

# Molecular Identification of Clinical Isolates of *Mycobacterium fortuitum* by Random Amplified Polymorphic DNA (RAPD) Polymerase Chain Reaction and ERIC PCR

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## ABSTRACT

**Backgrounds:** Non tuberculous mycobacteria (NTM) are of importance now-a-days due to their increasing virulence outbreaks and emerging antibiotic resistance. Since the most common NTM in Iran is reportedly *Mycobacterium fortuitum*, the present study was designed with the aim of molecular identification of clinical isolates of *M. fortuitum* to analyse their heterogeneity.

**Materials and Methods:** A total of 81 isolates of NTM isolated from various samples were collected. The clinical isolates were assigned to species *M. fortuitum* by using conventional and molecular methods. The DNA banding patterns of ERIC-PCR and RAPD-PCR were analysed by using Bionumeric 7.5 software.

**Results:** Out of 81 tested NTM, 36 strains of *M. fortuitum* were identified. 33 isolates were selected for molecular typing in this study. Based on RAPD and ERIC analysis, *M. fortuitum* isolates were divided into 3 and 6 clusters, respectively. Most of the isolates were distributed into types of II RAPD (20 members/ 60.6 %) and V (14 members/ 42.4% with sub cluster I& II) of ERIC. In RAPD analysis, the major fragments were 300 bp, followed by fragment 1000. In ERIC analysis, the major fragments were 280 bp followed by fragment 1200 bp.

**Conclusion:** In conclusion, though the results from this study represented higher discriminatory power of ERIC, however the combination of RAPD and ERIC analysis were able to sufficiently discriminate the genotypic diversity, infection control, and gain useful epidemiological information regarding *M. fortuitum* isolates.

**Keywords:** Molecular epidemiology, Non tuberculous *Mycobacteria*, Typing

## INTRODUCTION

Environmental *Mycobacteria* or Non tuberculous *Mycobacteria* (NTM) are of importance now-a-days due to their increasing virulence outbreaks and emerging antibiotic resistance [1]. They are common saprophytes of a wide variety of environmental reservoirs, such as soil, dust and water which act as opportunistic human pathogens in certain circumstances and often emerge in outbreaks. They have been reported to be associated with cutaneous infections [2]. Among NTM, rapidly growing mycobacteria (RGM), can cause localized cutaneous infections following trauma, as well as outbreaks of nosocomial infections including surgical site infections [3]. *Mycobacterium abscessus*, *Mycobacterium chelonae* and *Mycobacterium fortuitum*, are the most RGM common causes of human diseases [4]. *M. fortuitum* was first isolated from an amphibian source in 1905 and subsequently rediscovered as a human pathogen and named by Da Costa Cruz in 1938 [5]. *M. fortuitum* can cause a wide range of infections of lungs [6], skin, and soft tissue [7] comprises injection abscesses and wound infections [2,8], tendon sheath [9], breast abscess [10] and bone [11] in immunocompromised hosts including AIDS patients and in patients following cardiac bypass surgery or augmentation mammoplasty [5,12,13]. The molecular typing methods are used for diversity and outbreak investigations of *M. fortuitum*. Randomly amplified polymorphic DNA (RAPD) PCR; enterobacterial repetitive intergenic consensus (ERIC) PCR and 16S/23S rRNA internal transcribed spacer (ITS) genotyping are applied for comparing bacterial strains and epidemiological evaluation and investigation of polymorphism among various bacteril including NTM [3].

In our country, it has been reported that *M. fortuitum* is the most frequent NTM isolated from clinical samples [14]. So, this study was designed to investigate the genetic heterogeneity among *M. fortuitum* clinical isolates in Iran by using molecular methods of

RAPD- and ERIC-PCR which have been reported to be useful tools for typing and differentiation of species of *Mycobacteria*, including *M. fortuitum* [15,16].

## MATERIALS AND METHODS

### Bacterial isolates

In total 81 clinical isolates were received between March 2012 and December 2013 to the Laboratory of Infectious and Tropical Diseases Research center of Ahvaz Jundishapur University of Medical Sciences, Iran, collected in different centers in Iran including Masih Daneshvari Hospital, Tehran; Iran Pasteur Institute; two private laboratories in Tehran and Tuberculosis Reference Center of Khuzestan province, southwest of Iran.

### Identification of *M. fortuitum*

#### Conventional methods

The source of totally 81 clinical isolates was various samples including sputum, bronchial washing, pleural effusion, urine and wound. The following biochemical tests were performed for the clinical isolates for identification of RGM including *M. fortuitum*: staining for acid fast bacilli, *Mycobacteria* conventional phenotypic tests including growth on Lowenstein Jensen (LJ) medium (HiMedia, India) in 7 days or less, positive nitrate test, iron uptake and niacin production [17]. For quality control of the conventional tests, *M.bovis* BCG, *M. tuberculosis* H37Rv and *M. kansasii* (DSM44162) were used. Molecular methods can be used to identify isolates suspicious to be *M. fortuitum*.

### Molecular methods

#### DNA extraction

Chromosomal DNA was extracted using the method described by pitcher et al., with slight modification to improve the susceptibility

of cells to the standard digestion. 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 phenolchloroform and precipitated with isopropanol. The precipitate was washed in 70% ethanol, dehydrated, dissolved in 100 ml of Milli-Q water, and stored at -20°C until use [18].

### PCR amplification using genus specific primers

Amplification process was performed by using mycobacterial genus specific primers of MSGPF, 5'-CTGGTCAAGGAAGGTCTGCG and MSGPR, 5'-GATGACACCCTCGTTGCC. These primers can amplify a 228-base-pair fragment corresponding to a sequence from the 65-KDa heat shock protein gene (hsp65) [19].

The composition of PCR mixture (50 µl) consisted of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 200µM dNTPs, 10pmol of each primer, 0.5U of Taq DNA polymerase and 20ng DNA template and Millipore H<sub>2</sub>O up to 50 µl. The amplification conditions using PCR thermocycler (BIORAD, USA) were as follows: initial denaturation at 95°C for 5 min followed by 35 cycles of denaturation at 94°C for 15 sec, annealing at 58°C for 15 sec, and extension at 72°C for 30 sec and final extension at 72°C for 10 min. *M. tuberculosis* H37Rv, *M. bovis* BCG and *M. kansasii* (DSM44162) were used as control positive.

### PCR- restriction enzyme analysis (PRA)

PRA of hsp65 was performed as described by Kim et al., with minor modifications [20]. Briefly, a 644-bp fragment of hsp65 was amplified. PCR was performed in a volume of 50 µl containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 200µM dNTPs, 10pmol of each primer, 0.5U of Taq DNA polymerase and 50ng DNA template and Millipore H<sub>2</sub>O up to 50 µl. The reaction mixture was subjected to 30 amplification cycles of denaturation at 95°C for 60 sec, annealing at 62°C for 45 sec, and extension at 72°C for 90 sec followed by a 5-min final extension at 72°C. The PCR products were digested by three restriction enzymes of Ava II, Hph I, and Hpa II (Fermentas, Canada) as previously described [20]. Digested products were electrophoresed in 3% w/v agarose gel, stained with ethidium bromide and were visualized using gel documentation system (UV Tech, UK). The pattern was compared to the restriction map algorithm constructed by Kim et al and our inhouse database as well [20]. A 100 bp DNA ladder was used as size marker (Roche, Germany).

### Molecular typing of *M. fortuitum* isolates

#### RAPD-analysis

The 50ng extracted DNA from the isolates was subjected to RAPD-PCR analysis using primers 27F and 1525R as previously described [21], following the recommended optimization. The PCR products were separated by electrophoresis and photographed on gel documentation system. The major bands were identified and the previous published criterion was used for RAPD pattern interpretation [22].

#### ERIC-PCR

We used the amplification method using specific primers as previously described by Sechi et al., with minor modification [23]. In brief, the reaction mixture were performed in a volume of 50 µl containing 20 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 200mM dNTPs, 10pmol of each primer, 0.5U of Taq DNA polymerase and 50ng DNA template and Millipore H<sub>2</sub>O up to 50 µl. The programming conditions were as initial denaturation at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 45s, annealing at 52°C for 1 min and extension at 70°C for 1 min, with a final extension at 72°C for 10 min. Amplified products were separated by electrophoresis in 2% w/v agarose gels after staining with ethidium bromide.

Data were analysed using Chi-square test with the help of SPSS software (SPSS Incorporation, Chicago, USA).

### Clustering analyses

The DNA banding patterns of ERIC- PCR and RAPD- PCR were analysed using Bionumeric 7.5 software (Applied maths company, Sint-Martens-Latem, Belgium) with using dice similarity index for cluster analysis and the unweighted pair group average (UPGMA) described by Hunter and Gaston [24] for clustering of *M. fortuitum* isolates and Pearson coefficient, UPGMA, was used for Reproducibility of each clustering analyses.

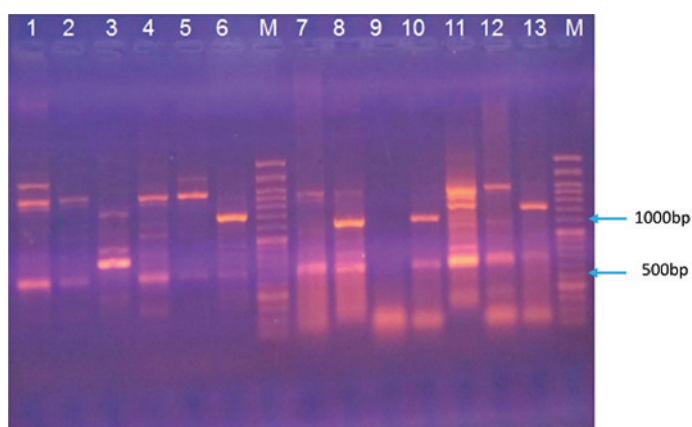
## RESULTS

The screened isolates in present study were isolated from 47 (58%) male and 34 (42%) female patients with confirmed NTM infection. The patient's ages were from 50 to 60 years with mean of 56.4. Thirty six (44.44%) out of total isolates were conventionally identified as *M. fortuitum* on the basis of rapid growing in less than 7 days and standard biochemical tests for RGM. The source of *M. fortuitum* isolates were as follows: Broncho Alveolar Lavage {BAL} (19 isolates {52.8%}), urine (9 isolates {25%}), pleural fluid (5 isolates {13.9%}), sputum (3 isolates {8.3%}).

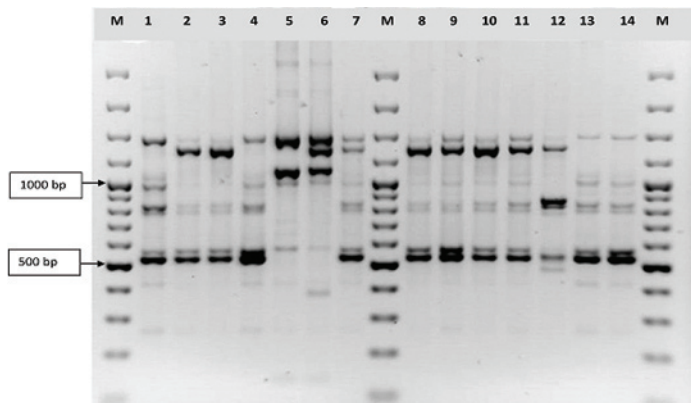
The presumptive *M. fortuitum* isolates were further analysed and verified as *Mycobacteria* using genus and group specific PCR targeting based on a 228-base-pair fragment from the 65-KDa heat shock protein gene.

Furthermore with the application of the PRA algorithm targeting 644 bp hsp65 DNA, all 36 isolates were clearly distinguished from other mycobacteria. All isolates had the characteristic Avall (501, 119 bp), HphI (254, 207, 97 bp) and HpaII (270, 161, 117 bp) PRA pattern of international type strain of *M. fortuitum* ATCC 6841T [20].

Thirty three isolates were selected for molecular typing in this study. Based on RAPD and ERIC analysis, *M. fortuitum* isolates were divided into 3 and 6 clusters, respectively. Most of the isolates were distributed into types of II RAPD (20 members/ 60.6%) and V (14 members/ 42.4% with sub cluster I & II) of ERIC and other types for RAPD were included: I (7 members/ 21%), III(6 members/ 18.2%, with sub cluster I& II) and for ERIC types: I (2 members/6 %), II (3 members/ 9%), III (6 members/18.2%), IV (6 members/ 18.2% with sub cluster I&II), VI (2 members/ 6%) [Table/Fig-1&2]. More than 70% of RAPD type I and II were isolated from BAL and sputum samples [Table/Fig-3]. In RAPD analysis, the major fragments were 300bp which was observed in many of RAPD types, followed by fragment 1000bp [Table/Fig-4] In ERIC analysis, the major fragments were 280bp which was observed in almost all types of ERIC-PCR, followed by fragment 1200bp [Table/Fig-5].



**[Table/Fig-1]:** ERIC PCR profiles of Iranian *Mycobacterium fortuitum* isolates. Slots from left to right: 1, S80; 2, S33; 3, S12; 4, S64; 5, S69; M, 100bp DNA size Marker; 7, S47; 8, S32; 9, S01; 10, S68; 11, S36; 12, S30; 13, S46.



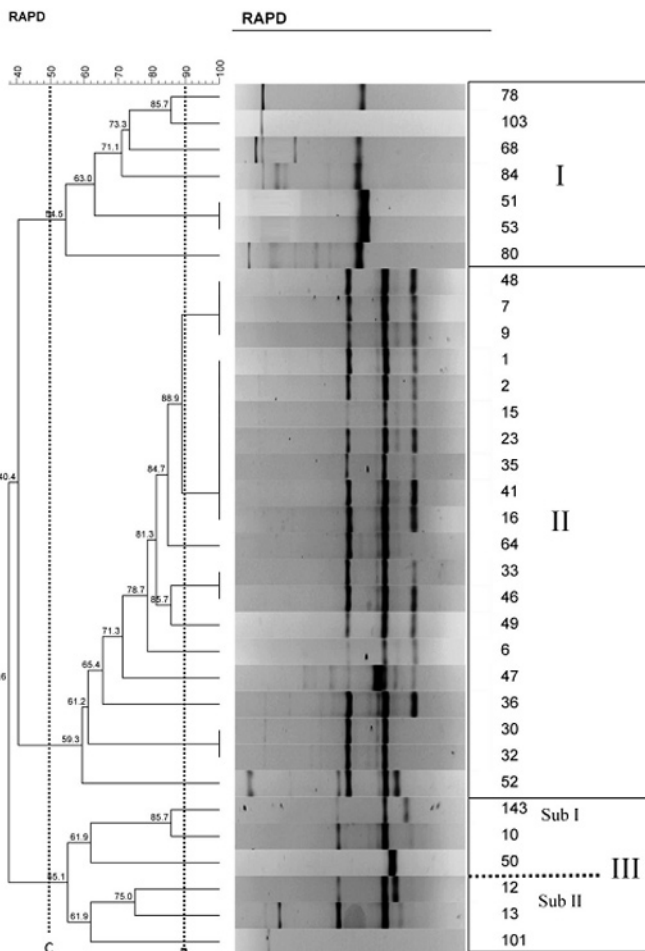
**[Table/Fig-2]:** RAPD PCR profiles of the amplified DNAs of Iranian *Mycobacterium fortuitum* isolates obtained by analysis with primers 27F and 1525 R. Lines M, 100 bp ladder; lines 1-14, *Mycobacterium fortuitum* isolates pattern

Strains	Sample source	Cluster	
		RAPD- PCR	ERIC- PCR
1	BAL	II	IV
2	Sputum	II	V
6	Sputum	II	IV
7	BAL	II	IV
9	BAL	II	V
10	BAL	III	V
12	BAL	III	III
13	Biopsy	III	IV
15	BAL	II	III
16	Blood	II	V
23	Soft tissue biopsy	II	IV
30	Sputum & BAL	II	II
32	Brain abscess	II	III
33	BAL	II	V
35	Sputum	II	V
36	Sputum & BAL	II	V
41	Blood	II	III
46	BAL	II	I
47	BAL	II	V
48	BAL	II	V
49	Sputum & BAL	II	V
50	Brain abscess	III	IV
51	BAL	I	III
52	BAL	II	V
53	Biopsy	I	V
64	Sputum	II	V
68	Sputum	I	III
78	Sputum	I	II
80	BAL	I	V
84	Brain abscess	I	V
101	BAL	III	I
103	BAL	I	II
143	Sputum	III	VI

**[Table/Fig-3]:** Characteristics and cluster analysis of 33 Clinical isolates of Iranian *M. fortuitum*  
Abbreviations: BAL, bronchoalveolar lavage

## DISCUSSION

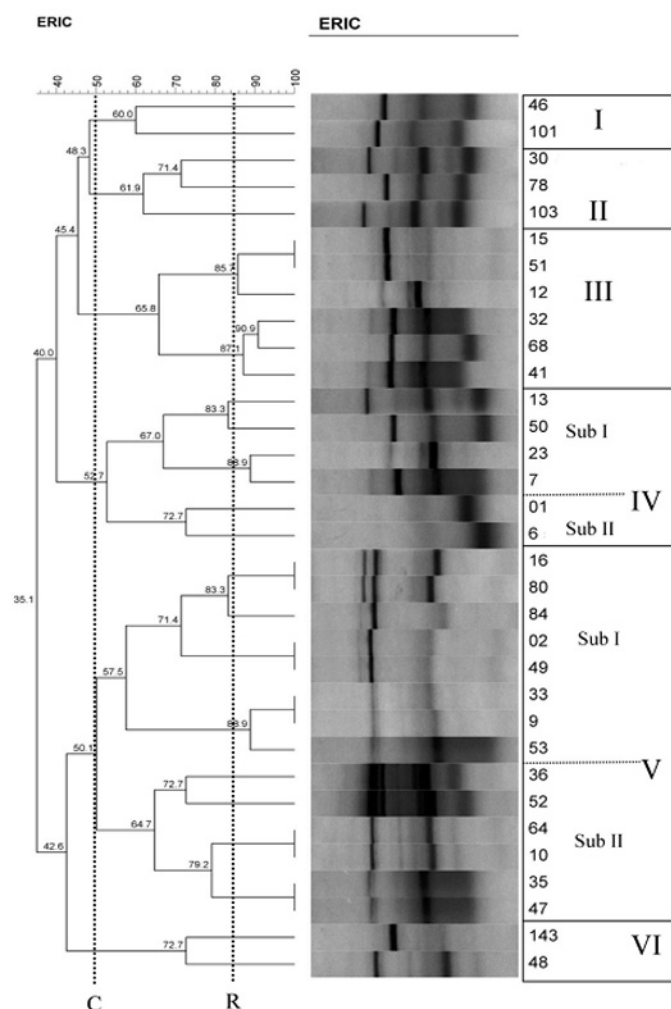
*M. fortuitum* group is a rapidly growing group of NTM more common in patients with genetic or acquired causes of immune deficiency [13]. For typing of *Mycobacteria*, several methods based on molecular biologic techniques have been introduced in recent decades including PRA, pulsed field gel electrophoresis (PFGE), RAPD and ERIC PCR. For instance, PRA schemes targeting hsp65 have been most widely used lately by some investigators with the explanation that this molecule is conserved in all *mycobacteria* and related strains, so it shows sufficient sequence variation to allow



**[Table/Fig-4]:** RAPD-PCR dendrogram of Iranian *Mycobacterium fortuitum* isolates. The vertical dotted lines indicate the cluster cut-off value (C= shows the 50% cut-off) and the cut-off point based on the reproducibility analysis (R)

mycobacterial differentiation at the species or strain level [20]. In this study PRA was successfully applied to molecularly detect *M. fortuitum* isolates and distinguishes them from other related NTM. Although the technique could not sufficiently recognize the genetic variation among the *M. fortuitum* isolates and the necessity for application of another genotypic method was felt and initially the candidate was RAPD- PCR, which shows sufficient sequence variation in the strains to allow differentiation at the species or strain level. However, there was some controversy among investigators for using the method. While Zhang et al., found the technique a useful tool for typing and differentiation of species of *Mycobacteria* [15], it was suggested by others that RAPD-PCR alone is not sufficiently discriminative to evaluate *M. fortuitum* isolates from outbreaks [3]. On the other hand, some other investigators emphasized on the priority of rep-PCR on RAPD and according to their findings, rep-PCR was able to provide improved reproducibility over RAPD and a discriminatory genotyping method for mycobacterial isolates evaluated [25]. So, in present study apart from using RAPD-PCR, we have designed to apply one more widely used method i.e. ERIC-PCR to have higher discriminatory power for our *M. fortuitum* isolates. Other investigators reported that ERIC marker could be used as an independent marker and shows higher discriminatory power compared to other typing methods [23].

With RAPD- and ERIC PCRs combination, cluster analysis showed that ERIC was able to divide isolates into more groups and sub cluster. Results from this study demonstrated that ERIC has the potential to be used as a screening tool and useful for rapid epidemiological typing tools of *M. fortuitum* infections and was recommended by the authors for initial screening with many strains of *M. fortuitum* are to be typed. From 3 and 6 genotypic clusters generated by RAPD and ERIC PCRs respectively, the majority of isolates were belonged to types II RAPD and V, III, IV



**[Table/Fig-5]:** ERIC-PCR dendrogram of Iranian *Mycobacterium fortuitum* isolates. The vertical black (C) line shows the 50% cut-off and the cut-off point based on the reproducibility analysis (R)

ERIC, this represents a high concordance of the results extracted from both techniques. However, considering the main and inferior fragments generated by RAPD- and ERIC-PCR, the results from ERIC was more discriminative and in general ERIC generated more uniform bands among the isolates with 2 major fragment bands of 280 & 1200 bp and at least 2 more minor fragment bands of 100 & 1000 bp. While in RAPD only 2 major fragment bands of 300 & 1000 bp were observed in almost all types and other fragment were mostly distributed in single genotypic types. We found a significant association between the type of sample and nature of generated genotype. Though the distribution of genotypes among different clinical samples showed great variation, however the isolates from BAL and sputum showed more uniformity in both genotypic techniques, since the major types isolated from these samples were type II and V, may be due to the fact that especially the number of BAL samples in this study was higher.

Although RAPD is relatively simple and useful for epidemiological analysis, standardization of the PCR conditions is very important for reproducibility as other investigators addressed [21]. Due to this, as one of the important issues observed in this work based on the experience of a previous study [22], was the necessity of standardizing the amount of DNA in each RAPD reaction mixture (50 ng of DNA) to ensure that non-specific bands were not present.

Similarity of > 50% in *M. fortuitum* isolates, showed high heterogeneity among them. Besides, the 100% similarity was found between some of the isolates as well, which may be due to a similar source. Our study showed that the same genetic types of *M. fortuitum* isolated from sputum and BAL of types II RAPD and V ERIC, occurred in different patients with 100% similarity in DNA

pattern, and it was possible to postulate that the same clone was present in the environment. The results also suggest that cross-colonization was probably occurred between same patients (with 100% similarity in DNA pattern).

## CONCLUSION

Though the results from present study represented higher discriminatory power of ERIC, however the combination of RAPD and ERIC analysis were able to sufficiently discriminate the genotypic diversity, infection control and gain useful epidemiological information regarding *M. fortuitum* isolates. Both techniques are simple, rapid, inexpensive, reproducible and are less complicated than most of other molecular typing methods such as PFGE or MLST. On the other hand, the cost of reagents for PFGE and MLST are expensive than the present typing techniques. Molecular epidemiology methods such as ERIC PCR, RAPD PCR and other PCR based techniques require only optimized and standard protocols and equipments available in most Institutes. Further studies of molecular characteristics of *M. fortuitum* are required among hospital staff and environment in the present region of the study to give us more extensive insight of actual epidemiology and state of infection-control by molecular epidemiology methods. The molecular epidemiological methods have proven to be useful for the detection and control of many outbreaks of infections by bacteria.

## ACKNOWLEDGEMENTS

We are grateful to the research affairs of Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran, to allow the work to be carried out in Infectious and Tropical Diseases Research center of the university and for their support. This article resulted from the MSc Microbiology thesis of Rasa Sheini Mehrabzadeh. Our special thanks go to Dr. Parvin Heidarieh and Dr. Nasrin Sheikhi for providing parts of the isolates.

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Date of Submission: **Jul 02, 2015**  
 Date of Peer Review: **Sep 16, 2015**  
 Date of Acceptance: **Oct 11, 2015**  
 Date of Publishing: **Dec 01, 2015**

**FINANCIAL OR OTHER COMPETING INTERESTS:** None.