



Molecular Characterization of Environmental Non-Tuberculous Mycobacteria Using PCR- RFLP Analysis of 441 Bp Heat Shock Protein 65 Fragments

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Abstract

Background: Non- Tuberculous Mycobacteria are environmental opportunistic pathogens that can be found in various terrestrial and aquatic habitats. There are an epidemiological links between species isolated in tap water and those isolated from patients. *hsp65* gene has more variability in its sequences, compared to the some more conserved genes in NTM, for identification of mycobacteria to species level. In this study, the prevalence of NTM in Isfahan City water samples was determined using culture, biochemical tests and PCR-RFLP analyses of *hsp65* gene.

Methods: Eighty-five water samples were collected and cultured. The mycobacterial isolates were identified by conventional biochemical tests. A 441 bp fragment of *hsp65* genes was amplified and digested by two restriction enzymes, BstEII and HaeII. Digested products were analyzed using polyacrilamid gel electrophoresis (PAGE).

Results: 25.9% of the water samples contained different species of NTM. Dominant isolates were *M. fortuitum* (26.7%), *M. delonae* like organism (13.3%) and *M. mucogenicum* (13.3%). Nineteen isolates of *Mycobacteria* were differentiated using *hsp65* genes PCR-RFLP. Three isolates could not be identified at the species level because their RFLP patterns were different from other known PCR-RFLP profiles. There were different *hsp65* gene PCR-RFLP profiles produced by digestion with BstEII and HaeIII.

Conclusion: This study showed that PCR-RFLP of *hsp65* gene in mycobacteria is more reliable method for identification of NTM at the specie level than conventional phenotypic methods ($P < 0.05$). In comparing of RFLP patterns of this study to other investigation, some minor differences were negligible.

Keywords: NTM, Environment, *hsp65* gene, PCR-RFLP

Introduction

Mycobacteria are a heterogeneous group of bacteria in terms of their genotypic features and disease association. These organisms can cause hypersensitivity, pneumonitis, asthma, and bronchitis, infection of skin, wounds and glands. Moreover, these infections are serious threat for cystic fibrosis patients. In recent years, Non- Tuberculous Mycobacteria (NTM) was reported as important agents of infection in immunosuppressive patients

(1). NTM can be isolated from different natural sources among them water (1- 4). These organisms have been isolated from hard conditions such as low pH and nutrients. Several species of NTM have been identified in different environments including public drinking water, pool, undrinkable tap water, water cooler etc. Therefore, water may act as an important NTM source for transmission to human. In some studies, the pres-

ence of NTM in water samples collected from different regions were determined (1, 2, 3, 5).

Traditionally, NTM has been detected in clinical and environmental samples by culture-based techniques; however, these techniques may not be well suited for environmental samples. Indeed, identification of NTM by culture and phenotypic characterization is widely used but it takes 4 to 6 weeks or longer for slow growing species and identification of some species may miss by biochemical methods. The rapid methods used for identification include high performance liquid chromatography (HPLC), DNA probes, restriction fragment length polymorphism (RFLP) using various target regions including heat shock protein 65 KD gene (*hsp65*), *ITS* and *tpo B* (1, 6, 7). Molecular methods linked to PCR are more reliable and faster for identification of NTM (1, 5). *hsp65* gene is used widely for identification of NTM to species level because of its variability compare to some other conserved genes such as 16S rRNA (6, 7). Therefore, it is suitable target for identification of NTM to species level than other methods (8).

To identify the presence of NTM species in water samples in Isfahan and demonstrate the usefulness of this protocol for identification and characterization of NTM, we applied culture-based techniques and PCR-RFLP targeting *hsp65* for characterization of 22 mycobacteria isolated from water samples in Isfahan, Iran.

Materials and Methods

Collection and preparation of the samples

Eighty five water samples were collected from different sources including mineral water (8.2%), dentistry unit water (10.6%), undrinkable tap water (14.1%), drinkable water supply (14.1%), haemodialysis unit water (8.2%), general pools (8.2%), river water (7.1%), water spout (11.8%), water cooler (11.8%), and water boiler (5.9%) in Isfahan, Iran. To 2-litres' sterile Erlenmeyer flasks, sodium thio-sulfate as antichlor and 0.04% Cetyl pridium chloride as antimicrobial agent were added. Sampling of water was done using the grab sampling method (9). At the sampling loca-

tion, total chlorine content was determined using DPD method (9, 10). Filled containers were tightly capped and delivered to the laboratory and processed the day of collection. Five hundred ml of samples were passed from 0.45 µm filters. The filters were transferred directly onto 7H10 Middle Brook solid media, include 15% OADC (Oleic acid, Albumin, Dextrose, Catalase). The plates were examined once a week for eight weeks. When the colonies appeared, they were subjected to acid-fast staining and acid-fast colonies were transferred to Lowenstein- Jensen (LJ) slant media and incubated in 37°C.

Phenotypic identification

The mycobacterial isolates were identified by the growth characteristics, including growth at 25, 37 and 42 °C, pigment production, semi quantitative catalase test, Tween 80 hydrolysis, arylsulfatase test (3 and 14 days), heat-stable catalase (pH 7, 68°C), pyrazin amidase (4 and 7 days), urease, nitrate reduction test and colony morphology. The phenotypic identification tests were repeated whenever mycobacteria identification was doubtful. Reference strains of *M. smegmatis* (PTCC 1307) and *M. fortuitum* (ATCC 6841) were used as control species in all steps of this study.

Species identification of NTM by PCR-RFLP

PCR-RFLP of the *hsp65* gene was undertaken to identify the species of isolates. Chromosomal DNA was extracted using CTAB (cetyl- Trimethyl Ammonium Bromide) method. PCR of the *hsp65* gene was performed using the forward primer for *hsp65*, Tb11 (5' ACC AAC GAT GGT GTG TCC AT 3') and the reverse primer, TB12 (5' CTT GTC GAA CCG CAT ACC CT 3') (11, 12). In a 25µl PCR mix, 2U of Taq polymerase, 10 mM Tris-HCl (pH 8.3), 1.5mM MgCl₂, 200mM dNTP, 20pmol of each primers and 2ng of DNA template were added. PCR was performed using initial denaturation at 95°C for 5min, 45 amplification cycles of 60s at 95°C, 60s at 56°C and 60s at 72°C and a final extension of 72°C for 7min in a Biometa Gradient thermocycler and an Eppendorf AG 22331. The PCR products were run on 1.5%

agarose gel. DNA bands were visualized by ethidium bromide staining and photographed (Fig. 1). The amplified products of *hsp65* gene regions were digested with two restriction enzymes of HaeIII and BstEII according to the recommendations of the manufacturers. The digested products were separated on 10% polyacrylamid gel electrophoreses (PAGE) and RFLP patterns were analyzed according to fragments sizes (11-14).

Results

Sources of samples, percentage of positive samples and NTM species that found in water samples are summarized in Table 1.

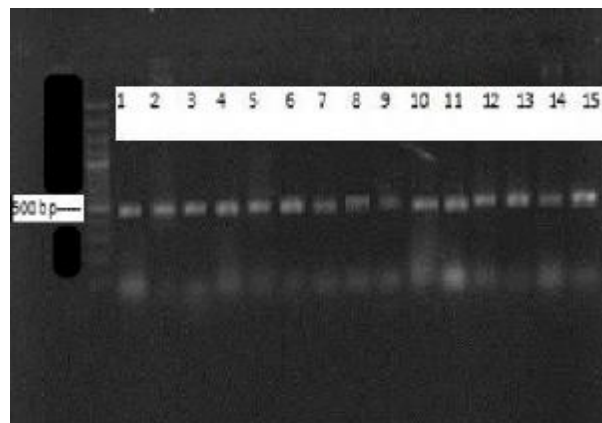


Fig. 1: Agarose gel electrophoresis of 441 bp *hsp 65* PCR products. First Lane: 100 bp DNA marker; lane 1: *M. smegmatis* (PTCC 1307); lane 2: *M. fortuitum* (ATCC 6841) and lanes 3 to 15: NTM isolates

Table 1: Rapid and slow growing mycobacteria isolated from different water sources in Isfahan

Water sources	NTM positive	Species of NTM
Water supply	(41.7%)	<i>M. gordanae</i> , <i>M. mucogenicum</i> , <i>M. fortuitum</i> ss. <i>fortuitum</i> , <i>M. chitae</i> , <i>M. neoaurum</i>
Undrinkable tap water	(50%)	<i>M. chelonae</i> , <i>M. fortuitum</i> , <i>M. mucogenicum</i> , <i>M. chelonae like organism</i> , 2 Unidentified species
Waterspout	(20%)	<i>M. mucogenicum</i> , <i>M. fortuitum</i> ss. <i>fortuitum</i>
Cold water dispenser	(50%)	<i>M. chelonae like organism</i> (2), <i>M. fortuitum</i> 3th variant(2), 1 Unidentified species
Dentistry unit	(22.2%)	<i>M. mucogenicum</i> , <i>M. fortuitum</i> 3th variant
Pool and baths	(28.6%)	<i>M. chelonae like organism</i> , <i>M. duvalii</i>
Haemodialysis center	0	-
Mineral	0	-
River	0	-
Hot water dispenser	0	-

Twenty-two mycobacteria were isolated and identified by growth characteristics and conventional biochemical tests. Dominant isolates were *M. fortuitum* (26.7%), *M. chelonae like organism* (13.3%) and *M. mucogenicum* (13.3%).

PCR-RFLP of the *hsp65* gene was undertaken to identify the species of isolates. A 441 bp fragment of *hsp65* genes was amplified (Fig. 1). After restriction digest of the PCR products with BstEII and HaeIII, the patterns of digested fragments were

analyzed by polyacrilamid gel electrophoresis (PAGE). Nineteen isolates of Mycobacteria were differentiated using *hsp65* genes PCR-RFLP (Table 2). Three isolates could not be identified at the species level because their patterns were different with the other known PCR-RFLP profiles. The patters of digested *hsp65* PCR products are shown in Fig. 2 .

Table 2: Fragment sizes of mycobacterial 441 bp *hsp65* PCR products after digestion by HaeIII and BstEII

BstEII Digestion	HaeIII Digestion	Mycobacterial isolates
No digestion	140/130/65/55	<i>M. duvalii</i>
305-325/130-140	145/70/60/45/40	<i>M. chelonae</i>
	140/65/55	<i>M. mucogenicum</i>
	140/70/60	<i>M. mucogenicum</i>
	145/100/70/60	Unidentified
	140/70/60	<i>M. mucogenicum</i>
	140/80/70/60	<i>M. mucogenicum</i>
	155/140/45/40	Unidentified
305-325/115-125	140/70/60/45/40	<i>M. chelonae like organism</i>
	140/80/65/45	<i>M. chitae</i>
	140/70	<i>M. chelonae like organism</i>
	170/140/70	<i>M. neoaurum</i>
	140/80	<i>M. chelonae like organism</i>
	140/75	<i>M. chelonae like organism</i>
	150/60	Unidentified
	135/115	<i>M. goodii (type III)</i>
	135/130	<i>M. fortuitum</i>
	155/135/70	<i>M. fortuitum</i> ss. <i>fortuitum</i>
155/135	<i>M. fortuitum</i> ss. <i>fortuitum</i>	
230-245/130-140/80-85	150/135/75/45	<i>M. fortuitum</i> 3th variant
	150/135/65	<i>M. fortuitum</i> 3th variant
	150/135/65	<i>M. fortuitum</i> 3th variant
	150/135/65	<i>M. fortuitum</i> 3th variant
	150/135/65	<i>M. fortuitum</i> 3th variant

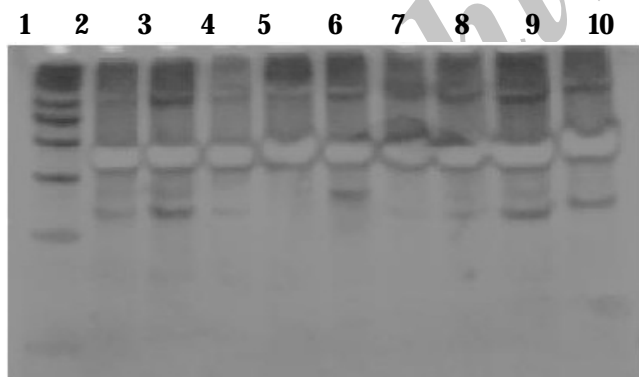


Fig. 2: Differential identification of NTM by digestion of *hsp65* 441-bp PCR amplicons by HaeIII, 1:50 bp ladder; 2: Unidentified, 3:*M. chelonae* like organism, 4:*M. chitae*, 5: *M. duvalii*, 6:*M. mucogenicum*, 7:*M. chelonae like organism*, 8:*M. chelonae like organism*, 9: *M. neoaurum*, 10: *M. chelonae like organism*

Discussion

NTM are widely distributed in the environment; soil, water and other natural reservoirs. The majorities of NTM are less harmful and not virulent particularly in humans with a normal immune system but some species cause serious infections especially in immunocompromised individuals. However, in recent years, NTM have emerged as a major cause of opportunistic infections. Therefore, it is necessary to perform risk analysis and identify the species of NTM that are present in the environment for understanding their incidence and making aware clinician. Phenotypic characterization including pigment production, growth rate and biochemical test algorithms has been used for the identification of Mycobacterium species. However, a number of advantages for the molecu-

lar techniques over conventional testing for identifying mycobacteria species are described (14- 20). In this study, 85 water samples were collected from different sources (Table 1). The prevalence of NTM was determined using culture, biochemical tests and PCR-RFLP analyses on *hsp65* gene. 25.9% of water samples contained one to three different species of NTM. Twenty-two NTM were isolated and 19 species identified by conventional methods. Dominant isolates were *M. fortuitum* (26.7%), *M. chelonae* like organism and *M. mucogenicum* (13.3%). The results analyses did not show any correlation between the mycobacteria presence in water samples and the range of the total chlorine concentrations ($P>0.05$) but the presences of NTM were correlated with the water sample temperature ($P<0.05\%$).

Covert and Rodgers isolated different species of NTM from 54% of ice samples and 35% of public drinking water (5). Shin and Lee showed that half of tap water samples in hospital environment are positive for mycobacteria (21). Argueta and Yoder reported that 25 of 121 food samples (20.6%) were positive for NTM (18). The results of this study showed that the incidence of NTM was similar to other geographical environments.

In this study, a 441 bp fragment of NTM *hsp65* gene was amplified and digested by BstEII and HaeIII and their patterns were analyzed on polyacrilamid gel. As shown in Fig. 2 and 3, there were different PCR-RFLP profiles. Nineteen isolates (86.4%) of NTM were identified to the species level. Three isolates presented profiles that were different from the known RFLP profiles and could not be identified that is agree with other studies (18- 21). Turenne and Tschetter showed that *hsp65* PRA was useful for identification of some species such as *M. gastri* and *M. kansasii* that cannot be identified by other methods (17). Wong and Yip, reported that PCR-RFLP targeting *hsp65* gene region could identify 74.5% of NTM (19). Telenti and Marchesi identified 10 NTM isolates to the species level using PCR-RFLP in which 439 bp PCR products were digested with BstEII and HaeIII (20). However, in this study, some isolates shown patterns that are different with the results of other studies (8, 11-15). Absence of standardi-

zation for all of NTM species may cause some confusing in pattern analysis especially in new species. In other hand, interpretation of bands is ambiguous for highly polymorphic species. Interpretation of the patterns sometimes leads to miss identification, because of the sequence base banding patterns, which does not conform the electrophoretic banding pattern exactly (8).

In this studym, 2 positive controls were used: *M. smegmatis* (Hae III: 154/129/63/45/41 and BstEII: 245/140/85) and *M. fortuitum* (HaeIII: 137/127/67/56 and BstEII: 229/129/82). In comparing of patterns of this study to other investigation, some minor different was negligible. For example HaeIII enzyme for *M. mucogenicum* produces segments size 139/65/58 bp (8) and in this study NTM 6, NTM 13 and NTM 18 (with same segments of BstEII enzyme products, 300-325/130-140), shown partly different in HaeIII products: 140/65/55, 140/70/60 and 140/80/70/60, but they identified as *M. mucogenicum*. Of course patterns of bands that reported in different researches sometimes varied by 10-12 bp different (5, 8, 14). In other example for NTM identified as *M. chelonae* in referents paper (13), HaeIII products segments were shown, 140/85 and 140/65 but in this study segments for NTM 140/80 and they were detected as *M. chelonae*. In addition, the bands lower than 50 bp difficultly were distinguished; however, bigger segments mostly were sufficient for primitive identification of species.

In conclusion, this study showed that PCR-RFLP of *hsp65* gene in mycobacteria is more reliable method for identification of NTM at the specie level than conventional phenotypic methods ($P<0.05$).

Ethical considerations

Ethical issues (Including plagiarism, Informed Consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc) have been completely observed by the authors.

Acknowledgments

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List of abbreviations

DPD method n, n- diethyl- p- phenylene diamine (DPD) colorimetric method

***hsp65* gene** Heat Shock Protein 65 gene

NTM Non- tuberculous Mycobacteria

PCR-RFLP Polymerase Chain Reaction- Restriction Fragment Length Polymorphism

PRA PCR-RFLP analyses

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