.5 to 0.8 mg/l[24]. Due to its acceptable retention time and subsequent maintenance of disinfection in water from the injection site to the site of consumption, the use of chlorine in water provides the unique properties of this material for being the most suitable water disinfection method[25]. What is essential at this point is to follow or better comply with the international guidelines on water chlorination. This amount of free residual chlorine kills bacteria such as *Enterobacteriaceae* (including *E. coli*), which is a contamination indicator of drinking water. Although increased levels of chlorine kill chlorine-sensitive bacteria, it may allow the growth of chlorine-resistant bacterial populations such as environmental mycobacteria[26].

In our study, we observed that the presence of NTM was associated with higher levels of turbidity (more than 1 NTU). Water turbidity is a result of suspended solids and sediments of different sizes and weights. Suspended particles in the water may enter the network system through different ways. For example, at the site of production and the depth of resource extraction, they can enter the pipes and subsequent distribution networks due to pump suction activity at the deepest point of the water source[27]. The presence of suspended solids and other particles is directly related to the presence of microorganisms in the water. These particles may cover microorganisms and act as a shield, reducing or even eliminating the influence of chlorine on these microorganisms. The greater the water turbidity, the higher chlorine dose needed to achieve a certain level[28]. Both soluble substances (chemical quality of water) and insoluble materials are involved in this process. Increased chlorine leads to the removal of more microorganisms with higher sensitivity to chlorine and increases the population of chlorine-resistant microorganisms like NTM. Therefore, based on our findings, in all areas where turbidity was more than 1 NTU, the levels of free residual chlorine were higher than the permissible limit due to injection of more chlorine, which increased the population of NTM[29].

This study showed that the presence of NTM in water is associated with acidity (pH). The normal pH of drinking water according to WHO guidelines is about 6.5-7.5[30]. Water with pH higher than this value is called alkaline water. The alkalinity of water is defined by and related to ions such as carbonate and bicarbonate. Generally, neutral pH (pH=7) is best for chlorine activity since the highest hypochlorous acid production occurs and the effect of chlorine is maximized at this pH. Free residual chlorine is less effective in alkaline water due to the low levels of hypochlorous acid. This is further proof of the need to increase the injection of chlorine to the drinking water and so, based on previous comments and for the same reasons, the population of bacteria that are insensitive or less sensitive to chlorine increases due to chlorine injection[31].

On the other hand, there is a direct correlation between water alkalinity and its corrosiveness. The more alkaline water is, the more corrosion it causes. This corrosion affects the internal lining of pipes and pipelines, distribution and transmission systems and subsequently increases water turbidity. It is evident that these factors are all part of a linked cycle and aggravate one another[32].

This study also revealed a significant relationship between the presence of NTM and the nitrate content of water. Nitrate (NO_3^-) is one of the major anions in drinking water. The amount of this anion is directly associated with surface and subsurface sewage (most of the time, not always) and also the common bacteria in water resources[33]. The maximum permissible level of nitrate in drinking water is 50 ppm[34]. The results of this research indicated that areas where nitrate levels were higher and approached the maximum allowed, had larger populations of microorganisms, including NTM. NTM are found everywhere, like all other environmental microorganisms. When the total population of environmental microorganisms in water increases, we can expect that the population of NTM will also increase[35].

The type of pipes and valves in the lines are also important in terms of the food supply due to gradual deterioration as well as the possibility of biofilm formation on their inner surfaces. Asbestos or galvanized pipes, as well as brass or aluminum valves can have a substantial influence on the microbial flora of drinking water. Although the porous structure of asbestos pipes makes their inner surface prone to the formation of biofilm, in the present study there was no significant relationship between the type of pipes and valves and the presence of NTM in drinking water[36].

In this study, we noted that older water resources were more likely to contain NTM. The best explanation for this may be the exhaustion of resources and increased soil permeability due to soil erosion and disruption of the ground layers around these water resources, which leads to penetration of NTM in the soil into water resources and the distribution networks through atmospheric precipitation[37,38]. Some studies have investigated the presence of NTM in the soil. For example, in a study in Iran, a total of 350 soil samples were collected from different parts of the city of Uremia and its suburbs, and mycobacteria were isolated from 65 (18.3%) specimens and *Mycobacterium fortuitum* was the most common species[38].

It would be logical to assume that the likelihood of waste water infiltration into water resources would be reduced as depth increases due to the earth's natural filtering properties, but statistical analysis of this study did not show a significant association between the presence of NTM and the depth of water resources[39,40] . The strengths of this study included screening the entire provincial region, analyzing a large number of samples, identifying some species for the first time in Iran, and accessing water resources and distribution networks. The limitation of this study was the lack of data regarding the origin of identified NTM species.

Conclusion

The key aspect of our study was the large number of samples from all 73 cities of Khorasan Razavi and the access to their distribution networks and water resources due to the support of the water and wastewater company in our province.

Although the complete elimination of these microorganisms from drinking water is not possible in general, some good management has been accomplished through interventions and proper utilization of water resources, such as compliance with laws related to privacy and preventing any human activity within the legal boundaries of water resources. The formation of sedimentary layers (biofilm) in distribution networks must be prevented through timely washing based on standard frequency proportional to the length and the population covered by the networks.

Furthermore, the replacement of valves and pipelines (where possible) could avoid their biological influence on the flora. Another management principle is to observe correct consumption patterns to prevent falling water tables and the associated geophysical changes, which lead to increased risk of wastewater infiltration into water resources.

Adequate levels of free residual chlorine must be maintained to prevent additional chlorine injection. Continuous monitoring of drinking water distribution networks and transmission lines for any defects and fractures in the networks is necessary to prevent wastewater infiltration.

In this study, we detected NTM in environmental water samples, which could pose a health risk concerning the drinking water supplies in the northeast of Iran. The identified NTM in our study are among the clinically important species in Iran as well as the Khorasan Razavi region, including *M. simiae*[41], *M. gordonae*, and *M. senegalense*[42].

In conclusion, although our results indicate that water contamination was not very severe in this area, further studies must be performed to investigate possible sources of contamination.

Ethics

Ethics Committee Approval: The ethical approval for performing this study was obtained from the Ethics Committee of the Mashhad University of Medical Sciences (921330).

Informed Consent: We did not have any patients and we took water samples, so informed consent was not applicable and we only mentioned the ethics statement of the study.

Peer-review: Externally and internally peer-reviewed.

Authorship Contributions

Concept: K.G., Design: K.G., Data Collection or Processing: H.A., Analysis or Interpretation: H.A., H.Z., Literature Search: H.A., Writing: H.A., H.Z.

Conflict of Interest: No conflict of interest was declared by the authors.

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