

Molecular Identification and Conventional Susceptibility Testing of Iranian Clinical *Mycobacterium fortuitum* Isolates

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Abstract

Objective(s)

Rapidly growing mycobacteria (RGM) are capable of producing diseases in humans. Since mycobacteria vary in their susceptibility, precise identification is critical for adoption of correct drug therapy. The main aim of this study was molecular identification and evaluation of antimicrobial susceptibility pattern of Iranian clinically isolated *Mycobacterium fortuitum*.

Materials and Methods

A total of 72 presumptively identified isolates of clinical atypical mycobacteria collected by Isfahan Research Center for Infectious Diseases & Tropical Medicine during 2006-2008 were included in the current study. A combination of conventional and molecular tests was applied to identify the isolates. Molecular methods including genus and group specific PCR and PCR-Restriction Algorithm (PRA) based on *hsp65* gene were applied to achieve exact identification of mycobacterial strains. Antimicrobial susceptibility testing on *M. fortuitum* isolates was performed by in-house prepared broth microdilution test.

Results

Out of 72 collected atypical mycobacteria isolates, we identified 25 strains of *M. fortuitum*. All strains had the specific molecular markers of mycobacterial identity and similar species specific PRA pattern of the international type strain of *M. fortuitum*. Drug susceptibility testing showed that the *M. fortuitum* isolates are sensitive to amikacin, sulfamethoxazole and ciprofloxacin (100%), imipenem (92%), clarithromycin (76%), cefoxitin (56%) and doxycycline (16%).

Conclusion

Molecular identification of atypical mycobacteria based on 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 and should be given proper considerations when clinical samples are processed.

Keywords: Broth microdilution, Identification, *Mycobacterium*, Susceptibility test, Heat shock protein gene (*hsp65*)

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Introduction

Rapidly growing mycobacteria (RGM) are ubiquitous in nature. The *Mycobacterium fortuitum* group including *M. fortuitum*, *M. peregrinum*, *M. fortuitum* third biovariant complex, *M. porcinum*, and *M. mageritense*, *M. chelonae*, and *M. abscessus* are the species of RGM most often associated with human diseases. These organisms cause a variety of localized or disseminated diseases, particularly pulmonary as well as primary skin and soft tissue infections (1).

Mycobacterial susceptibility testing is important for the management of patients with tuberculosis and those with a disease caused by certain non-tuberculous mycobacteria. Since mycobacterial species vary in their susceptibilities to antimicrobial agents, identification of atypical mycobacteria to the species level is critical in therapeutic outcome (2, 3).

Classical identification of mycobacteria based on phenotypic tests may take several weeks, and often fail to provide a precise identification. During last decade, several molecular methods have been introduced for mycobacterial identification. Of these methods, PRA is preferred because of its rather simplicity and rapidity. PRA scheme targeting heat shock protein 65 (*hsp65*) has been most widely used, since this molecule is conserved in all mycobacteria, while showing sufficient sequence variation to allow mycobacteria differentiation at the species level (4).

In our country, Iran, It has been reported that *M. fortuitum* is the most frequent atypical mycobacteria isolated from clinical samples (5), however, to our knowledge there was not sufficient evidence to indicate the role it might play in Iranian clinical samples. Furthermore, we were unable to track down any information regarding the susceptibility pattern of Iranian *M. fortuitum* strains. For these reasons, we decided to show a more precise picture of *M. fortuitum* infection in pulmonary and extra pulmonary diseases of Iranian patients from some parts of Iran and assess the antibiotic susceptibility of the isolates to current therapeutic drugs.

Materials and Methods

Organisms

A total of 72 isolates of atypical mycobacteria originating from clinical samples collected by Isfahan Infectious Diseases and Tropical Medicine Research Center (isolated from Isfahan), Pasture Institute of Iran (isolated from Tehran) and Isfahan Public Health Center (isolated from Isfahan) during 2006-2008 were included in the current study. Clinical isolates were initially identified as presumptive mycobacterial strains. The clinical sources of isolates were sputum, bronchial washing, pleural effusion, urine and skin wound.

Identification of *M. fortuitum* isolates

Conventional methods

All isolates were investigated by conventional methods consisting of acid fast staining and analysis of phenotypic characteristics, i.e., growth on Lowenstein-Jensen (LJ) medium at 25 °C, 37 °C and 45 °C, arylsulfatase activity, pigment production, growth rate (<7 days), nitrate reduction, growth on MacConkey agar without crystal violet, tolerance to 5% NaCl, iron uptake, existence of urease, tellurite reduction, semi quantitative and catalase activity at 68 °C and niacin production tests. *M. tuberculosis* H37Rv, *M. bovis* BCG and *M. kansasii* (DSM 44162) were used as control strains for phenotypic tests. Isolates suspicious to be *M. fortuitum* were further investigated by molecular methods (6-8).

Molecular methods

DNA extraction

Purified DNA was extracted by standard phenol-chloroform extraction method (9). In brief, after thermal inactivation, the bacterial cells were treated with lysozyme and digested with proteinase K in the presence of sodium dodecyl sulfate. The high quality DNA was purified with phenol-chloroform and precipitated with isopropanol. The precipitate was dehydrated and dissolved in 50 µl of sterile double-distilled water and stored at -70 °C.

Genus and species specific PCR

We used broad-spectrum genus and species specific PCR primer pairs (MGSf-

5'CTGGTCAAGGAAGGTCTGCG-3', MGSr-5' GATGACACCCTCGTTGCCAAC-3') and optimal amplification conditions as follows; the reaction mixture (50 µl) consisted of 2 µl of DNA template (20 ng), 5 µl of 10X PCR- buffer with MgCl₂, 1 µl of mixed deoxynucleoside triphosphates (10 mM), 4 µl of each forward and reverse primers (10 pmol), 0.5 µl of Roche-Taq DNA polymerase (5 U/µl) and 33.5 µl of PCR Grade Water. The PCR program consisted of 30 cycles of denaturing at 95 °C for 15 Sec, annealing at 58 °C for 15 sec, and extension at 72 °C for 30 sec using Eppendroff PCR system (10). *M. tuberculosis* H37Rv and *M. kansasii* (DSM 44162) were used as positive controls. A sample of complete mix plus DNA-free water was used as negative control.

PCR-RFLP (PRA)

The isolates were investigated by *hsp65*-PRA for precise identification of *M. fortuitum*. Having confirmed the successful amplification of the 644-bp PCR fragment, the product was subjected to restriction enzyme digestion by *Hph*I, *Hpa*II and *Ava*II enzymes. All reagents were obtained from Fermentas (Fermentas Inc, Germany). Digestion of the 644 bp *hsp65* fragment by enzymes was performed following the manufacture's protocol (4). After digestion, products were electrophoresed in a 3% agarose gel. We used 50 bp DNA size marker to estimate the molecular weight sizes of the fragments. *M. tuberculosis* H37RV and *M. kansasii* (DSM 44162) were included in all experiments for interpretation of the PRA profiles. The size of the restriction fragments was generally species specific (4).

Antimicrobial drug sensitivity test

Inoculum preparation

Each isolate was sub-cultured onto a sheep blood

agar plate and incubated at 30 °C for 72 hr. Three to five similar colonies were transferred to a tube containing 4.5 ml of Middlebrook 7H9 (from Difco, BBL™, US) and glass beads. The turbidity was adjusted until it matched that of a 0.5 McFarland standard by visual examination.

The growth suspensions were mixed vigorously on a vortex for 15 to 20 sec. The final inoculum (approximately 5×10⁵ CFU/ml) was prepared by transferring 50 µl of the suspension to a tube containing 10 ml of cation-adjusted Mueller-Hinton broth (from Merck; Darmstadt, Germany) and inverting the tube 8 to 10 times prior to use.

Antimicrobial agents

The antimicrobial agents used were amikacin (A1774), cefoxitin (C4786), ciprofloxacin (17850), imipenem (I0160), clarithromycin (C9742), doxycycline (D9891) and sulfamethoxazole (S7507) purchased from Sigma Aldrich Corp (Germany). We prepared in-house cation adjusted microdilution trays. The final range of concentrations of antimycobacterial agents in the wells were as follows: 1 to 128 mg/ml for amikacin, 2 to 256 mg/ml for cefoxitin, 0.125 to 16 mg/ml for ciprofloxacin, 0.03 to 64 mg/ml for clarithromycin, 0.25 to 32 mg/ml for doxycycline and 1 to 64 mg/ml for imipenem and sulfamethoxazole. Each flat bottomed tray also contained a positive growth control well. Having prepared a two-fold serial dilution of each antibiotic agent they were divided into the wells. The microdilution trays were sealed and stored at -70 °C temperature until they were used. MIC values and an interpretation based on the breakpoints for RGM are listed in Table 1.

Table 1. Suggested broth microdilution breakpoints for rapidly growing mycobacteria by NCCLS

Antimicrobial agents	MIC (µg/ml) for category		
	Susceptible	Intermediate	Resistant
Amikacin	≤16	32	≥64
Cefoxitin	≤16	32-64	≥128
Ciprofloxacin	≤1	2	≥4
Clarithromycin	≤2	4	≥8
Doxycycline	≤1	2-8	≥16
Imipenem	≤4	8	≥16
Sulfamethoxazole	≤32	-	≥64

Molecular Identification of *M. fortuitum* Isolates

Preparation of the assay plates

Aliquots of 0.01 ml of final inoculum per well were dispensed into the assay plates. The inoculated trays were covered with an adhesive seal and incubated at 30 °C in ambient air. A sheep blood agar plate was also inoculated with a loopful of the final inoculum to check the purity. The trays were first examined after 72 hr by looking for macroscopic growth with an indirect light source. If no macroscopic growth was visualized, the trays were re-incubated and read daily thereafter (for up to 5 days) until moderate growth was visible. For all antibiotics but sulfamethoxazole, the MIC was defined as the lowest concentration that completely inhibited macroscopic growth. For sulfamethoxazole, the endpoint or MIC was defined as the concentration of the drug in the well with approximately 80% inhibition of growth compared to the growth in the control well with no drug (11-13). Susceptible and resistant breakpoints are listed in Table 3.

Results

In the current study out of a total of 72 atypical mycobacteria, 25 clinical isolates (21 isolates from Isfahan and 4 from Tehran) were identified as *M. fortuitum*. The sources of isolates were as follows: bronchial washes (13 isolates), urine (6 isolates), pleural fluid (3 isolates), sputum (2 isolates) and skin wound (1 isolate). All isolated strains showed biochemical characteristics consistent with *M. fortuitum* complex. However, the isolates were not identified to the biovariant level.

All Iranian isolates showed phenotypic characteristics consistent with *M. fortuitum*, i.e., rapid growing (<7 days) at 25, 31, 37 °C and at 45 °C, no pigment production, growth on MacConkey agar without crystal violet and principal biochemical properties of catalase, tween hydrolysis, and arylsulfatase positivity.

The presumptive *M. fortuitum* Iranian isolates were further analyzed and verified as mycobacteria using genus and group specific PCR targeting a 228 bp fragment (Figure 1). Furthermore with the application of the PRA algorithm targeting 644 bp *hsp65* DNA, twenty five isolates were clearly distinguished

from other mycobacteria. All isolates had the characteristic *Ava*II (501, 119 bp), *Hph*I (254, 207, 97 bp) and *Hpa*II (270, 161, 117 bp) PRA pattern of international type strain of *M. fortuitum* ATCC 6841T (Figure 2).

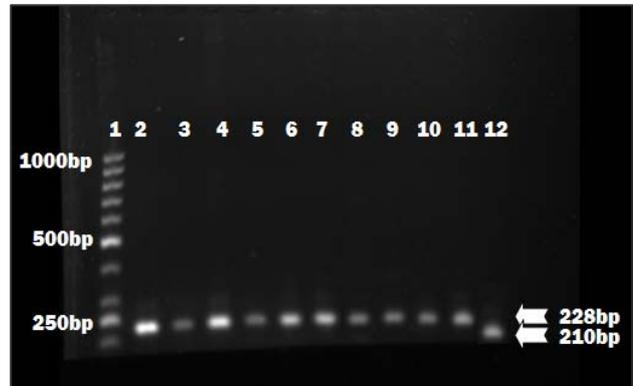


Figure 1. Genus specific amplification of atypical mycobacteria. Lanes 2-10; *M. fortuitum* clinical isolates, Lane 11: *M. kansasii* DSM 44162, Lane 12: *M. tuberculosis* H37Rv strain. White arrows indicate group specific fragments for *M. fortuitum* and *M. tuberculosis* respectively. Lane 1: The 50 bp DNA size marker.

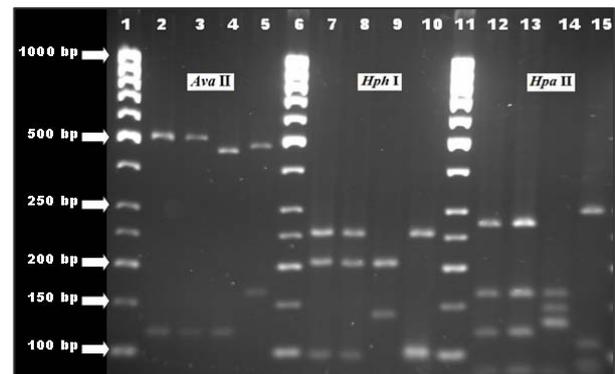


Figure 2. The differentiation of *M. fortuitum* clinical isolates from other mycobacterial species by *hsp65* PRA based on digestion of a 644 bp amplified fragment with *Ava*II, *Hph*I and *Hpa*II. Lanes 2, 3, 7, 8, 12 & 13: *M. fortuitum*; Lanes 4, 9 & 14: *M. kansasii* DSM 44162 and Lanes 5, 10 & 15: *M. tuberculosis* H37Rv. Lanes 1, 6, 11 & 16: The 50-bp DNA size marker.

Following precise identification of *M. fortuitum* isolates, they were subjected to standard susceptibility testing based on National Committee on Clinical Laboratory Standards (NCCLS) recommendations. All isolates grew well in the broth medium and produced sharp, easily discernible growth endpoints. The susceptibility pattern of *M. fortuitum* strains investigated by this study and MIC₅₀ (MIC at which 50% of the isolates tested are inhibited), MIC₉₀ and MIC values are shown in Table 2.

Table 2. Drug susceptibility testing of *M. fortuitum* isolates.

Drug	No.(%) of isolates			MIC ($\mu\text{g/ml}$)		
	Susceptible	Intermediate	Resistant	Range	50%	90%
Amikacin	25 (100)	0 (0)	0 (0)	1-2	1	2
Sulfamethoxazole	25 (100)	0 (0)	0 (0)	1-32	1	16
Cefoxitin	14 (56)	3 (12)	8 (32)	4-256	16	128
Doxycycline	4 (16)	11(44)	10 (40)	0.25-32	8	16
Clarithromycin	19 (76)	2 (8)	4 (16)	0.06-16	1	4
Ciprofloxacin	25 (100)	0 (0)	0 (0)	0.125-0.25	0.125	0.25
Imipenem	23 (92)	2 (8)	0 (0)	1-16	1	4

Discussion

In the United States and many other countries it has been reported that *M. avium* complex is the most common atypical mycobacteria isolated from clinical samples (13). However in a multi-country retrospective survey by Martin-Casabona *et al* (2004) which included data from 14 countries including Iran, it was reported that the five most frequently isolated Non-tuberculous mycobacterial (NTM) species were *M. avium* complex, *M. goodii*, *M. xenopi*, *M. kansasii* and *M. fortuitum* respectively. These five species accounted for 87.6% of total NTM isolated (5).

This report has also compared the results from different countries and concluded that except for Iran, Turkey, Belgium and the Czech Republic, the isolation rate of *M. avium* complex was above 20%. Interestingly, it has been showed that *M. fortuitum* was most frequent in Iran and Turkey comprising more than 53.9% and 33.9% of all NTM isolates, respectively (5).

Despite increasing clinical significance of *M. fortuitum*, we found no information about drug susceptibility pattern of Iranian isolates. This was the reason why we decided to implement the current study. However, since our study included a small sample from certain regions of Iran the results cannot represent the whole country profile and might be interpreted as a pilot study.

All our investigated isolates were susceptible to amikacin, ciprofloxacin and sulfamethoxazole. However susceptibility rate to imipenem, clarithromycin, cefoxitin and doxycycline were rather lower, i.e. 92%, 76%, 56% and 16% respectively. According to a document published in 2007 by American Thoracic Society *M. fortuitum* isolates were 100% susceptible to amikacin, ciprofloxacin

ofloxacin, sulfonamides and imipenem, 80% to clarithromycin and 50% to cefoxitin and doxycycline (6). There are several papers from different countries in which susceptibility rates of *M. fortuitum* isolates to amikacin, imipenem, ciprofloxacin, clarithromycin, sulfonamides and cefoxitin are reported (96-100%), (62-98%), (62-95%), (30-84%), (6-85%) and (19-55%) respectively (1, 14-15).

Based on literature review, different susceptibility patterns for currently prescribed antibiotics with the exception of amikacin had been reported from different countries (1, 14-15). In comparison to other studies, our results showed that susceptibility pattern of *M. fortuitum* clinical isolates to amikacin, sulfamethoxazole, clarithromycin, cefoxitin and ciprofloxacin were in agreement with the rate reported by American thoracic society document, but susceptibility to doxycycline (16%) was much lower than that of obtained by this study.

Some investigators believe that differences in antibiotic resistance rates in RGM may be the result of population or geographical differences or, probably, the method of susceptibility testing (15, 16). Despite limitations of current testing methods for RGM and lack of correlation between *in vitro* susceptibility and therapeutic responses, one might assume that *in vitro* resistance would foresee treatment failure (6, 16). However, there are large prospective studies assessing the correlation of *in vitro* susceptibility with therapeutic outcome indicating that susceptibility results for atypical mycobacteria should be interpreted cautiously.

Conclusion

In conclusion, we would like to emphasize that correct identification of *M. fortuitum* species needs a combination of phenotypic and

molecular tests. Furthermore, determination of susceptibility patterns of clinical isolates of *M. fortuitum* is subject to accurate identification of the isolates to the species level.

In addition, since susceptibility patterns in RGM are unforeseeable with possible geographic differences; we are unable to recommend any antibiotic drug of choice for clinical management of patients. On the other hand, in view of *M. fortuitum* environmental source, further studies might include environmental isolates to have a better understanding of susceptibility patterns and

resistance mechanism of *M. fortuitum*.

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