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ORIGINAL ARTICLE **OPEN ACCESS**

Isolation and Molecular Identification of *Mycobacterium fortuitum* isolates from Environmental water and clinical samples at different regions of Iran

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ABSTRACT
Mycobacterium fortuitum belongs to the nontuberculous mycobacteria (NTM) that causes diseases resembling pulmonary tuberculosis, lymphadenitis, skin and soft tissues infection, and disseminated infection. It occurs worldwide and is usually found in natural and processed water, sewage and dirt. Recent outbreaks of nosocomial infections caused by organisms identified as the M.fortuitum complex suggest that species identification is epidemiologically important. The purpose of this study was to investigate the contamination of surface water as well as clinical specimens of M.fortuitum by means of molecular and phenotypic methods.Water samples were collected from different regions of Iran (Tehran city and some local parts in north and south of Iran). After enrichment using filtration, all samples were cultured on Lowenstein-Jensen (LJ) medium at temperatures of 37℃. Conventional bacteriology method was used for identification of mycobacteria.The PCR- RFLP analysis (PRA)*was used to targethsp65 genes and the restriction patterns of amplicons (441bp) was used for identification after digesting withBstEII and HaeIII endonucleases. The petroff 's method was used to isolate the organism from the clinical specimens. All clinical specimens were identified at the species level as described for the environmental samples. Based on the growth rate of isolates, the results of biochemical test and patterns of PRA, of54 NTM isolates from environmental water; 30 (55.55%) were identified as M.fortuitum. Moreover, in clinical samples 12 of 19 isolates (63.15%) were identified as M. fortuitum. PRA is a reliable and rapid approach which can identify mycobacterial strains to the species level. Our study showed that M.fortuitum plays a significant role in pulmonary and extrapulmonary infection in patients. On the other hand, the data obtained in our study revealed that, M.fortuitum is the predominant isolated NTM from water. It seems there is a correlation between clinical and environmental samples*
Keywords : *molecular Identification, Mycobacterium fortuitum, Water – clinical sample*

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INTRODUCTION

Environmental mycobacteria also referred to as atypical mycobacteria or non tuberculous mycobacteria (NTM) are common saprophytes in all natural ecosystems, such as water, soil, food and dust [1].Although members of the Mycobacterium tuberculosis complex (MTC) are responsible for the majority of mycobacterial infections worldwide, environmental opportunistic infections due to non-tuberculous mycobacteria (NTM) are increasingly becoming more of a public health challenge [2]. In developing countries, it may be difficult to assess the prevalence of NTM infections, mainly due to the fact that identification of the species involved is generally not done, and these diseases are often under-diagnosed or misdiagnosed as tuberculosis [2,3]. Absence of evidence regarding person to person transmission indicates the importance of environment as an infection source [4]. Therefore studying the possible sources, as well as common species in each geographic area is required. Several species of NTM have been identified in different environments including public drinking water, pool, tap water, water cooler

etc [5,6]. Therefore, water may act as an important NTM source for infection transmission to human and further mycobacteriological studies of water may contribute to our knowledge about their ecology and epidemiology [7]. Classical identification of mycobacteria based on phenotypic tests may take several weeks, and often fail to provide precise identification [8] .Published case reports from Iran show the pathogenic potential of this mentioned species [3]. During last decade, several molecular methods have been introduced for mycobacterial identification. Of these methods, *PCR- RFLP analysis (PRA)* is preferred because of it's rather simplicity and rapidity [8].Restriction enzyme analysis of PCR products of specific genesgenerates mostly species-specific DNA patterns, and provides a comparatively cheap alternative over DNA sequencing. In particular, PRA of part of the gene encoding the 65-kDa heat shock protein (*hsp65*) has been widely used for diagnostic purposes [3]. This method is based on the amplification of a 441-bp fragment of the *hsp65*gene present in all mycobacteria, followed by digestion of the PCR product with the restriction enzymes *BstEII* and *HaeIII*. By combining both restriction patterns, a species assignment is possible based on comparison with patterns described in published algorithmsor availablefrom an Internet database [http://app.chuv.ch/prasite][9,12].

Amongst the species *M.fortuitum*is one of the candidates to be studied. Epidemics of hospital-associated infections caused by *M.fortuitum*after surgeries, in hemodialysis unit and in eye,dermal and many other infections show the real importance of this group of mycobacteria in the health system (10,11). It is believed that NTM have paramount value to create a variety of infectious and emerging diseases in individuals; both immune-deficient and cases with normal immune systems have been reported. The purpose of this study was to investigate the contamination of surface water, as well as clinical specimens of *M. fortuitum* by means of molecular and phenotypic methods.

MATERIALS AND METHODS

A total of 852 clinical specimens were cultured to possibly isolate non-tuberculous mycobacteria. The specimens were digested and decontaminated by petroff 's (using 4% NAOH) method (13).Totally, 166 samples were collected from different regions of Iran (Tehran city and some local parts in north and south of Iran). These included industrially polluted water, sewage, garbage, general pools, river water and water spout. All samples were collected in sterile bottles and transported on ice to the laboratory and were examined at 12 to 18 hours. For decontamination of water, cetylpyridinium chloride (CPC) method was carried out(7). Briefly, CPC was added to the sample to give a final concentration of 0.01%. The mixture was shaken for 30 s. After an exposure for 30 min, the samples were immediately filtered through cellulose acetate membrane filter (50 mm, pore size 0.45µm) and rinsed in 100 ml of sterile water to remove residual CPC (7,14,16). A strip 10mm wide was then aseptically cut out from the center of the filter and placed on the medium (15). After inoculation, the media were incubated at 37°C for 12 weeks and checked for growth every 2 - 3 days.

At the end of the 12 weeks incubation period, all isolates were investigated by conventional methods consisting of acid fast staining and analysis of phenotypic characteristics, i.e., arylsulfatase activity, pigment production, growth rate (<7 days), nitrate reduction, growth on MacConkey agar without crystal violet, existence of urease, semi quantitative and catalase activity and niacin production tests (1).

Preparation of bacterial DNA – Two to three loops of growth from freshly grown mycobacterial cultures from LJ medium were harvested and transferred to 400 µl of sterile nuclease-free distilled water. The tube was vortexed briefly, boiled for 40 min and centrifuged to settle the cell debris (17). A 10 µl each of the supernatant was used as genomic DNA template for PCR**.**

PCR of the *hsp65* gene was performed using the forward primer for *hsp65*, Tb11 (5′ACC AAC GAT GGT GTG TCCAT 3') and the reverse primer, TB12 (5' CTT GTC GAA CCG CAT ACC CT 3') (10). The PCR products were run on 1.5% agarose gel and visualized with transluminator after staining with ethidium bromide.

Identification of *Mycobacterium* species was based on Restriction enzyme analysis of PCR products digested with *BstEII* and *HaeIII* restriction enzymesaccording to Telenti*et al*(18).

RESULTS AND DISCUSSION

Out of the 852clinical samples, 19 mycobacterial isolates (2.23%) were gained. The sources of the isolates were as follows: bronchial washes, urine, soft tissue, fluid, sputum.

Totally, 166 samples were examined for isolation of mycobacteria from water. The mean pH was 7± 0.50 and the water samples' temperature was 5-25°C. Out of 166 water samples, only 54 samples (32.53%) yielded NTM.

54 atypical mycobacteria were isolated from environmental samples; based on growth rate and conventional biochemical, *M.fortuitum* was identified with the relative frequency of 55.55 %(n=30). Moreover; in clinical samples of the study, 19 atypical spices were isolated of which 63.15% (n=12) were

M.fortuitum. All 42 isolates showed phenotypic characteristics, consistent with *M. fortuitum*, i.e., rapid growing (<7 days) at 37°C, no pigment production, growth on MacConkey agar without crystal violet and principal biochemical properties of catalase, Tween hydrolysis, and arylsulfatase positivity, the isolates were not identified to the biovariant level.

Table1.Clinical information of Iranian isolates of NTM

Origin clinical sample	No. of NTM isolates obtained (%)	*Mycobacterium fortuitum*No. (%)
fluid	3	2
soft tissue	1	1
urine	1	-
sputum	8	6
bronchial washes	6	3

Table 2.Environmental information of Iranian isolates of NTM

Water source	No. of samples	No. of NTM isolates obtained (%)	*Mycobacterium fortuitum*No. (%)
Tehran city	74	37	24
North of Iran	62	8	4
South of Iran	40	9	2

The presumptive *M.fortuitum* isolates were further analyzed and verified as mycobacteria using genus and group specific PCR targeting a 441 bp fragment (Figure 1).Furthermore, with the application of the PRA algorithm targeting 441 bp*hsp65* DNA, 42isolates were clearly distinguished from other mycobacteria. A 441 bp fragment of *hsp65* genes was amplified and digested by *BstEII* and *HaeIII*. The digested fragments separated on 3% agarose gel and RFLP patterns were analyzed according to fragment sizes. All 42 isolates had the PRA *hsp65* pattern characteristic of *M.fortuitum*, with*BstEII*restriction fragments of 240/ 120 and 85 bp and *HaeIII* fragments of 145 /120 /60 bp (Figure 2).

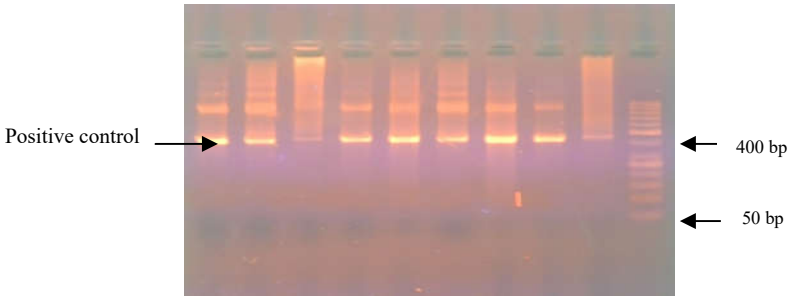

Figure1. Genus specific amplification of NTM. Lanes 2-9; *M. fortuitum* isolates, Lanes 1; Positive control, Lane 10: The 50 bp DNA size marker.

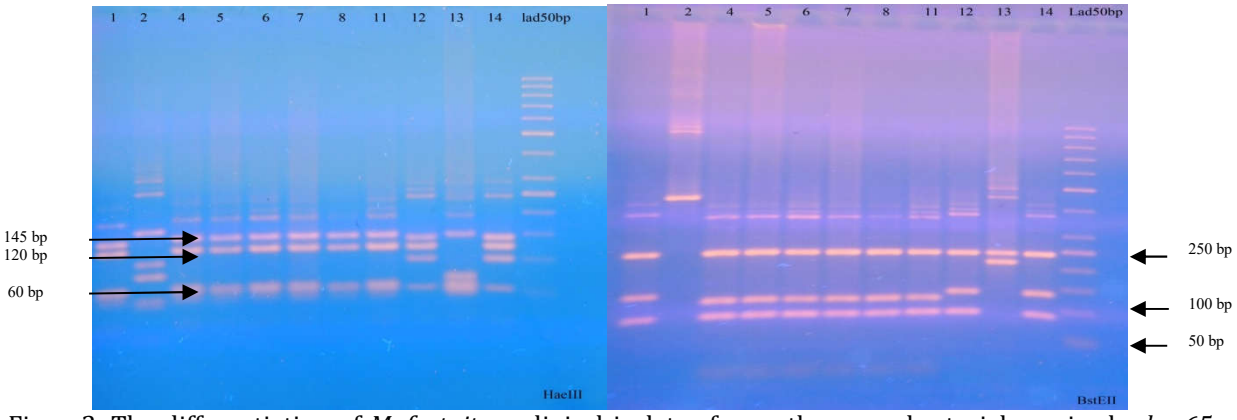

Figure2. The differentiation of *M. fortuitum* clinical isolates from other mycobacterial species by *hsp65* PRA based on digestion of a 644 bp amplified fragment with *BstEII* and *HaeIII*. Lanes 4, 5, 6, 7, 8 & 11: *M.fortuitum*, Lanes 15: The 50-bp DNA size marker.

In this study, a combination of conventional and molecular tests was applied to identify the isolates. There was complete agreement of identification results between conventional phenotypic methods and *hsp65* PRA.

Increase in outbreak of infections with Nontuberculous Mycobacteria (NTM) has become the major concerns of health systems of different countries. There are reports on the clinical significance of *M. fortuitum* as the most frequent rapidly growing mycobacteria and water as its main source of infection. Although exposure to NTM does not occur frequently but immune-compromised individuals are at high risk of being infected with *M. fortuitum* from water source [19]. Emerging infections with this organism include skin and soft tissue infection lymphadenitis, pulmonary infections, catheter-related infections and disseminated infection [20, 21]. This organism can also be transferred following trauma, injections, and augmentation mammoplasty and ultrasound hydrolipoclasia. Furthermore, in the field of orthopaedic surgery, *M.fortuitum* infection has been encountered only following prosthetic arthroplasty or fracture surgery with internal fixation [22,23].

Water has been proven as important source for these opportunistic mycobacteria [9 34, 24].This fact was illustrated by *M. fortuitum* furunculosis following footbaths and disseminated infection in leukemia patients [25]. Warm water reservoirs are infested with *M. fortuitum*, such as; foot spas which are caused infections when people use them for pedicure. [22, 26].

In the present study, of 166 water specimens collected from different sources, 30 were positive for *M. fortuitum* in culture. This suggests that the risk of water contamination with this organism is high. On the other hand, a high rate of clinical specimens were also positive for *M. fortuitum* (63.15%) which indicates that environmental contacts through water probably is the cause of many infections with this organism in human.

Generally our study shows the importance of water contamination as a potential pathogenic source for patients with immune deficiency and suggests that more serious measures must be taken by health authorities to disinfect the water sources at the health care facilities.

NTM are involved in causing different infections and some of them cause diseases similar to tuberculosis. Treatment of patients infected with NTM is different from those infected *M. tuberculosis*. Therefore, correct and rapid identification of mycobacteria at species level is important for treatment. Identification of NTM by biochemical methods is time consuming and the interpretation of the results is a complex process. In contrast, molecular methods such as PRA targeting conserved loci such as *hsp*65 gene of different species have improved the sensitivity. There was agreement between the results of conventional phenotypic methods and hsp65 PRA for identification of *M. fortuitum* in this study. Both methods indicated that 55.55% of identified environmental species and 63.15% of clinical specimens were *M. fortuitum*. Comparing species abundance in this study is in agreement with the previous findings in Iran [27, 28]. In Korea and U.S.A and in many parts of the world, *Mycobacterium avium* Complex (MAC) is the most common cause of infection among NTM [29, 30, 31].

In this study we have shown that among methods of species identification in mycobacterium using PRA on colony obtained from different mycobacterium with replication of 441 bp was faster and more precise in compare with biochemical methods and if this method to be used on clinical specimen it helps in correct diagnosis, treatment and control of tuberculosis by preventing possible errors in determining species and also by reducing time of diagnosis and on the other hand it provides more precise statistics of outbreak of different MTM among clinical specimens. Since determining outbreak of different NTM in environmental specimens such as water in different geographical regions have always been of high importance, using PRA in this case can also pave the way for problems in species identification.

There are many factors that affect recovery of mycobacteria from water. These factors are including decontamination methods and climate conditions. Given the increased prevalence of immune deficiency diseases in the community and the presence of Mycobacteria species in environmental samples such as different aquatic sources, awareness and gaining more knowledge about species dispersion is essential.

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