

Heterogeneity of Iranian clinical isolates of *Mycobacterium fortuitum*

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ABSTRACT

Background and Objectives: The increase of infections caused by nontuberculous mycobacteria (NTM) is receiving increasing attention worldwide. *Mycobacterium fortuitum* is encountered with increasing frequency in clinical laboratories of Iran.

Materials and Methods: Sequence variation of 48 *M. fortuitum* clinical isolates, were investigated by sequence analysis of the 16S-23S Internal Transcribed Spacer.

Results: Twelve different sequence types (sequevar) were identified by sequence analysis of ITS region. Seven previously described sequevar including MfoA, MfoB, MfoC, MfoD, MfoE, MfoF and MfoG identified. Five novel sequevar namely MfoH, MfoI, MfoJ, MfoK and MfoL that were distinctly different from the previously described sequevar were detected among different clinical strains of *M. fortuitum*, from Iran.

Conclusion: This study showed that the ITS region possesses high discriminatory power between the clinical isolates up to the clonal level. The results also suggest the possibility of the existence of predominant clone of *M. fortuitum* in affected patients in Iran. The data also point to the conclusion that a large variety of *M. fortuitum* clone can produce disease although certain clones seem to be predominant.

Keywords: *Mycobacterium fortuitum*, ITS, diversity

INTRODUCTION

To date, the genus *Mycobacterium* comprises over 160 species (<http://www.bacterio.cict.fr/>). Several species other than *M. tuberculosis*, nontuberculous mycobacteria (NTMs), are becoming increasingly

recognized as significant human pathogens (1). In the last decade, the rapid development of molecular techniques has led to a great increase in our knowledge about mycobacterial identification and typing (2). Methods for identification and typing of mycobacteria include nucleic acid probes (3), PCR hybridization with species-specific probes (4, 5), PCR restriction fragment length polymorphism analysis (6, 7) and nucleic acid sequencing (8). Identification of *Mycobacterium* species by conventional methods including growth characteristics and biochemical tests are time-consuming and often not unequivocal in their interpretation (2, 8). Currently, the widely

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accepted strategy formulated to improve methods of mycobacterial strain identification includes analysis of the gene encoding 16S rRNA, however this technique is unable to differentiate members of several closely related species called complex groups a result of small number of polymorphic positions within the 16S rRNA (8- 10).

Several studies have shown that sequencing of 16S-23S Internal Transcribed Spacer (ITS) gene sequencing could help to differentiate and identify the closely related mycobacterial species (11, 12). Studies aiming at the assessment of heterogeneity within *M. fortuitum* group have used different approaches, from phenotypic methods, conventional typing such as PFGE and ERIC, *hsp65*-PRA and sequence based approaches (13- 17).

Taxonomic status of species belongs to *M. fortuitum* group clearly addressed by sequencing methods (9). However, the possible correlation between the pathogenicity of the clinical isolates of *M. fortuitum* subsp. *fortuitum* in the host and their genotypes has not been extensively evaluated. Distinct ITS sequences namely sequevar found in the *M. avium* complex demonstrated that the ITS region is suitable for differentiating strains within some mycobacterial species and has the potential to be used as a marker to distinguish clinically relevant subspecies (11, 12, 18).

In this study the ITS sequences of 48 Iranian *M. fortuitum* clinical isolates from different regions were investigated to understand the genetic diversity of the strains and their presumptive relationships with different clinical presentation of disease caused by this organism in Iran.

MATERIALS AND METHODS

Bacterial strains. Mycobacterial strains investigated in the present study included 48 clinical isolates of Iranian *M. fortuitum*, which had been isolated in or referred to our laboratory, at Research Center for Infectious Diseases, Ahvaz, Iran. Case subjects were considered for inclusion if they met the 1997 American Thoracic Society criteria for NTM disease (19). Table 1 summarises patients' histories. The strains were collected or recovered from the symptomatic patients during 2010-2013.

Identification of isolates by phenotypic tests. Isolates were initially screened using conventional

phenotypic tests including standard morphological and biochemical assays previously established for identification of mycobacteria (20).

DNA extraction and purification. Chromosomal DNA was extracted using a method of Pitcher *et al.* (21) with a slight modification to facilitate the susceptibility of cells to the standard digestion. In brief, after thermal inactivation, a pretreatment of biomass with lipase (Type VII; final concentration, 2 mg/ml [Sigma]) and a further treatment with proteinase K (100 pg/ml) and sodium dodecyl sulfate (final concentration, 0.5% [wt/vol]) were applied. The DNA was purified by phenol chloroform-isoamyl alcohol and precipitated with isopropanol. The precipitate was washed in 70% ethanol, dehydrated and dissolved in 100 µl of Milli-Q water and stored at -20°C freezer until use.

Identification of isolates to species level. The identity of the isolates as *M. fortuitum* was confirmed by PCR restriction fragment length polymorphism analysis (PRA) of 441 base pairs region of the 65-kDa heat shock protein gene (*hsp65*) by two restriction endonuclease including *BstEII* and *HaeIII* as described previously (16). Representative isolates (randomly selected strains) from each genotype based on ITS region further identified by 16S rRNA sequencing (8).

ITS gene sequencing. All *M. fortuitum* clinical isolates were subjected to the ITS region amplification and sequencing following the procedures described herein (22). Amplification of the full ITS segment was performed with primers L (5'-GCTGGATCACCTCCTTCT-3') from conserved sequence at the 3' end of the 16S rRNA (from position 1525 to position 1543 on the *Escherichia coli* 16S rRNA) and R (5'-CTGGTGCCAAGGCATCCA-3') which deduced from conserved sequence of 23S rRNA 5' sequences (position 23 to position 40 on the *E. coli* 23S rRNA). Mixture reaction (50 µl) containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 200 µM (each) deoxynucleoside triphosphate (dATP, dGTP, dCTP, and dUTP), 10 pmol of each primer, 0.5U of Taq DNA polymerase, and 5 µl (50 ng) of extracted DNA using recommended thermal profile. The purified PCR products were directly sequenced with the forward 16S-1511f and reverse 23S-23r primers using an ABI 3100 genetic

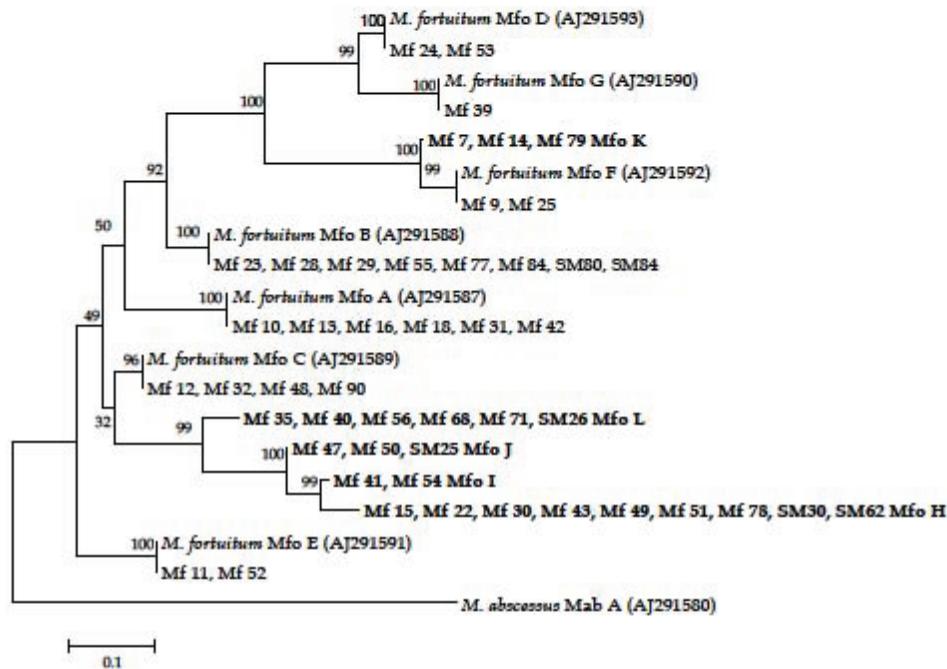


Fig 1. Distance matrix tree showing the divergence of ITS sequences of the Iranian clinical isolates of *M. fortuitum*. All alignment positions which are occupied by residues were used for the calculation of binary distance values. The topology of the tree was evaluated and corrected according to the results of maximum-parsimony and maximum-likelihood analyses. The bar represents 0.1 estimated sequence divergence.

analyzer and a BigDye Terminator cycle sequencing kit by SEQLAB Company (Germany).

Data analysis of ITS gene sequences. The obtained sequences of ITS region of each strain were aligned with the published ITS region sequences of *M. fortuitum* type strains and clinical strains (retrieved from GenBank™ database) using the jPhydit software package according to primary-structure (23). Comparative analyses of ITS region was performed with distance matrix, maximum-parsimony, and maximum-likelihood methods as implemented in the Mega4 program (24). Tree topologies was tested by bootstrap analysis on 1000 resampling.

The GenBank accession numbers of ITS sequences of clinical isolates of *M. fortuitum* determined in this work are as follows: KF366424 - KF366435.

RESULTS

Based on growth characteristics, 48 isolates were rapidly growing mycobacteria (RGM). The isolates were initially classified into Runyon's groups IV (20) and further identified by *hsp65*-PRA method as *M. fortuitum* (Table 1).

Analysis of the *hsp65* gene by PRA (PCR restriction fragment length polymorphism analysis) (explain PRA for first time) demonstrated identical electrophoretic patterns from clinical isolates (*BstEII* pattern 235/120/85 and *HaeIII* pattern 145/120/60/55) to that *M. fortuitum* pattern (<http://app.chuv.ch/prasite>). Randomly selected strains from each genotypes of ITS region, identified as *M. fortuitum* species by 16S rRNA sequence analysis.

PCR amplification of the ITS region with the primers L and R resulted in detection of a single band of approximately 380 bp. No variation in product length was considerable between strains.

Twelve different sequence types (sequevar) were identified based on ITS region. Of these 7 had been previously described and these include MfoA, MfoB, MfoC, MfoD, MfoE, MfoF and MfoG identified (11) but five novel sequevars designated as MfoH, MfoI, MfoJ, MfoK and MfoL were identified among different Iranian clinical strains of *M. fortuitum*. These novel sequevar were distinct from the first group.

Pairwise comparisons between the previously reported sequevar and clinical isolates displayed higher sequence variation between *M. fortuitum* strains. A dendrogram based on maximum parsimony

Table 1. Characteristics of 48 Clinical isolates of Iranian *M. fortuitum*.

Strains (Mf)	Sample source	G/A*	PMH	Main symptoms	Chest X-ray	Diagnosis by clinical findings	ITS sequevars*
7	Leg abscess	F (66)	Healthy	Subcutaneous abscesses	ND	Tb	K
9	BAL	F (64)	Kidney transplant recipient	Fever, cough	Irregular nodular lesions	Tb	F
10	BAL	M (59)	COPD	Fever, cough	Cavitation	Tb	A
11	Wound infection (2)	F (62)	HIV	Fever, weight loss	NA	Tb	E
12	BAL	M (34)	Chronic lymphocytic leukemia	Dyspnea, cough	Pleural effusion	Tb	C
13	Biopsy	M (19)	Soft tissue abscess	Subcutaneous nodules	ND	Tb	A
14	Blood	M (44)	BMT	Fever, weight loss	ND	ND	K
15	BAL	F (71)	Chronic bronchitis	Dyspnea, cough	Bilateral involvement	Tb	H
16	Blood	F (47)	BMT	Fever, weight loss	ND	Fungal infection	A
18	Sputum (3)	F (66)	COPD	Dyspnea	Consolidative with pleural effusions	Tb	A
22	BAL	F (71)	HIV	Fever, cough	Pleural effusions	Tb	H
23	Soft tissue biopsy	M (45)	Healthy	-	-	ND	B
24	Oral ulcers	M (32)	Pamphlgus	Oral ulcers	-	ND	D
25	Blood	F (47)	HIV	Fever	ND	ND	F
28	BAL	F (72)	Recurrent CMV, HIV	Fever, cough	Cavitation	Tb	B
29	BAL	F (71)	Healthy	Fever, cough	Infiltrates	Tb	B
30	Sputum and BAL	M (42)	HIV	Fever, general weakness and dysuria	Infiltrates	Tb	H
31	BAL	F (65)	Kidney transplant recipient	Fever, cough	Diffuse pneumonic infiltrates	Tb	A
32	Brain abscess	M (32)	Brain abscess	FUO	NA	Nocardiosis	C
35	Leg discharges (2)	M (49)	DM	Swelling left leg, purulent wound discharge	ND	Mycetoma	L
39	BAL	F (67)	Tb	Fever, cough	Cavitation	Tb	G
40	BAL	F (31)	Healthy	Fever, chest pain, cough	Nodule	Tb	L
41	Blood	M (66)	Mitral valve prosthesis	Post operative fatigue, chest pain	ND	ND	I
42	BAL	F (81)	Neo	Fever, chest pain, cough	Infiltrate	Tb	A
43	Sputum (3)	M (62)	Ischemic heart disease	Fever, cough	Small irregular nodular lesions	Tb	H

analysis reflecting the ITS sequence-based clustering of all test strains of *M. fortuitum* is shown in Fig. 1. Within the consensus tree, twelve clusters with distinct branches among the *M. fortuitum* reference strains could be defined.

Of 48 test strains, 25 (52%) grouped and formed distinct clusters with previously reported sequevars (11). The branches was supported with highest bootstrap value (100%) (Fig. 1).

Among previously reported sequevars, MfoB (8 isolates, 16.8%) was the most frequently encountered, followed by MfoA (6 isolates, 12.5%) and MfoC (4 isolates, 8.3%). Two isolates (4%) belong to each sequevars MfoD (Mf 24, Mf 53), MfoE (Mf 11, Mf 52) and MfoF (Mf 9, Mf 25) were identified. One isolate (Mf 39), showed identical ITS sequence, to that of reported for MfoG.

Of 48 test strains, 23 (48%), represented novel sequevars and took five well-supported and distinct

positions on the ITS tree (Fig. 1). Among the new sequevars reported in this study, MfoH (9 isolates, 18.7%) was the most frequently encountered followed by MfoL (6 isolates, 12.5%), MfoJ and MfoK (4 isolates, 8.3%) and MfoI (2 isolates, 4%).

Twenty-eight isolates (58%) were recovered from the patients with pulmonary disease (Table 1). However, the significant association between *M. fortuitum* sequevars and pulmonary disease was not detected.

DISCUSSION

The main aim of this study was to access genetic diversity of *M. fortuitum* clinical isolates using ITS sequence analysis. Due to high polymorphism, ITS region has been reported as alternative target for molecular identification of mycobacteria to species level (11, 12, 18). ITS region also showed potential

Table 1. Continued ...

47	BAL	M (67)	Chronic bronchitis	Fever, cough	Diffuse pneumonic infiltrates	Tb	J
48	BAL	M (59)	Chronic bronchitis	Fever, cough	Diffuse pneumonic infiltrates	Tb	C
49	Sputum, BAL	M (42)	Follicular non-Hodgkin Lymphoma	Dyspnea, cough	Bilateral involvement	Tb	H
50	Brain abscess	M (59)	Brain abscess	FUO, weight loss, headache	CT scan, cerebral abscess	Nocardiosis	J
51	BAL	F (47)	Healthy	Fever, chest pain, weight loss	Cavitation	Tb	H
52	BAL	F (55)	Healthy	Fever, chest pain, cough	Nodule	Tb	E
53	Biopsy	F (18)	Skin graft recipient	Weakness, soft tissue abscess	NA	Nocardiosis	D
54	Biopsy	F (28)	Soft tissue abscess	Subcutaneous nodules	ND	Mycetoma	I
55	BAL	F (81)	Tb	Fever, cough	Cavitation	Tb	B
56	Blood	M (32)	Liver transplant recipient	FUO	ND	ND	L
68	Brain abscess	F (64)	Brain abscess	FUO, weight loss, headache	CT scan, cerebral abscess	Nocardiosis	L
71	Sputum (3)	F (64)	Tb, HIV	Fever, cough	Cavitation	Tb	L
77	Brain abscess	F (50)	Multifocal brain abscesses	FUO, headache	CT scan, cerebral abscess	Nocardiosis	B
78	Sputum	M (88)	Neo	Chest pain	Cavitation	Tb	H
79	Bone marrow biopsy	M (14)	Hemophili	Fever, Dyspnea, cough	ND	Tb	K
84	BAL	F (73)	Neo	Fever, chest pain, weight loss	Infiltrate	Tb	B
90	Sputum (4)	M (48)	Neo	Fever, cough, chest pain	Cavitation	Tb	C
SM25	BAL	M (63)	Liver transplant recipient	Fever, cough	Small irregular nodular lesions	Tb	J
SM26	BAL	F (42)	Tb, HIV	General weakness, dysuria	Cavitation	Tb	L
SM30	BAL	F (21)	Follicular non-Hodgkin Lymphoma	Fever, cough	Diffuse pneumonic infiltrates	Tb	H
SM62	Blood	F (46)	Kidney transplant recipient	FUO	ND	ND	H
SM80	BAL	F (73)	Neo	Fever, chest pain, weight loss	Infiltrate	Tb	B
SM84	BAL	F (40)	Neo	Fever, chest pain, weight loss	Infiltrate	Tb	B

Abbreviations: G/A, gender/age; PMH, past medical history; F, female, ND, not determined; BAL, bronchoalveolar lavage; M, Male; COPD, chronic obstructive pulmonary disease; HIV, human immunodeficiency virus; NA, not available; BMT, bone marrow transplantation; CMV, cytomegalovirus, FUO, fever unknown origin, DM, diabetes mellitus; Tb, tuberculosis; Neo, neoplasia, CT, computed tomography.

*, ITS sequevars from A to G has been reported previously (9) and H to L reports as new genotypes base of ITS sequences.

utility for strain differentiation among some species of mycobacteria (11, 12, 17). Some studies have reported a correlation of the genomic variants and particular host range in mycobacteria, indicating the usefulness of genetic markers including ITS, IS901, major polymorphic tandem repeat (MPTR) and *hsp65* in the subspecies differentiation (11, 12, 16-18, 22, 25, 26). For example, Stout *et al.* (2008), found a significant association between *M. avium* sequence types of ITS region and *M. avium* pulmonary disease (27).

Different variant of *M. fortuitum* also had been characterized by *hsp65*-PRA or other genetic marker (11, 12, 14-16) but relevance and clinical importance of these genotypes have been poorly studied. *M. fortuitum* is most frequently encountered species of NTM that usually implicated in chronic infectious diseases caused by mycobacteria in the Iranian clinical settings (28). Different sequevars reported

here, were recovered from wide types of clinical samples including BAL, Blood, Bone marrow biopsy, brain abscess, leg abscess, oral ulcers, sputum and wound infection.

This study demonstrates that the ITS of the *M. fortuitum* exhibits high variations, base substitutions and insertion or deletion. Present study suggests that higher degree of variation at ITS region is valuable for discriminating of clinical strains of *M. fortuitum*. However, no association was found between any particular sequence types and case status.

In conclusion, the ITS region of the genus *Mycobacterium* exhibits a high variation which is discriminative for related taxa. The marker has sufficient power to differentiate and separate clinical strains of *M. fortuitum*. This study showed that the ITS region possesses high discriminatory power between the clinical isolates up to the clonal level. The results also suggest the possibility of the

existence of predominant clone of *M. fortuitum* in affected patients in Iran. The data also point to the conclusion that a large variety of *M. fortuitum* clone can produce human disease although certain clones seem to be predominant.

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