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Original Article

Molecular typing of Iranian mycobacteria isolates by polymerase chain reaction-restriction fragment length polymorphism analysis of 360-bp *rpoB* gene

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Abstract

Background: Diagnosis and typing of *Mycobacterium* genus provides basic tools for investigating the epidemiology and pathogenesis of this group of bacteria. Polymerase chain reaction (PCR)-restriction fragment length polymorphism analysis (PRA) is an accurate method providing diagnosis and typing of species of mycobacteria. The present study is conducted by the purpose of determining restriction fragment profiles of common types of mycobacteria by PRA method of *rpoB* gene in this geographical region.

Materials and Methods: Totally 60 clinical and environmental isolates from February to October, 2013 were collected and subcultured and identified by phenotypic methods. A 360 bp fragment of the *rpoB* gene amplified by PCR and products were digested by MspI and HaeIII enzymes.

Results: In the present study, of all mycobacteria isolates identified by PRA method, 13 isolates (21.66%) were *Mycobacterium tuberculosis*, 34 isolates (56.66%) were rapidly growing Nontuberculosis Mycobacteria (NTM) that including 26 clinical isolates (43.33%) and 8 environmental isolates (13.33%), 11 isolates (18.33%) were clinical slowly growing NTM. among the clinical NTM isolates, *Mycobacterium fortuitum* Type I with the frequency of 57.77% was the most prevalent type isolates. Furthermore, an unrecorded of the PRA pattern of *Mycobacterium conceptionense* (HeaIII: 120/90/80, MspI: 120/105/80) was found. This study demonstrated that the PRA method was high discriminatory power for identification and typing of mycobacteria species and was able to identify 96.6% of all isolates.

Conclusion: Based on the result of this study, *rpoB* gene could be a potentially useful tool for identification and investigation of molecular epidemiology of mycobacterial species.

Key Words: Molecular typing, mycobacteria, polymerase chain reaction-restriction fragment length polymorphism analysis, *rpoB* gene

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INTRODUCTION

Mycobacterium genus includes more than 160 distinct species that are mostly involved in human infections.[1] Mycobacteria are responsible for considerable human morbidity and mortality worldwide. Some species, such as *Mycobacterium tuberculosis* are the primary cause of mortality in individual adult's infections.[2]

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This successful human bacterial parasite has infected one-third of the world population and each year causes more than 8.8 million new cases of infection and kills closely 2 million people.[3,4] The World Health Organization also estimates that between 2000 and 2020, 35 million people will die from tuberculosis (TB).[2,5]

Nontuberculous Mycobacteria (NTM) were only known as environmental bacteria. However, they have recently been identified as important pathogens due to the increased prevalence of immunodeficiency diseases. NTM, have more than 100 species but only around 15 being cause of a wide variety of human infections.[6,7] They are one of the cause for nosocomial and occupational infections and induce pneumonitis, hypersensitivity, asthma and bronchitis in workers exposed to liquid water, metal and aerosols.[8] This group of mycobacteria also causes opportunistic infections, particularly in patients who are immune compromised.[9] With regarding the widespread prevalence of AIDS, infection with NTM as well as TB has increased in many parts of the world more than last decades.[10] Hence, clinical diagnosis and treatment of NTM infections have become a challenge for clinicians.[11]

Diagnosis and typing of *Mycobacterium* genus provides a basis for investigating their epidemiology and pathogenesis. Regarding to the prevalence of mycobacteria infection in Iran and because of the neighborhood of Iran with countries that are among 22 high-burden countries and the countries with high prevalence of multidrug-resistant TB, increasing attention to mycobacteria diseases and introducing molecular epidemiology of mycobacteria seems would be necessary to deal with this challenge. In other hands, because there are different treatments strategies for various related disease of mycobacteria species; notice the frequency of species in each region would be useful in different geographic regions in adopting a promising method of control and treatment by physicians.[12]

Mycobacteria are routinely identified to the species level by the phenotypic approaches, but these phenotypic-based methods have some limitations in the diagnosis including being time-consuming, requirement of living organism, confusing and sometimes incorrect results.[12,13] Today, molecular approaches like sequencing, INNO-LiPA mycobacteria v2, and AccuProbe are used for precise and rapid identification of the species, however, high cost of these methods and also the requirement for special facilities impose limitations on their application in developing countries.[14-16] Common typing methods like bacteriophage typing and serotyping are replaced by ribotyping, polymerase chain reaction (PCR)-based methods, fingerprinting plasmid, and analysis of restriction fragments of chromosomal DNA by pulse-field gel electrophoresis in the last decades.[17] Among molecular approaches for typing, analysis PCR-restriction fragment length polymorphism (RFLP) is an accurate and inexpensive method providing diagnosis and typing of species and subspecies of mycobacteria.[14,18] In order to typing mycobacteria by PCR-RFLP analysis (PRA) method, different genes such as *16SrRNA*, *rpoB*, *dnaj,* and *hsp65* were targeted.[9,14] The variable regions of *rpoB* gene that is encoding the β subunit of RNA polymerase enzyme are proposed as proper gene for phylogenic analysis, determination of inter-species diversity and diagnosis of bacteria specially ones with close relativity.[18,19] In this study, the PRA profiles and inter-species diversities of Iranian clinical and environmental mycobacteria isolates was determined and their profiles was compared with the global patterns and relevant information about the epidemiology and genetic basis of Iran isolates was provided.

MATERIALS AND METHODS

Mycobacteria strains and species

Totally 60 mycobacteria isolates were obtained from February to October, 2013 at the *Mycobacterium* collection of Department of Microbiology, Isfahan University of Medical Sciences and Tuberculosis Center of Isfahan. Of these 60 specimens, 40 were respiratory specimens, including sputum, bronchoalveolar lavage, and bronchial wash specimens, 11 were nonrespiratory and 9 specimens were environmental sample such as water due to break up the mucin, each respiratory specimen was treated with a same volume of 3.5% NaOH in a 50-ml centrifuge tube and, followed by vortexing for 30 s. specimens decontaminated after the tubes were incubated at room temperature for 15 min. Sterile phosphate buffer was added to stop the digestion decontamination process, and then the tube were mixed by inversion, and the tubes were centrifuged at 3000 × *g* for 15 min. The supernatant was discarded, and the remaining pellet was resuspended in 3.0 ml of sterile phosphate-buffered saline. Nonrepirotry after the homogenies processed in the same way sampling of water was done using the grab sampling method. To 2 L' sterile Erlenmeyer flasks, sodium thio-sulfate as antichlor and 0.04% Cetyl pridinium chloride as an antimicrobial agent were added. Five hundred milliliters of samples were passed from 0.45 μm filters. The filters were transferred directly onto 7H10 Middlebrook solid media, include 15% oleic acid, albumin, dextrose, catalase.

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Primary identification of isolates by conventional methods

The mycobacteria species were subcultured on LJ media and Middlebrook 7H9. The isolates were identified by primary conventional methods including acid-fast staining, colony characteristics, 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), pyrazinamidase (4 and 7 days), urease, nitrate reduction test and colony morphology. The reference strains used in this study were *M. tuberculosis* H37Rv (ATCC 27294) and *Mycobacterium fortuitum* (ATCC 49403).

Polymerase chain reaction-restriction fragment length polymorphism analysis

Preparation of genomic DNA

Fresh cultures of bacteria were prepared on 7H10 medium and then DNA was extracted using cetyltrimethylammonium bromide method.[20] Purified DNA was stored at – 70°C for subsequent experiments.

Polymerase chain reaction amplification of rpoB

The reaction was performed in a thermal cycler from PCR Hybid (Omnigene). Partial *rpoB* gene (360 bp) was amplified using primers (RPO5') 5-TCAAGGAGAAGCGCTACGA-3' and (RPO3') 5'-GGATGTTGATCAGGGTCTGC-3'.[11] Each 25 µl PCR mixture contained 2 µl DNA supernatant (5 ng genomic DNA(, 1 µl of each primer (10 pmol/ml), 1.25 µl $MgCl_2$ (1.5 mM), 0.5 µl dNTP (200 mM), 0.25 µl Taq polymerase (500U), 2.5 µl × 10 Buffer. The PCR program was performed in a thermocycler (Eppendorf) including: 94°C for 5 min, 35 cycle of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min, and a final step at 72°C for 7 min. The PCR products were run on 1.5% agarose gel.

Restriction fragment length polymorphism

The amplified products of *rpoB* gene regions were digested with two restriction enzymes of MspI and Hea III according to recommendations of the manufacturers. Fourteen µl of PCR products were digested in a 20 µl reaction volume containing 2 µl of × 10 reaction buffer supplied by manufacture, 3.5 µl dH_2O and 5U MspI enzyme and were incubated at 37°C for 4 h. A similar condition was used for the Hea III enzyme. Finally, samples were run on 4% metaphor agarose gel, and fragment sizes were determined based on the restriction patterns first described by Lee *et al*.[11] For undetermined isolates based on the reference pattern or methods also sequence analysis was performed.

RESULTS

Of 60 isolates, out of 13 isolates (21.66%) identified as *M. tuberculosis* and 47 isolates (78.33%) as NTM by phenotypic method. Among of 47 NTM isolate, 38 isolates (80.85%) were clinical and 9 isolates (19.14%) were environmental [Table 1].

Of 60 mycobacteria isolates identified by PRA method, 13 isolates (21.66%) were *M. tuberculosis*, 34 isolates (56.66%) were rapidly growing NTM that including 26 clinical isolates (43.33%) and 8enviromental isolates (13.33%), 11 isolates (18.33%) were slowly growing NTM which were clinical isolates.

Based on PRA of *rpoB* gene, among 34 rapidly growing isolates, 32 isolates were *M. fortuitum* Type I; 1 isolate *Mycobacterium smegmatis* and 1isolate *Mycobacterium conceptionense*. An unrecorded pattern profile of *M. conceptionense* was identified [Figure 1a].

Among 11 slowly growing isolates, *Mycobacterium avium*, 1isolate; *Mycobacterium intracellular*

Table 1: List of mycobacteria strains and their sources identified by phenotypic tests and fragment sizes of mycobacterial 360 bp rpoB PCR products after digestion by HaeIII and MspI

Isolates	RFLP Pattern		Phenotypic method No. and sources
	HeaIII	MspI	
MTBC	250/100	175/80/60/40	13 clinical isolates
M. fortuitum type I	120/90/80	175/105/70	25 clinical and 6 environmental isolates+1 strain *M. fortuitum* ATCC 49403
M. gordonae type I	210/95/90	145/95/40/30	8 clinical and 1 environmental isolates
M. gordonae type II	330	145/100/40	
M. gordonae type IV	270	145/95/40/30	
M. avium	270	105/80/40	1 clinical isolate
M. kansasii type I	205/90	175/60/40/30	2 clinical isolates
M. smegmatis	200/95/45	190/145	1 environmental isolate
M. conceptionense	120/90/80	120/105/80	1 environmental isolate
M. intracellular type I	180/90	175/105/80	1 clinical isolates

M. fortuitum: *Mycobacterium fortuitum*, *M. gordona*: *Mycobacterium gordona*, *M. avium*: *Mycobacterium avium*, *M. kansasii*: *Mycobacterium kansasii*, *M. smegmatis*: *Mycobacterium smegmatis*, *M. conceptionense*: *Mycobacterium conceptionense*, *M. intracellular*: *Mycobacterium intracellular*, PCR: Polymerase chain reaction

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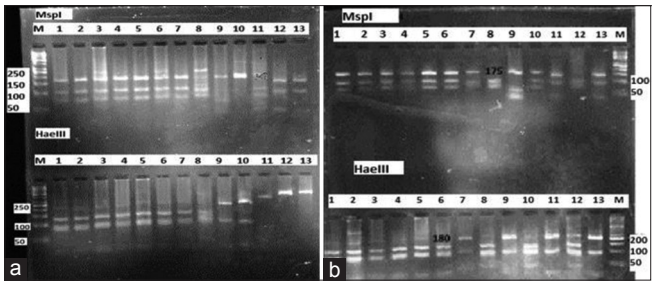

Figure 1: (a) Result of polymerase chain reaction-restriction fragment length polymorphism analysis (PRA) studied mycobacteria lanes: M, size marker (50-bp); 1–7, *Mycobacterium fortuitum* Type I; 8, unknown 9, *Mycobacterium kansasil* Type L; 10, *Mycobacterium smegmatis*, 11, *Mycobacterium avium*, 12–13, *Mycobacterium gordonae* Type II. (b) Result of PRA studied mycobacteria lanes: M, size marker (50-bp); 1–6, *Mycobacterium fortuitum* Type I; 7, *Mycobacterium intracelluare*; 8, *Mycobacterium conceptionese*, 9, *Mycobacterium kansasii* Type 1; 10, *Mycobacterium fortuitum* Type I, 11–13, *Mycobacterium gordonae* Type I

subtype I, 1 isolate; *Mycobacterium kansasii* Type I, 2 isolates; *Mycobacterium gordonae* Type I, 4 isolates, Type II, 2 isolates and Type IV, 1 isolate were identified. Two isolates that were identified as *M. gordonae* by phenotypic methods were not identified by PRA because their profiles were not recorded in any of the last reported studies [Figure 1b]. After sequencing of 360 bp *rpoB* gene, they were identified as *Actinosynnema mirum*. The PCR-RFLP profiles of all isolates are listed in Table 1. *M. fortuitum* (ATCC 49403) shown its specific pattern (HaeIII: 70/100/12O, MspI: 80/90/175).

DISCUSSION

In the present study, based on PRA of *rpoB* gene, among the clinical NTM isolates, *M. fortuitum* Type I with the frequency of 57.77% was the most prevalent type isolates. Other dominant clinical isolates were *M. gordonae* Type I with the frequency of 8.88% and *M. gordonae* Type II, *M. kansasii* Type I, both with the frequency of 4.44%. In 2013, Choi *et al.*, reported *M. intracellular* Type I, *M. avium*, M. abscessus with the frequency of 26.7%, 25% and19.5% respectively, as the species with higher frequency.[21] In 2012, Ong *et al*. reported *M. fortuitum* Type I, *M. chelonae/M. abscessus*, *M. avium*, *M. fortuitum* Type II and *M. gordonae* Type I with 30%, 22.85%, 10%, 11.42%, and 8.57%, respectively, as NTM species with higher frequency.[15] The PRA pattern in *M. Intracellular* subtype, I in the present study, was MspI: 175/105/80 while in other studies reported MspI: 175/100/80 and 170/95/80.[15,22] These slight differences are ignorable as sometimes in different studies; the 10-12-bp variations are due to the not exactly identical procedures and standards.[11,21,22] PRA patterns of *M. kansasii* Type I (HeaIII: 90/205, MspI: 30/40/60/175) and *M. avium* (HeaIII: 270, MspI: 40/80/105) in this study are identical to some studies and different from the profile of some others.[15,22] For example indifferent pattern, Macheras *et al*., showed that profiles of *M. kansasii* Type I and *M. avium* are MspI: 105/70/60/45/40; HaeIII: 210/80/60 and MspI: 180/95/40; HaeIII: 180/90, respectively.[23] In the present study, an unrecorded pattern of the profile of *M. conceptionense* (HeaIII: 120/90/80, MspI: 120/105/80) was identified which is not reported in other recorded studies. This result represents a wide inter-species diversity between mycobacteria in different geographical areas.

In this study, the applied method for identification and typing of isolates showed high discriminatory power and was able to identify 96.6% of all isolates. In 2012, Kazumia reported that the sequence of the gene *rpoB* improves diagnostic system and precise identification of mycobacteria species.[12] In 2010, Ong *et al*. applied PRA method for *rpoB* and *hsp65* genes for typing simultaneously and showed that PRA of the *rpoB* gene was able to identify 85.6% of all isolates and is more efficient than *hsp65* gene for identification and typing of rapidly growing mycobacteria.[15] Based on the results of Lee *et al*. in 2000, mycobacteria can be detected by PRA of *rpoB* gene to the level of species and subspecies. They reported that *M. gordonae*, *M. kansasii*, *M. fortuitum,* and *M. gastri*can be identified by this method too while *16SrRNA* has some limitations in these cases.[11] In some studies, PRA for *16SrRNA*, *hsp65*, and *dnaj* genes were also conducted but need other restriction enzymes, which made the interpretation of the patterns very difficult. Additionally, some studies confirm that *rpoB* is a powerful identification tool for identifying and typing of NTMs specially rapid growing mycobacteria and a powerful tool for investigating the inter and intra-species relativity.[22-28] In cases of strains that cannot be identified completely by one or two enzymes, a third enzyme can be helpful.[11] With regard to the fact that There are fewer restriction patterns documented in the rpoB PRA[11] this study showed that can be useful to improving the small database by introduced the new pattern in conclusion, based on result of this study, *rpoB* gene could be a potentially useful tool for rapid identification and investigation of molecular epidemiology of mycobacterial species.

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