Identification of nontuberculous mycobacteria isolated from hospital water by sequence analysis of the *hsp65* and *16S rRNA* genes

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ABSTRACT

Nontuberculous mycobacteria (NTM) have emerged as an important cause of opportunistic nosocomial infections. NTM has frequently been isolated from hospital water distribution systems. The aim of this study was to survey the risk of NTM infections and determine the prevalence of NTM species in the hospital water distribution systems in Tabriz, Iran. One hundred and twenty samples of water from different sources of Tabriz hospitals were collected. The samples were filtered through 0.45-μm pore size membranes and decontaminated with 0.01% cetylpyridinium chloride. The sediment was inoculated onto Lowenstein–Jensen medium and incubated for 8 weeks. For identification to the species level, partial sequence analysis of the *hsp65* and *16S rRNA* genes were used. NTM were detected in 76 (63.3%) of 120 samples. Potentially pathogenic mycobacteria and saprophytic mycobacteria were isolated. *Mycobacterium gordonae* was the only single species that was present in all types of water. The prevalence of NTM in Tabriz hospitals' water compared with many investigations on hospital waters was high. This indicates that the immunocompromised patients and transplant recipients are at risk of contamination which necessitates considering decontamination of water sources to prevent such potential hazards.

Key words | *16S rRNA*, hospital water, *hsp65*, nontuberculous mycobacteria

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INTRODUCTION

Water is one of the most important reservoirs of the nontuberculous mycobacteria (NTM). Isolation of NTM from potable water samples was first reported in the early 1900s (Eilertsen 1969; Collins *et al.* 1984). Potable water, water coolers, ice and ice machines, hemodialysis supply network, showers, dental unit water, tub immersion, and water baths are noticeable water reservoirs in the hospitals (Vaerewijck *et al.* 2005). Being tolerant to a wide range of pH and temperature, being resistant to chlorine and able to form biofilms are shown to be important factors contributing to the survival, colonization and persistence of NTM in water distribution systems (Vaerewijck *et al.* 2005). Some NTM species such as *M. kansasii*, *M. lentiflavum*, *M. avium*, and *M. intracellulare* are potentially pathogenic and are associated with human diseases (Lewis *et al.* 1960). The most common diseases caused by NTM are lymphadenitis in children and pulmonary diseases in adults. Children with cancer, solid organ transplant and hematopoietic stem cell transplant recipients and immunocompromised persons are the main victims (Al-Anazi *et al.* 2014). Risk factors for human mycobacterial infections are chronic obstructive pulmonary disease, changes in lung and chest architecture, α-1-antitrypsin deficiency, cystic fibrosis, heterozygosity for CFTR mutations, gastric reflux disease and immunodeficiency due to HIV infection, cancer or chemotherapy (Kim *et al.* 2005b; Koh *et al.* 2007). The most

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frequent *Mycobacterium* species present in potable water and hospital water distribution systems are *M. avium*, *M. chelonae*, *M. fortuitum*, *M. gordonae*, *M. kansasii*, and *M. xenopi* (Vaerewijck *et al.* 2005; Genc *et al.* 2013).

Recent progress in sequencing of conserved genes such as *16S rRNA*, *hsp65*, *rpoB*, *dnaj*, *sodA* and the 16S-23S internal transcribed spacer (ITS) region, brought more accurate and faster recognition of NTM at the species level. In the late 1980s, use of the *16S rRNA* gene became a 'gold standard' for identification of bacteria (Woese 1987). In the past decade, the use of polymerase chain reaction (PCR) and sequencing of *16S rRNA* gene has played a central role in the discovery of new bacteria. With the use of *16S rRNA* sequencing, 215 novel bacterial species have been discovered during 2001 to 2007 (Woo *et al.* 2008). However, this genetic marker also has some limitations (having very similar and sometimes the same sequences) in a number of the mycobacteria species, especially in rapidly growing mycobacteria (RGM). Also, two copies of the *16S rRNA* gene (with different sequences) can be found in the same mycobacteria (Reischl *et al.* 1998). The other conserved genes such as *hsp65* and *rpoB* have been used for more accurate identification of very closely related species. In 1993, Telenti *et al.* developed a molecular method for NTM differentiation based on amplification of a 441 bp of the 65 kDa heat-shock protein gene (*hsp65*) by PCR and restriction enzyme analysis (PRA) (Telenti *et al.* 1993). However, identifications with this gene are usually based on PCR PRA; identifications based on the *hsp65* sequence have also been performed (Ringuet *et al.* 1999). This gene, which is present in all mycobacteria, is more variable than the *16S rRNA* gene sequence and is therefore potentially useful for the identification of genetically related species. The *hsp65* sequence analysis has also been used successfully for differentiation of mycobacterium species and this genetic marker has been a better identification tool (Kim *et al.* 2005a; Escobar-Escamilla *et al.* 2014).

More recent data have shown that the prevalence of NTM diseases in hospitalized patients has increased (Billinger *et al.* 2009). Unlike tuberculosis, transmission of NTM to humans has mostly been associated with environmental sources, and nosocomial infections with NTM are most commonly associated with the contamination of hospital water distribution systems (Wallace *et al.* 1998). NTM had been isolated from clinical specimens of the hospitalized patients, which for diagnosis had been sent to Mycobacteriology Research Center (MRC) in Tabriz University of Medical Sciences (TUOMS). In spite of the importance of NTMs, no other research has been done in this region of Iran. The present study was a research project in TUOMS. TUOMS intends to launch a bone marrow transplantation (BMT) and kidney transplantation center with 300 beds, and this study aimed to survey the risk of NTM infections in this area and determine the prevalence of NTM in the hospital water distribution systems in Tabriz, northwest of Iran.

MATERIALS AND METHODS

Study design and sample collection

In this study, a total of 120 water samples were collected from different sources of target hospitals between August 2015 and April 2016 in Tabriz, Iran. Sterile flasks were used to collect the samples; each sample was removed after 1 min of free flow. Samples were transported to the laboratory and processed in the day of collection. The samples consisted of shower water ($n = 21$), water cooler (bottle-less water coolers, which were connected to a water supply, and bottled water coolers, which require delivery of water in large bottles by hospital personnel, $n = 34$), potable faucet water (piped drinking water, $n = 33$), non-potable faucet water (tap water used for irrigation of the trees and lawns on the hospital campus, $n = 17$) and hemodialysis supply network ($n = 15$).

Bacterial culture

About 400 mL of samples were filtered through membranes with 0.45 μm pore size and 30 mm diameter (Millipore, PES Syringe Filter, Orange Scientific, Belgium) at the laboratory of Microbiology of TUOMS. Each filter was aseptically transferred into a separate sterile 15 mL tube containing 2 mL sterile distilled water (SDW). For each tube, 2 mL of 0.01% cetylpyridinium chloride (CPC, cetylpyridinium chloride monohydrate for synthesis; Merck 84008, Merck KGaA, Darmstadt, Germany) was added. The tubes were vortexed for 15 min at 3,000 rpm, membranes were aseptically removed, and the tubes were centrifuged at 6,000 rpm for 15 min at room temperature. The supernatant

was discarded and 400 µl SDW was added to sediment in order to dilute the residual CPC. The sediment was inoculated onto Lowenstein–Jensen medium and incubated in duplicate cultures at 30 °C and 37 °C for 8 weeks. Mycobacteria growth was controlled weekly. The Ziehl–Neelsen staining technique was used to confirm the suspected colonies to be acid–alcohol-resistant bacilli.

DNA extraction

DNA was extracted as described by van Soolingen *et al.* (1991). In short, a loop-full of mycobacterial cells was suspended in 500 µl of 1X TE buffer (10 mM Tris-HC1, 1 mM EDTA, pH 8.0) and was heated at 80 °C for 20 min in order to lyse the cells. Then, 50 µl of lysozyme (final concentration of 1 mg/ml) was added and incubated for 1 h at 37 °C; 70 µl of 10% sodium dodecyl sulfate (W/V) and 6 µl of proteinase K (at a 10 mg/ml concentration) were added, and the mixture was incubated for 10 min at 65 °C. Following an incubation for 10 min at 65 °C, 80 µl of N-Cetyl-N,N,N-trimethylammonium bromide was added. An equal volume of chloroform-isoamyl alcohol (24:1, vol/vol) was added, and the mixture was vortexed for 10 s. After centrifugation for 5 min at 12,000 rpm, isopropanol was added in the volume of equal to almost 60% of the supernatant volume in order to precipitate the nucleic acids. After 30 min incubation at −20 °C and centrifugation for 15 min at 12,000 rpm, the pellet was washed with 70% cold ethanol and then the air-dried pellet was dissolved in 20 µl of 0.1 X TE buffer (1 mM Tris-HCI, 0.1 mM EDTA, pH 8.0). The extracted DNA samples were stored at −20 °C prior to the next steps.

PCR amplification of 16S rRNA and hsp65 genes

This study used the sequence analysis of the *hsp65* and *16S rRNA* genes to identify the NTM. A fragment *hsp65* gene and a fragment *16S rRNA* gene were amplified with primer sets according to Telenti *et al.* (1993) and Mendum *et al.* (2000), respectively. Primer sets are shown in Table 1. PCR reactions were performed using 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 0.2 mM dNTP mix, 0.1 U μl^{-1} Taq polymerase, 0.5 µM of each of the primers, DNA template and nuclease free water. PCR cycle conditions for amplification of the two genes (*hsp65* and *16S rRNA*) were as follows: 95 °C for 4 min followed by 35 cycles of 94 °C for 30 s, 65 °C for 30 s, and 72 °C for 50 s, and then final extension at 72 °C for 10 min. To control the accuracy of the PCR, DNA from *M. tuberculosis H37Rv* and nuclease free water (Sinaclon, BioScience) were used as positive and negative control, respectively. Analysis of 3 µl of the PCR products (amplicon) was done by electrophoresis on 1.5% gel agarose. After electrophoresis and gel staining with GelRed™ DNA stains, the fragments were visualized under UV light in the gel documentation system (Gel Doc, ATP Co).

PCR products were purified with a QIA quick PCR purification kit (QIAGEN, Germany) and analyzed by Sanger sequencing (Macrogen Cor., Korea). DNASTAR Lasergene Software (version 7.1) was used to analyze the sequencing results for *16S rRNA* and *hsp65* genes. The sequences were compared with similar sequences of the organisms in Gene Bank using the BLAST online software of the National Center for Biotechnology Information (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Statistical analysis

Descriptive tests like frequency and mean were applied to analyze the data using SPSS v.16 software (SPSS Inc., Chicago, IL, USA).

Table 1 | Primer sets for amplification of *hsp65* and *16S rRNA* genes

Target gene	Primer sequence	Amplified region	Comments
hsp65	Tb11: 5′-ACCAACGATGGTGTGTCCAT-3′ Tb12: 5′-CTTGTCGAACGCATACCCT-3′	441 bp	Primer set common for all mycobacteria
16S rRNA	5′-ATGCACCACCTGCACACAGG-3′ 5′-GGTGGTTTGTCGCGTTGTTC-3′	473 bp	Reference (Mendum *et al.* 2000)

RESULTS

The results of bacterial culture indicated that the drinkable water distribution systems in the examined hospitals are colonized by potentially pathogenic mycobacteria (PPM) such as *M. kansasii*, *M. chelonae*, *M. fortuitum*, *M. simiae* and saprophytes such as *M. gordonae*. Seventy-six out of 120 water samples (63.3%) collected from different sources of Tabriz hospitals were contaminated with NTM.

Eighty-seven NTM colonies were isolated from water samples. Some water samples yielded more than a single isolate. Of those, 40 NTM were isolated from water coolers, 18 NTM were isolated from non-potable faucet water, 15 NTM were isolated from potable faucet water, 12 NTM were isolated from hot water, and two NTM were isolated from hemodialysis supply networks.

Thirty-nine (39/87, 44.83%) NTM were RGM and 48 (48/87, 55.17%) NTM were slowly growing mycobacteria (SGM). Most of the RGM were *M. mucogenicum* (10/39, 25.64%) and *M. fortuitum* (9/39, 23.08%). Other RGM were *M. abscessus* (17.95%), *M. chelonae* (12.82%), *M. phocaicum* (10.25%), *M. peregrinum* (7.70%) and *M. novocastrense* (2.56%). Most of the SGM was *M. gordonae* (27/48, 56.25%). Other SGM were *M. simiae* (20.83%), *M. kansasii* (14.58%), *M. paragordonae* (6.25%) and *M. marinum* (2.09%). Most of the species grew at 30 °C. *Mycobacterium avium* Complex (MAC), which are most commonly associated with human diseases, were not found in any of the samples. The occurrence of NTM in water cooler reservoirs was high; nearly 88.23% (30/34) of samples collected from water cooler reservoirs were positive for NTM. *M. gordonae* was the only single species present in the all sources of water. *M. phocaicum* was isolated only from the water cooler. The frequency of NTM is summarized in Table 2.

Bacterial identification through gene sequencing

The *hsp65* gene sequence has high genetic heterogeneity compared with *16S rRNA* gene and can be used to identify the species which cannot be obviously differentiated by analysis of the *16S rRNA* gene. In the present study, for instance, regarding *16S rRNA* analysis, *M. phocaicum* showed 99% similarity to *M. mucogenicum* and hence they could not be differentiated using *16S rRNA* gene sequencing. However, using *hsp65* gene sequencing it was possible to differentiate *M. phocaicum* and *M. mucogenicum.* In the present study, a short sequence of the *16S rRNA* gene

Table 2 | Frequency of NTM isolated from different water sources of Tabriz hospitals

	Sources of water					
Mycobacterium species	**Hot water** ***n*** **= 21 (17.50)**	**Water cooler** ***n*** **= 34 (28.34)**	**Potable faucet water** ***n*** **= 33 (27.50)**	**Non-potable faucet water[a]** ***n*** **= 17 (14.16)**	**Hemodialysis supply network** ***n*** **= 15 (12.50)**	**Total 120**
M. abscessus	1	4	1	1	0	7 (8.04)
M. chelonae	0	3	1	1	0	5 (5.75)
M. phocaicum	0	4	0	0	0	4 (4.60)
M. fortuitum	1	4	1	3	0	9 (10.34)
M. gordonae	4	9	6	7	1	27 (31.03)
M. kansasii	3	2	1	1	0	7 (8.04)
M. marinum	0	0	0	1	0	1 (1.15)
M. mucogenicum	2	5	1	2	0	10 (11.50)
M. novocastrense	0	1	0	0	0	1 (1.15)
M. paragordonae	0	1	1	0	1	3 (3.45)
M. peregrinum	0	2	1	0	0	3 (3.45)
M. simiae	1	5	2	2	0	10 (11.50)
Total	**12**	**40**	**15**	**18**	**2**	**87 (100)**

[a]Collected from tap water in hospital campus.

was analyzed; therefore, most mycobacteria species were identified with the sequence analysis of the *hsp65* gene. Although the analysis of the *16S rRNA* gene is prevalent, it still has several limitations. Therefore, a combination of *hsp65* with *16S rRNA* gene analysis is an effective method for identification of *Mycobacterium* species.

In the present study, we found 12 species of NTM in water samples, of which the most common was *M. gordonae*. The molecular phylogenetic and molecular evolutionary analysis of these species based on the *hsp65* gene sequencing were conducted using MEGA7 software (Kumar *et al.* 2016) and the representative result is shown in Figure 1. *Mycobacterium tuberculosis* H37RV sequence was used as the out-group species. The NTM isolates represented in this study are indicated by *.

DISCUSSION

NTM are often isolated from drinkable water, shower aerosols, hemodialysis centers and hospital potable water distribution systems (Vaerewijck *et al.* 2005; Shin *et al.* 2007; Briancesco *et al.* 2010; Sartori *et al.* 2013; Thomson *et al.* 2013). The nosocomial infections caused by NTM have been indicated to be associated with the contamination of hospital water distribution systems (Wallace *et al.* 1998; Kauppinen *et al.* 1999; Kline *et al.* 2004). Costrini *et al.* (1981) investigated 19 patients with pulmonary disease caused by *M. xenopi* and then this organism was isolated from hospital water distribution systems. Studies have revealed that the number of hospital infections caused by NTM has increased for reasons such as inadequate sterilization and disinfection. Nosocomial infections caused by NTM have been recognized for more than 40 years (since 1975) and there continues to be a problem (Robicsek *et al.* 1978).

NTM contamination has been reported in different sources of water in various countries. Khosravi *et al.* (2016) analyzed 258 hospital water samples collected from different sites of teaching hospitals in three cities of Khuzestan province in Iran. In their study, 77 hospital water samples (29.8%) were positive for NTM. The authors were isolated 70.1% (52/77) RGM and 32.4% (25/77) SGM. In their study *M. fortuitum* (34/77, 44.1%) and *M. gordonae* (13/77, 16.8%) were the most common inhabitant species

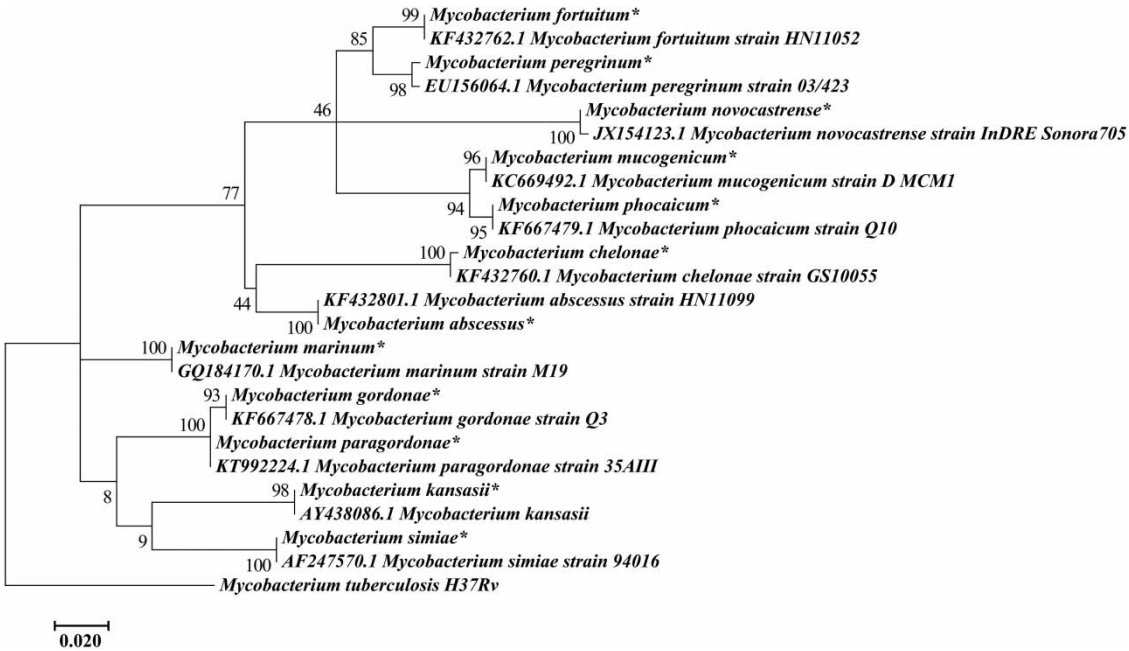

Figure 1 | Phylogenetic tree of the partial *hsp65* gene sequences of 12 species of NTM (RGM and SGM) using the neighbor-joining method with K2P distance correction model and MEGA7 software (Kumar et al. 2016). The support of each branch, as determined from 1,000 bootstrap samples, is indicated by percentages at each node. *Mycobacterium tuberculosis* strain H37Rv was used as the out-group. The scale bar represents a 0.02 difference in nucleotide sequences.

recovered from hospital water samples. Some species of NTM such as MAC, *M. simiae*, *M. lentiflavum*, *M. phocaicum* and *M. novocastrense* were isolated, too. The authors used *hsp65*-PRA and sequence analysis of the *rpoB* techniques. In another study from Iran, *M. lentiflavum* (28.2%) and *M. paragordonae* (21.1%) were reported to be the dominant species in hospital water samples (Azadi *et al.* 2016). In their research, 71 NTM (30 rapidly growing and 41 SGM) were recovered from 148 water samples collected from 38 hospitals in Isfahan province. The authors used PCR amplification of the *hsp65* and sequence analysis of the *16S rRNA*.

In the present study, 87 NTM colonies (39 RGM and 48 SGM) were isolated from 76/120 (63.3%) water samples, and our recovery percentage was higher than previous studies (Peters *et al.* 1995; Hussein *et al.* 2009; Genc *et al.* 2013; Azadi *et al.* 2016; Khosravi *et al.* 2016). This might be due to the isolation method applied in this study, which was highly sensitive and effective. A number of researchers failed to isolate NTM from drinking water (Vaerewijck *et al.* 2005). This may be because of the temporary presence of NTM in the water, its absence, or the use of inappropriate isolation methods. Therefore, use of suitable and effective isolation methods can directly influence the rate of reported NTM.

MAC, which are most commonly associated with human diseases, were isolated from hospital water samples in the southwest of Iran (Khuzestan), but not found in the northwest of Iran (Tabriz). Instead, other PPM species including *M. abscessus*, *M. chelonae*, *M. fortuitum*, *M. kansasii*, *M. marinum*, *M. mucogenicum*, and *M. simiae*, which are related to human disease (Wallace *et al.* 1998), were isolated. RGM species are important agents of nosocomial infections, and Tabriz hospital water supplies were rich in these organisms (39/87, 44.83%). In this study, *M. gordonae* was the most encountered *Mycobacterium* that included 31.03% of the isolates. *M. gordonae* is capable of causing clinically significant diseases in both immunocompetent and immunocompromised individuals (Mazumder *et al.* 2010). This research indicated that the hospital water distribution systems could be considered as a significant source of nosocomial infections.

The distribution of NTM analyzed by previous studies has been shown to be variable. In Turkey, Genc *et al.* (2013) analyzed 160 hospital water samples collected from two hospitals in Istanbul. Based on their report, 33 water samples (20.6%) were positive for NTM. In their study, *M. lentiflavum* (60.3%), *M. gordonae* (30.3%) and *M. peregrinum* (9.1%) were the most common NTM recovered from hospital water samples. The authors used GenoType *Mycobacterium* assay. Hussein *et al.* (2009) investigated 93 hospital water samples (49 cold water and 44 warm water) collected from four hospitals in Germany. Based on their results, NTM were recovered from 21 of 49 (43%) cold and 32 of 44 (73%) warm water samples (in total 53/93, 57%). In their study, *M. chelonae*, *M. flavescens*, *M. frederiksbergense*, *M. gordonae*, *M. moriokaense*, *M. mucogenicum*, *M. vaccae*, and *M. xenopi* were isolated. The authors used the sequence analysis of the *16S rRNA* technique. In the study by Briancesco *et al.* (2010), 60% of the household water samples and 73% of the hospital water samples were positive for NTM. In their research, common isolated species were *M. intracellulare* and *M. genavense*. Comparable to these studies, we also isolated *M. mucogenicum* and *M. gordonae* as the most frequent RGM and SGM species, respectively. However, *M. gordonae* was the most frequent mycobacteria isolated from various sources of hospital water, indicating its high prevalence in such reservoirs which can be due to its limited need for nutrition and high tolerance for chlorine. *M. gordonae* is 330 times more resistant to chlorine compared with *Escherichia coli* (Le Dantec *et al.* 2002).

Shin *et al.* (2007) also investigated the prevalence of NTM in a hospital environment. These authors collected 150 samples from different parts of hospitals, of which 105 samples were water. They isolated saprophytic mycobacteria (*M. gordonae*) and PPM (*M. mucogenicum*, *M. kansasii*, *M. chelonae*) from faucet water and shower water. In their study, *M. gordonae* was the most prevalent (44/60, 73.3%), and among SGM, *M. gordonae* was the only single species isolated from water samples. *M. mucogenicum* was the second most prevalent (10/60, 16.7%). The main method in their study was PCR-restriction fragment length polymorphism (PCR-RFLP) with *rpoB* gene, but the authors used the *hsp65* and *rpoB* sequence analysis for identification of unidentified NTM. NTM were also isolated from 46.7% (56/120) of hospital hot water samples in the Czech Republic (Sebakova *et al.* 2008), from 20.4% (10/49) of hospital tap water samples

in China (Chang *et al.* 2002), and from 50/118 (42.4%) hospital water samples in Berlin, Germany (Peters *et al.* 1995). Sartori *et al.* (2013) analyzed 210 samples of water collected from five different sites of a hemodialysis center. In their study, 51 NTM (24.3%) including *M. lentiflavum*, *M. gordonae*, *M. szulgai*, *M. kansasii* and *M. gastri* were isolated. In another study, (11/110, 10%) NTM including *M. gordonae* (4), *M. kansasii* (3), *M. gastri* (3) and *M. lentiflavum* (1) were isolated from a hemodialysis center water system (Montanari *et al.* 2009). The method used in both studies was *hsp65*-PRA.

In the present study, prevalence of NTM in the hemodialysis center was 2/15 (13.3%). This is an alert and shows that the hemodialysis supply network can be considered a reservoir for transmission of NTM among patients with chronic kidney disease.

The majority of authors (Hussein *et al.* 2009; Briancesco *et al.* 2010; Genc *et al.* 2013; Hashemi-Shahraki *et al.* 2013; Khosravi *et al.* 2016) used membrane filtration followed by decontamination with CPC. Shin *et al.* (2007) used 1% sodium hydroxide as decontaminant with the centrifugation method. Sartori *et al.* (2013) used 4% sulfuric acid as decontaminant with membrane filtration. Furthermore, it should be mentioned that classification of NTM to the species level by conventional methods is time consuming, and sometimes leads to incorrect results. Therefore, molecular techniques such as DNA sequencing and PRA have been developed to address this challenge, especially use of the *hsp65*, *16S rRNA*, and *rpoB* genes (Telenti *et al.* 1993; Domenech *et al.* 1994; Lee *et al.* 2000). The 32 KDa protein gene (Soini & Viljanen 1997), *dnaj* gene (Takewaki *et al.* 1994), superoxide dismutase gene (Zolg & Philippi-Schulz 1994), *secA1* gene (Zelazny *et al.* 2005), and the ITS of the 16S-23S rRNA gene (Roth *et al.* 1998) are other target genes for the identification of mycobacteria by PCR-based sequencing.

The majority of authors had used Telenti's PRA method (Telenti *et al.* 1993) for identification of NTM species. Another accurate technique for identification of NTM is sequence analysis of the *hsp65* gene (Ringuet *et al.* 1999). The sequencing of this gene has been used successfully for differentiation of *Mycobacterium* species (Kim *et al.* 2005a). Escobar-Escamilla *et al.* (2014) concluded that the *hsp65* gene sequencing is a better identification tool for differentiation of *Mycobacterium* species and is useful to complement diagnosis of NTM. These authors used the *hsp65* sequence analysis to recognize 10% of the isolates that were not identified by PRA.

All results were presented to infection control committees and to the head of development committees to raise awareness of the risk of NTM for BMT patients. This survey showed a high risk of NTM infections in the study area and there needs to be awareness about the risk of these infections for BMT patients. Therefore doctors have to consider these infections and hospital managers have to improve water quality and screening systems before establishing new transplantation wards in their hospitals.

CONCLUSION

The prevalence of NTM in Tabriz hospital waters compared with several other investigations was high (63.3% in contrast to 20.6% in Turkey, 29.8% and 48% in Iran, 42.4% and 57% in Germany). The results of this study indicate that in water disinfection strategies, NTM should be considered as part of the normal microbiological flora in hospital water distribution systems. This indicates that immunocompromised patients, transplant recipients, and patients with leukemia and neutropenia at Tabriz hospitals are at risk of contamination, which calls for more effective water disinfection procedures. Therefore, we recommend that the water used for these patients be filtered. For prevention of probable NTM diseases in immunocompromised patients, antimicrobial prophylaxis is recommended. Also, the results of several studies show that distribution and frequency of NTM species in various geographical areas is different. Moreover, partial sequence analysis of *16S rRNA* gene alone is not sufficient for the identification of NTM species. The analysis of more than one gene can be an effective way for differentiation between closely related species. In this regard, our study showed that sequence analysis of the *hsp65* gene is an accurate, reliable, and useful means of identification of NTM.

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