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Species diversity and molecular characterization of nontuberculous mycobacteria in hospital water system of a developing country, Iran

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ABSTRACT

Background: Hospital environment is of crucial importance in cross-transmission of opportunistic pathogens to the patients. Nontuberculous mycobacteria have the remarkable capability to withstand the adverse condition of hospital environments and pose a potential threat to the health of patients. The current study aimed to assess the frequency and diversity of mycobacteria in hospital water of a developing country using a combination of conventional and molecular methods.
Methods: A total of 148 hospital water samples collected from 38 hospitals were analyzed for the presence of mycobacteria using standard protocols for isolation and characterization of the isolates. The conventional tests were used for preliminary identification and Runyon's classification, the PCR amplification of *hsp*65 gene and sequence analysis of 16S rRNA were applied for the genus and species identification.
Results: A total of 71 [48%] isolates including 30 rapidly growing and 41 slowly growing mycobacteria were recovered. The three most prevalent species were *M. lentiflavum*, 28.2%, *M. paragordonae*, 21.1%, and *M. fredriksbergense*, 9.8%, followed by *M. simiae* and *M. novocastrense*, 7%, *M. canariasense* and *M. cookii* like, 5.6%, *M. setense*, 4.2%, *M. fortuitum* and *M. gordonae*, 2.8%, and the single isolates of *M. austroafricanum*, *M. massiliense*, *M. obuense*, and *M. phocaicum* like.
Conclusion: The results of our study show that the hospital water resources, drinking or non-drinking can be the reservoir of a diverse range of mycobacteria. This reaffirms the fact that these organisms due to intrinsic resistance to common antiseptic and disinfectant solutions persist in hospitals and create a threat to the patient's health and in particular to those that suffer from weakness of immunity.
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1. Introduction

Bacteria of the genus *Mycobacterium* consist of more than 175 officially recognized species [1]. The genus includes non-pathogenic organisms as well as opportunistic pathogens known as environmental mycobacteria, atypical or nontuberculous mycobacteria [NTM], and highly successful human pathogens such as *M. tuberculosis*.

The impact of NTM infections has been in particular serious in individuals with the immunocompromised condition being associated with opportunistic life-threatening infections in AIDS and transplanted patients [2,3]. Nevertheless, an increased incidence of pulmonary diseases and nosocomial infections in immune-competent population underlined the importance of NTM on human health [4–7]. The ubiquitous nature of NTM allows their persistence in water and aquatic biofilms. This capability within hospitals environments such as water pipes and medical devices or other healthcare units represents a threat to human health since they respond to variation in the environment such as oxygen deprivation, heat or cold shocks, pH changes, exposure to toxins and antibiotics by exhibiting altered growth and characteristics that favor the onset and spread of HAI [8,9].

Although many of the opportunistic infections associated with mycobacteria have been reported to be common in developed countries [10–12], to our knowledge, there has been few extensive reports on the diversity and potential inhabitants of NTM in hospital environments from developing countries [13,14].

The identification of NTM is traditionally based on the

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conventional phenotypic structures such as what has been recommended by Runyon's identification and classification. Molecular tests that are currently applied provides a conclusive approach to the identification of NTM. The molecular test strategy most often used is PCR-based RFLP (PRA) and sequence analysis of variable regions within microbial conserved genes, i.e., 16S rRNA or *rpo*B and *hsp*65 [15,16].

The aim of this study was to appraise the diversity of NTM recovered from environmental samples of hospitals and the phenotypic and molecular profiles of these isolates. The information from this study might be useful for raising the awareness among hospital authorities involved in infection control about these neglected but highly important bacteria. Moreover, the result of our study presented here would give a better picture of the extent of NTM adapted to hospital environments to the degree that they can survive long enough to transmit to a human host.

2. Materials and methods

2.1. Sampling and decontamination

From September 2011 to December 2012 a total of 148 water samples were collected from water systems of 38 hospitals in Isfahan province. The samples were processed based on standard procedures [17]. In summary, each 1000 cc water sample was transported at 4 °C to the laboratory and processed within a maximum period of 24 h. Initially water samples were decontaminated with cetylpiridinium chloride (CPC) 0.005%, for 15 min, and vacuum filtered using cellulose nitrate filters (0.45 μm, Sartorius AG, Gottingen, Germany) the filters were rinsed and macerated in tubes containing 15 ml of distilled water. Almost 100 μL aliquots of dissolved filters transferred into tubes of Löwenstein-Jensen (LJ) media, and incubated at a temperature between 25° and 42 °C in an atmosphere of 5% CO_2.

The details of water samples tested during the research period are presented in Table 1.

2.2. Conventional identification

The isolates were characterized phenotypically by the use of conventional phenotypic and biochemical tests and classified into Runyon groups [18]. The tests included Ziehl-Neelsen (ZN) staining and colony characteristics as well as standard biochemical assays, i.e., growth rate, growth at 25 °C, 32 °C, 37 °C and 42 °C, semi-quantitative and heat-stable (68 °C) catalase production, pigment production, tween opacity, nitrate reduction, urease activity, tellurite reduction, niacin accumulation, pyrazinamidase, and tolerance to 5% NaCl.

2.3. Molecular identification

2.3.1. DNA extraction and purification

The method of Pitcher was used for DNA extraction with some modifications [19]. For the lysis of mycobacterial cells, a pretreatment with lipase (2 mg/ml) was carried out followed by cell wall disruption with a higher concentrations of lysozyme (200 mg/ml final concentration) and proteinase K (300 μg/ml final concentration) in the presence of sodium dodecyl sulfate (SDS) and additional treatment with guanidium isothiocyanate solution. The extracted DNA was purified with phenol-chloroform-isoamyl alcohol (25:24:1, vol/vol/vol) and chloroform-isoamyl alcohol (24:1, vol/vol) and precipitated with sodium acetate and ethanol at −20 °C. Precipitated DNA was washed with 70% ethanol and resuspended in 100 μl of Milli-Q water.

2.3.2. Molecular identification of mycobacterial isolates

The water isolates identified phenotypically as *Mycobacterium* were further analyzed to the genus and species level using a panel of molecular tests that included a genus-specific PCR based on a 228-bp fragment of the 65-kDa heat shock protein as recommended by Khan and Yadav [20] and the amplification and direct sequence analysis of 16S rRNA gene for species identification as described previously [21]. Sequencing was performed by Bioneer Company (South Korea). The sequences were aligned manually with the existing sequences of mycobacteria retrieved from GenBank™ database and analyzed using the Blast program in GenBank and the jPhydit program [22].

2.3.3. Nucleotide sequence accession numbers

The GenBank accession number for the 16S rRNA sequencing of environmental isolates of Iranian mycobacteria determined in this study are listed below. *M. lentiflavum* (KF019694), *Mycobacterium* sp. AW6 (JX566888), *M. simiae* (KF028776), *M. frederiksbergense* (KF019696) *M. austroafricanum* (KF019697), *M. canariasense* (KF188706), *M. novocastrense* (JX566889), *M. setense* (KF019693), *M. obuense* (KF028777) and *M. phocaicum* like (KF019699).

3. Results

In this study, a total of 71 (48%) mycobacterial isolates were recovered from 148 water samples collected from hospitals. The positive samples came from the 25 of 38 hospitals. No mycobacteria were detected in any of the analyzed samples taken from the other 13 hospitals. A total of 20 isolates were recovered from water collected from shower heads and patients' rooms, 40 isolates from tap water of in patient's rooms or departments, and 11 isolates from water resources used for irrigation of greenery in hospital outdoor spaces.

The recorded temperature, pH, and total dissolved solids (TDS) for the water samples collected from hospitals' indoor areas were 12–44 °C; 6.6 to 8 and 350–700 mg/L, respectively.

The chlorine residual concentrations of water samples collected from hospitals' indoor and outdoor areas were between 0.4 and 0.6 ppm and zero respectively. The water used for irrigation of greenery is not used for drinking or washing and is used only for green space irrigation.

The details water samples and the mycobacteria isolated are presented in Table 1.

Based on morphological, culture and biochemical properties and the genus specific marker, i.e., the presence of a 228-bp fragment of the *hsp*65, all 71 isolates were identified as *Mycobacterium* of which 5 isolates fit into Runyon group I, 41 isolates in Runyon group II and 25 isolates in Runyon group IV (Table 1).

The 16S rRNA gene sequencing of the isolates revealed that all isolates had nucleotide signatures of mycobacteria at positions 70–98 (A–T), 293–304 (G–T), 307 (C), 328 (T), 614–626 (A–T), 631(G), 661–744 (G–C), 824–876 (T–A), 825–875 (A–T), 843 (C), and 1122–1151 (A–T) [14,15]. In addition, the rapidly growing isolates shared the short helix 18 located at position 451–482 that is characteristic of the rapidly growing mycobacteria. In contrast, the slowly growing isolates except for the isolated belong to *M. simiae* complex (*M. simiae and M. lentiflavum)* characterized by an extended helix shared by slowly growing mycobacteria.

Based on phenotypic and molecular data, the isolates belong to 11 validated species and 2 unknown or potentially novel species. The three most prevalent mycobacterial species isolated from hospital water resources were *M. lentiflavum*, 20 isolates (28.3%), *M. paragordonae*, 15 isolates (21.2%), and *M. fredriksbergense*, 7 isolates (9.8%) (Table 1). Theses followed by *M. simiae* and *M. novocastrense*, 5 isolates each (7%), *M. canariasense* 4 (5.7%),

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