

# Genetic diversity of Iranian clinical isolates of *Mycobacterium tuberculosis*

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## SUMMARY

In the current study we aimed to execute a rather less complicated molecular typing method, i.e. the random amplification of polymorphic DNA (RAPD) analysis to find the heterogeneity of Iranian strains of *Mycobacterium tuberculosis*. The isolates comprised a total of 96 strains of *M. tuberculosis* collected from clinical specimens of patients in Isfahan and Tehran. The isolates were assigned to the species *M. tuberculosis* by the key conventional and molecular methods. They were then subjected to RAPD analysis by four arbitrary primers, namely, the primers 27F, 1525R, MS-GF and INS-2. They were then evaluated for the number and intensity of the band patterns. The RAPD profiles of the Iranian isolates showed a degree of heterogeneity which varied based on the primer used. However, analysis of the isolates by primer INS-2 revealed the highest degree of diversity yielding 31 distinguishable RAPD types. RAPD analysis provides a rapid and easy means of identifying heterogeneity among the *M. tuberculosis* isolates. This typing system might be considered a valuable alternative molecular typing for countries with limited resources provided that the reproducibility and reliability of the method is carefully assured.

**KEY WORDS:** *Mycobacterium tuberculosis*, RAPD, Typing

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## INTRODUCTION

Since the late 1980s, molecular methods have been gradually developed to study the genetic diversity of *Mycobacterium tuberculosis* strains (Mathema *et al.*, 2006). Several molecular approaches such as restriction fragment length polymorphism (RFLP) based on small transposable element IS6110, the direct repeat (DR), the GC-rich repetitive sequence (PGRS), variable numbers of tandem repeat loci (VNTR) and single-nucleotide polymorphism (SNP) have been developed for molecular fingerprinting of *M. tuberculosis* isolates (van Embden *et al.*, 1993; Yang *et al.*, 1996; Heyderman *et al.*, 1998; Sola *et al.*, 2003;

Gutacker *et al.*, 2006; Supply *et al.*, 2010). Most of these methods require high expertise, a well-equipped laboratory and relatively complex protocols to clearly differentiate the various TB strains. In the current study we aimed to resolve the genetic diversity of Iranian isolates of *Mycobacterium tuberculosis* by using a rather less complicated molecular typing method, namely, RAPD analysis (Weish *et al.*, 1990).

## MATERIALS AND METHODS

### Isolates

A total of 96 isolates of *M. tuberculosis* corresponding to 96 independent patients with confirmed tuberculosis infection referred to or isolated in our laboratory over a period of 6 years between 2002 to 2008 were investigated. The isolates originated from a variety of clinical specimens comprising sputum (51 cases), gastric

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washing (14 cases), bronchoalveolar aspirate (13 cases), urine (15), lymph nodes biopsy (2 cases) and skin tissue biopsy (1 case).

### Primary isolation and conventional identification

The species identification of the isolates was achieved by standard conventional and molecular methods (Kent *et al.*, 1985; Ben Kahla *et al.*, 2011). The susceptibility tests were performed using the National Committee for Clinical Laboratory Standards (NCCLS) proportion method (NCCLS, 1994) on Löwenstein-Jensen (LJ) medium containing rifampin (RIF) 40 mg/L, isoniazid (INH) 0.2 mg/L, ethambutol (EMB) 2 mg/L and streptomycin (SM) 40 mg/L.

### Preparation of genomic DNA for PCR

Intact chromosomal DNA was extracted using the method of Pitcher *et al.*, 1989, with a slight modification to ease the cell wall disruption as previously described (Shojaei *et al.*, 2000). The DNA was purified by phenol chloroform-isoamyl alcohol. The precipitate was washed in 70% ethanol, dehydrated and dissolved in Milli-Q water and stored in a -20°C freezer until use.

### Molecular identification

To confirm the identity of the clinical isolates a 245-base-pair sequence of IS6110 specific for the members of *M. tuberculosis* complex was targeted and amplified by polymerase chain reaction using the specific primers INS-1 and INS-2 as described previously (Eisenach *et al.*, 1990).

### RAPD analysis

The extracted DNA from the isolates was subjected to PCR-RAPD analysis following the optimized procedure recommended by Burucoa *et al.*, 1999. In brief, the PCR amplifications for RAPD fingerprinting were carried out with the randomly chosen primers from our library, i.e., 27F (5'-AGAGTTTGATCCTGGCTCAG-3'), 1525R (5'-AAGGAGGTGATCCAGCC-3'), MSGF (5'-CTG-GTCAAGGAAGGTCTGCG-3') and INS-2 (5'-GCGTAGGCGTCCGGTACAAA-3') in a low-stringency PCR amplification.

The PCR products were separated by electrophoresis and photographed on a Uvidoc gel documentation system. DNA fragment sizes in the RAPD patterns were calculated using

DNAfrag 3.03 package (Nash, 1991). The identity of the RAPD patterns was defined on the basis of the similarity of numbers and matching positions of the major bands by means of the GelCompar 3.1 software (Applied Maths, Kortrijk, Belgium). Only major bands were considered and band intensity was not used as a criterion.

## RESULTS

Of the 96 patients with confirmed tuberculosis infection, 50 (52%) were female, 47 (49%) were between the ages of 50 and 60 years, 16 (16.7%) were between the ages of 40 and 50 years, 16 (16.7%) were between the ages of 30 and 40 years, 10 (10.4%) were between the ages of 20 and 30 years, 7 (7.2%) were between the ages of 5 and 20 years. Thirty nine patients were found to have a history of various immunosuppressive syndromes, including cystic fibrosis (12 cases), chronic obstructive pulmonary disease (7 cases), malignancy (3 cases), diabetes mellitus (5 cases), bronchitis (5 cases), or HIV infection (7 cases), while the remainder of the patients had no apparent history of such disorders.

Out of 96 clinical samples, 81 tested specimens were positive by direct microscopy analysis of acid-alcohol fast staining. However, on the basis of culture, growth rate, biochemical properties and molecular testing the identity of all 96 isolates was confirmed as *M. tuberculosis*.

Based on the result of in vitro drug susceptibility testing to the first-line antituberculosis drugs, mono-resistance to ethambutol, streptomycin and isoniazid was detected in 38 (39.5%), 32 (33.3%) and 3 (3.1%) strains respectively. Mono-resistance to rifampin was not detected. Resistance to two drugs including ethambutol and streptomycin was detected in 18 (18.7%) strains. Resistance to isoniazid and rifampin (MDR) was found in 2 (2%) strains.

The RAPD analysis performed with DNA from the Iranian *M. tuberculosis* isolates, and primers 27F, 1525R and MSGF was found to generate less informative banding patterns. However, the RAPD analysis using primer INS-2 generated altogether 31 different patterns for the isolates studied here. These profiles designated RAPD types 1 to 31 (Figure 1). The RAPD profiles consisted of three to eleven amplicons ranging from 400 to



the rare single line of descent (Figure 2). The reference *M. tuberculosis* H37Rv strain formed a unique RAPD type pattern which was distinctly separate from the Iranian isolates.

## DISCUSSION

Our results showed that molecular RAPD fingerprinting can discern isolates of *M. tuberculosis*. Compared with other typing methods for TB strains such as pulse-field gel electrophoresis, or hybridization with the universal or specific probes (van Embden *et al.*, 1993; Singh *et al.*, 1999), this procedure yields greater polymorphism, is technically simpler and faster, and requires no radioactive tracers (Weish *et al.*, 1990; Linton *et al.*, 1994; Linton *et al.*, 1995; Richner *et al.*, 1997; Harn *et al.*, 1997; Burucoa *et al.*, 1999). It has been reported that the degree of polymorphism obtained by RAPD is almost the same as that obtained by RFLP (Linton *et al.*, 1995). However, RAPD is faster and technically less complicated than most other molecular typing methods (Weish *et al.*, 1990; Burucoa *et al.*, 1999). Moreover, in RAPD analysis no DNA sequence information is necessary (Weish *et al.*, 1990). In addition, a smaller amount of purified DNA (<50 ng) is required than for methods such as RFLP (Weish *et al.*, 1990; Linton *et al.*, 1994). This is of practical significance as, in our experience, DNA isolation from mycobacteria is almost unlikely without shearing.

Although RAPD is relatively simple and useful for epidemiological analysis, standardization of the PCR conditions is very important for reproducibility (Burucoa *et al.*, 1999). For instance in our experiment it was necessary to standardize the amount of DNA in each RAPD reaction mixture (50 ng of DNA) to ensure that non-specific bands were not present.

And last but not least, even with this standardization, reproducible patterns were difficult to acquire, and duplicate analyses were unavoidable for the true profile differences to be evaluated.

In the current study the RAPD analysis based on the primer INS-2, resulted in 31 RAPD types for 96 isolates. We classified *M. tuberculosis* strains as either clustered (containing  $\geq 3$  members), small group (2 members) or unique. Of 96 *M. tuberculosis* isolates, 68 (70.8%) grouped into clus-

ters, 12 (12.5%) distributed into small groups and 16 (16.7%) had unique RAPD profiles. The RAPD types of 8 and 9 which included 33 (34.3%) of all isolates prevailed. The high degree of diversity of RAPD patterns among *M. tuberculosis* isolates suggests that the chance occurrence of identical RAPD fingerprints among unrelated cases would be unlikely. Thus, we might infer that the cases of tuberculosis with identical RAPD fingerprints, such as RAPD types 2, 6, 8, 9 and... are suspected to be due to epidemiologically related strains while the cases with unique RAPD fingerprints, such as RAPD types 3, 7, 11, 13, and ... are likely due to remote infection or recurrent tuberculosis.

## CONCLUSION

Despite the fact that the advance in molecular methods has offered new typing choices for *M. tuberculosis* strains, a full understanding of the typing results requires close collaboration between the laboratory and the TB control department in the health sector. Having integrated the molecular typing results with reliable conventional epidemiologic TB surveillance, the effectiveness of investigation would be maximized. This in turn, contributes to the success of evidence-based tuberculosis control programs.

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