

Molecular characterization of nontuberculous mycobacteria in hospital waters: a two-year surveillance study in Tehran, Iran

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ABSTRACT

Microbiological control of hospital waters as one of the main sources of nontuberculous mycobacteria (NTM) is important for the prevention of NTM-associated illness. This study aimed to investigate the prevalence of NTM in the hospital water systems of Tehran, Iran. A total of 218 samples from different hospital waters (i.e., tap water and medical devices such as humidifying cup of oxygen manometer, dialysis devices, nebulizers, and dental units) were included in this study. Phenotypic and molecular tests were used to identify the isolated organisms to species level. Of 218, 85 (39.0%) samples at 37 °C and 87 (40.0%) samples at 25 °C were identified as NTM. Using *hsp65*-sequencing method, *Mycobacterium lentiflavum* was the most frequently encountered, followed by *M. gordonae* and *M. paragordonae*. No significant difference was seen in frequency and species in mycobacteria isolated at 37 °C and 25 °C temperatures. Humidifying cup of oxygen manometer had the most contaminated water among the investigated water distribution systems in hospitals. Isolation of NTM from hospital water sources is a serious public health problem in Iran and merits further attention by health authorities. Establishment of microbiological monitoring systems for hospital waters and expanding the number of facilitated laboratories are strongly recommended.

Key words | hospital, Iran, nontuberculous mycobacteria, oxygen manometer, water

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INTRODUCTION

Infections caused by nontuberculosis mycobacteria (NTM) have been recently reported as an important public health problem in many parts of the world (Prevots & Marras 2015). NTM infections are rarely spread from person to person and are mainly acquired from environmental sources including water, drinking water supplies, soil, etc. (Prevots & Marras 2015). They can cause serious infections in immunocompromised patients, including pulmonary disease which may resemble tuberculosis (TB) as well as cervical lymphadenopathy and skin and soft tissue infections (Griffith *et al.* 2007). NTM are often resistant to disinfectants and antibiotics, including anti-TB drugs, and treatment for

one species may not be effective for others (Soni *et al.* 2016; Aguilar-Ayala *et al.* 2017). As NTM are usually resistant to disinfectants, it is not uncommon to isolate NTM from water distribution systems (Crago *et al.* 2014). NTM colonization and persistence within a hospital water distribution system may lead to dissemination and infection in patients (Vaerewijck *et al.* 2005; Castillo-Rodal *et al.* 2012; Mullis *et al.* 2013; Crago *et al.* 2014; Falkinham 2015, 2016). In countries such as Iran, where TB is endemic, we have suggested that there is an increasing prevalence of NTM disease, which is of concern (Nasiri *et al.* 2015, 2018a, 2018b). While monitoring hospital water distribution systems for

NTM is not a routine practice in Iran and because of the significant impact that these infections may have on patients, the investigation of NTM seems to be important. Thus, the present study aimed to evaluate the frequency of NTM in hospital waters in Tehran, Iran.

MATERIALS AND METHODS

Sample collection

A total of 218 samples from January 2016 to December 2017 was collected from tap water and medical devices (such as oxygen manometers, dialysis devices, nebulizers, and dental units) of different wards of the six hospitals in Tehran, the capital of Iran. Approximately 50 mL of each sample was collected in a sterile glass bottle, transferred to the laboratory in an icebox and analyzed within 24 hr. Stranded procedure recommended by the Centers for Disease Control and Prevention (CDC) was used to produce water for hemodialysis machines (Sehulster *et al.* 2003).

Sample preparation and culture

Water samples were centrifuged for 30 min at 3,000 rpm. The sediment (3 mL) was transferred to two sterile containers, and was decontaminated by our method (1.5 mL of NaOH 1% and SDS 3%) for 30 min at room temperature. Phenolphthalein and phosphoric acid 40% was used for the neutralization step. The samples were then centrifuged for 30 min at 3,000 rpm. The supernatant was discarded and the sediments of each treated sample were used to prepare a Ziehl–Neelsen smear and inoculated into two separate batches of Lowenstein Jensen (LJ) medium containing cyclohexamide 0.5 g and incubated at temperatures 37 °C and 25 °C for two months (Khosravi *et al.* 2016).

Phenotypic identification of mycobacteria

All mycobacterial isolates were grown on LJ medium and examined for growth rate, macroscopic and microscopic morphological features, growth at different temperatures, and also a set of biochemical tests including Tween 80 hydrolysis, nitrate reduction, niacin production,

arylsulfatase, urease production, tellurite reduction, salt tolerance, and catalase production according to standard procedures (Nasiri *et al.* 2018a, 2018b).

Molecular assignment of isolates to NTM

IS6110-based polymerase chain reaction (PCR) assay was used for differentiation of NTM from *Mycobacterium tuberculosis* complex. Genomic DNA, for *IS6110*-based PCR assay, was extracted using QIAamp DNA Mini Kit (QIAGEN, USA) according to kit instructions. A 123 bp fragment of insertion element *IS6110* of the *M. tuberculosis* complex was used as a target and amplified using previously described PCR primers (Eisenach *et al.* 1990). Genomic DNA of *M. tuberculosis* H37Rv (ATCC27294) and *M. fortuitum* (ATCC 49404) were used as positive and negative controls, respectively. The assay is negative for NTM species due to the absence of *IS6110* (Eisenach *et al.* 1990).

hsp65-PCR restriction analysis (PRA)

An approximately 441 bp fragment of *hsp65* gene was amplified by PCR using two specific primers Tb11 (50-ACCAA CGATGGTGTGTCCAT-30) and Tb12 (50-CTTGTCGAA CCGCATACCCT-30). PCR products were digested with 5 U of restriction enzyme *Hae*III and *Bst*II for 24 hours at 37 °C (Telenti *et al.* 1993). The pattern of digested products was analyzed using 8% polyacrylamide gel. *M. fortuitum* (ATCC 49404) and double distilled water were used as positive and negative control in all PCR experiments, respectively. Species identification was performed using algorithms proposed by Roth *et al.* (2000) and Telenti *et al.* (1993).

PCR and sequencing of *hsp65* and *rpoB*

A 441 bp fragment of *hsp65* gene was amplified by PCR with two specific primers Tb11 (5'-ACCAACGATGGTGTGTC-CAT-3') and Tb12 (5'-CTTGTC GAACCGCA-TACCCT-3') (Telenti *et al.* 1993). The cycling condition was 95 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 62 °C for 30 s, and 72 °C for 1 min and finalized with 72 °C for 5 min. Distilled water was used as a negative control. PCR products of *hsp65* genes were analyzed on 1% agarose gel electrophoresis. For confirmation of results, a number of

samples were randomly examined for *rpoB* gene by using two specific primers MycoF (50-GGCAAGGTCACCCCG AAGGG-30) and MycoR (50-AGCGGCTGCTGGGTGAT CATC-30) (Adékambi *et al.* 2003).

Analysis of sequence data

The obtained sequences for each isolate were aligned separately and compared with all existing relevant sequences of mycobacteria retrieved from GenBank database at the NCBI website via the nucleotide BLAST search.

RESULTS

Out of 218 samples from different water sources of hospitals in Tehran, 85 (39.0%) samples at 37 °C and 87 (40.0%) samples at 25 °C were identified as NTM using conventional

and molecular methods (Tables 1 and 2). Using *hsp65*-PRA and sequencing methods, *M. lentiflavum* (n: 72, 84.7%) was the most frequently encountered, followed by *M. gordonae* (n: 11, 13.0%) and *M. paragordonae* (n: 2, 2.3%) at 37 °C (Table 3). No significant difference was seen in frequency and species in mycobacteria isolated at temperatures of 37 °C and 25 °C. Randomly selected NTM isolates were also confidently identified by *rpoB* gene.

The frequency of NTM species in different hospital water sources are presented in Tables 4 and 5. Humidifying cup of oxygen manometer had the most contaminated water among the investigated water distribution systems in the hospitals.

DISCUSSION

There are several reports of nosocomial infections caused by waterborne NTM, indicating that this route of transmission

Table 1 | Frequency of mycobacterial isolates in water sources of hospitals incubated at 37 °C

Hospital (No. of samples)	Water samples (n = 218)			Total (%)
	Positive (%)	Negative (%)	Contaminated ^a (%)	
No. 1 (43)	8 (3.7)	8 (3.7)	27 (12.3)	43 (19.7)
No. 2 (27)	19 (8.7)	1 (0.5)	7 (3.2)	27 (12.4)
No. 3 (25)	10 (4.6)	9 (4.1)	6 (2.8)	25 (11.5)
No. 4 (51)	3 (1.4)	45 (20.6)	3 (1.4)	51 (23.4)
No. 5 (65)	39 (17.9)	17 (7.8)	9 (4.1)	65 (29.8)
No. 6 (7)	6 (2.7)	0	1 (0.5)	7 (3.2)
Total	85 (39)	80 (36.7)	53 (24.3)	218 (100)

^aContaminated by bacteria other than NTM.

Table 2 | Frequency of mycobacterial isolates in water sources of the hospitals incubated at 25 °C

Hospital (No. of samples)	Water samples (n = 218)			Total
	Positive (%)	Negative (%)	Contaminated ^a (%)	
No. 1 (43)	6 (2.7)	8 (3.7)	29 (13.3)	43 (19.7)
No. 2 (27)	22 (10)	1 (0.5)	4 (1.85)	27 (12.4)
No. 3 (25)	7 (3.25)	12 (5.5)	6 (2.7)	25 (11.5)
No. 4 (51)	5 (2.3)	41 (18.8)	5 (2.3)	51 (23.4)
No. 5 (65)	40 (18.4)	19 (8.7)	6 (2.7)	65 (29.8)
No. 6 (7)	7 (3.25)	0	0	7 (3.2)
Total	87 (39.9)	81 (37.2)	50 (22.9)	218 (100)

^aContaminated by bacteria other than NTM.

Table 3 | Frequency of different species of *Mycobacterium* isolated at 37 °C and 25 °C identified by *hsp65* gene

Mycobacterium species	Number of isolates (%)	
	37 °C	25 °C
<i>Mycobacterium lentiflavum</i>	72 (84.7)	70 (80.4)
<i>Mycobacterium gordonae</i>	11 (13)	14 (16)
<i>Mycobacterium paragordoniae</i>	2 (2.3)	2 (2.3)
<i>Mycobacterium fortuitum</i>	0 (0)	1 (1.1)
Total	85 (100)	87 (100)

may cause significant impact on patients (Crago *et al.* 2014). This study found that an unexpected number of the examined isolates throughout the hospital water distribution systems

over the two-year surveillance were NTM, in particular *M. lentiflavum* and *M. gordonae*. Although *M. lentiflavum* and *M. gordonae* are generally considered non-pathogenic, there are cases describing infections caused by these mycobacteria. Similar findings were reported by Khosravi *et al.* (2016) in Khuzestan province of Iran, in which of 77 culture-positive mycobacteria isolated from 258 hospital water samples, *M. fortuitum* and *M. gordonae* were among the most prevalent isolates. Likewise, in other studies conducted in Iran, *M. gordonae* and *M. lentiflavum* were among the most prevalent NTM isolated from water samples (Bahram *et al.* 2012; Moghim *et al.* 2012; Azadi *et al.* 2016). Our results were also relatively similar to other investigations on hospital waters in the world (Angenent *et al.* 2005; Hussein *et al.* 2009;

Table 4 | Frequency of mycobacterium species in different water sources of hospitals incubated at 37 °C

Water sources	Number of samples	Number of positive samples	NTM species (based on analysis)
Oxygen manometer (humidifying cup)	66	38	<i>M. lentiflavum</i> (34), <i>M. gordonae</i> (4)
Tap water	51	26	<i>M. lentiflavum</i> (20), <i>M. gordonae</i> (4), <i>M. paragordoniae</i> (2)
Dialysis devices	69	6	<i>M. lentiflavum</i> (4), <i>M. gordonae</i> (2)
Water distillation unit	5	1	<i>M. lentiflavum</i> (1)
Nebulizers	13	5	<i>M. lentiflavum</i> (5)
Humidifier	4	4	<i>M. lentiflavum</i> (3), <i>M. gordonae</i> (1)
Shower heads	6	2	<i>M. lentiflavum</i> (2)
Drinking fountain	2	2	<i>M. lentiflavum</i> (2)
Dental units	1	1	<i>M. lentiflavum</i> (1)
Neonatal incubator	1	0	

Table 5 | Frequency of mycobacterium species in different water sources of hospitals incubated at 25 °C

Water sources	Number of samples	Number of positive samples	NTM species (based on analysis)
Oxygen manometer (humidifying cup)	66	34	<i>M. lentiflavum</i> (29), <i>M. gordonae</i> (5)
Tap water	51	32	<i>M. lentiflavum</i> (24), <i>M. gordonae</i> (6), <i>M. paragordoniae</i> (2)
Dialysis devices	69	5	<i>M. lentiflavum</i> (3), <i>M. gordonae</i> (2)
Water distillation unit	5	1	<i>M. lentiflavum</i> (1)
Shower heads	6	5	<i>M. lentiflavum</i> (5)
Humidifier	4	2	<i>M. lentiflavum</i> (1), <i>M. fortuitum</i> (1)
Nebulizers	13	5	<i>M. lentiflavum</i> (5)
Drinking fountain	2	2	<i>M. lentiflavum</i> (2)
Dental units	1	1	<i>M. lentiflavum</i> (1)
Neonatal incubator	1	0	

Genc *et al.* 2013). In a study in Turkey, a total of 160 water samples from hot and cold water in two hospitals was examined (Genc *et al.* 2013). Out of 33 isolated strains, 20 (60/6%) were *M. lentiflavum* and the remainder were *M. gordonae* (30/3%) and *M. peregrinum* (9/1%) (Genc *et al.* 2013). Furthermore, in our recent publication we showed that *M. gordonae* was among the dominant pathogens recovered from clinical samples, highlighting the role of hospital tap water as a vector for transmission of the bacteria (Nasiri *et al.* 2018a, 2018b).

Due to the risk of NTM-contaminated hospital water supplies for nosocomial infections, preventive measures should be considered. For water supplies, it is suggested to prevent water stagnating in water supplies and to regularly disinfect regions at risk (i.e., reservoirs and drinking fountains) (Dailloux *et al.* 1999). Furthermore, in the tap water used to rinse haemodialyzers, the use of water inlet filters is recommended (Dailloux *et al.* 1999). For healthy people, a number of common measures should be indicated, namely, disinfection of wounds in the case of an accidental injury and protecting them from water.

M. gordonae was the second most common NTM in our study. This mycobacterium is usually found in water, soil, and raw milk and is known as saprophytic and a rarely pathogenic bacterium in humans (Wolinsky 1979; Jarikre 2011). *M. gordonae* (the tap water bacillus) has been reported responsible for infections including skin and soft tissues infections (Gengoux *et al.* 1987; Modilevsky *et al.* 1989), lung disease (de Gracia *et al.* 1989), and liver and peritoneal infections (Kurnik *et al.* 1985). Based on recent studies, *M. gordonae* is one of the most common mycobacterium isolated in hospital waters (Angenent *et al.* 2005; Genc *et al.* 2013).

M. lentiflavum, another prevalent NTM in the current study, was first described in 1996 (Springer *et al.* 1996). This mycobacterium has been shown to grow in a wide range of temperatures (22 to 37 °C), and can cause human disease in both immunocompetent and immunocompromised individuals (Springer *et al.* 1996; Cabria *et al.* 2002; Tortoli *et al.* 2006; Marshall *et al.* 2011). In a study conducted in Australia in 2001 and 2008, the relationship between genotype and geographical similarities between *M. lentiflavum* isolates from patients and drinking water was evaluated (Marshall *et al.* 2011). Based on their results, genotypes of

the environmental clone of *M. lentiflavum* were close to the strains that were isolated from patients. This finding suggests that drinking water can be the source of infection for *M. lentiflavum*. Similarly, in the study of Torvinen *et al.* (2004) over 90% of the mycobacteria isolated from water belonged to *M. lentiflavum* and *M. gordonae*. Likewise, in South Korea, Lee *et al.* (2008) showed that 65% of the samples obtained from drinking water were *M. lentiflavum*. Although for setting up the decontamination method we evaluated several protocols, we should mention that due to the method used for decontamination, some species might not have been isolated.

The rising number of NTM isolated from hospital waters in Iran may have several negative effects on public health status. Importantly, most TB laboratories in Iran are not equipped to perform NTM species identification; thus, culture reported positive for TB may likely be the result of laboratory contamination with environmental NTM. Consequently, NTM may be misdiagnosed as TB which results in unnecessary anti-TB treatment (Nasiri *et al.* 2015).

Another important finding in our study is the contamination of medical devices such as oxygen manometer (humidifying cup), nebulizers and hemodialysis fluid with mycobacteria which is a significant health risk for hospitalized patients. Recently, Heidarieh *et al.* (2016) in Iran indicated that a diverse bacterial community, containing predominantly mycobacteria, was detected in a hospital's hemodialysis distribution system. Due to poor immune system in these patients, microbiological monitoring of water used for hemodialysis and other medical devices is very important for the prevention of NTM-associated illness (Montanari *et al.* 2009; Heidarieh *et al.* 2016). Recently, transmission of *M. chimaera* from heater-cooler units during cardiac surgery has been reported (Sax *et al.* 2015; Sommerstein *et al.* 2016). Thus, it is recommended that standard protocols for the evaluation of microbial contamination should be used for regular monitoring and identification of NTM in medical devices.

CONCLUSION

Isolation of NTM from hospital water sources is a serious public health problem in Iran and merits further attention

by health authorities. The establishment of microbiological monitoring systems for hospital waters, and expanding the number of facilitated laboratories are strongly recommended.

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