

## Evaluation of Molecular Diversity of *Mycobacterium tuberculosis* Strains By Polymorphisms in RD Regions

Ehsan Sharifipour<sup>1</sup>, Mohammad Javad Nasiri<sup>1\*</sup>, Parissa Farnia<sup>1</sup>, Mohadeseh Mozafari<sup>1</sup>, Shiva Irani<sup>2</sup> and Ali Akbar Velayati<sup>1</sup>

<sup>1</sup>Mycobacteriology Research Centre, National Research Institute of Tuberculosis and Lung Disease (NRITLD), Shahid Beheshti University of Medical Sciences, Tehran, Iran

<sup>2</sup>Department of Biology, Science and Research Branch, Islamic Azad University, Tehran, Iran

### Abstract

**Background:** The region of differences (RDs) polymorphisms is a potential molecular epidemiology method to distinguish origins of *Mycobacterium tuberculosis* (MTB). Till date, 68 RDs were identified in MTB. This study was designed to investigate highly polymorphic regions (RD1, RD150 and RD181) among MTB strains isolates from Iranian Pulmonary tuberculosis patients (PTB).

**Method and materials:** A total of the 190 MTB isolates were identified by conventional and molecular methods. Thereafter, Spoligotyping and RD typing (RD1, RD150 and RD181) were applied to genotype these strains.

**Results:** The most frequent spoligotype belong to Haarlem (n=72, 37.8%) followed by CAS (n=37, 19.4%), T1 (n=18, 9.4%) and Beijing (n=11, 5.7%) lineages. Deletion in RD181 was only identified among Beijing lineage.

**Conclusion:** Deletion in RD181 was prominent among the prevalent Beijing strains in Iran. In this regard, this deletion marker can be used in identification, phylogeny, and molecular epidemiology of the Beijing genotype.

**Keywords:** *Mycobacterium tuberculosis*; Region of differences; Iran

### Introduction

Tuberculosis (TB) is recognized as a major public health problem in nearly all parts of the world. According to the World Health Organization (WHO), among 8.7 million incident case of TB in year 2011, 7.7% occurred in the Eastern Mediterranean Region [1]. Iran is among the leading countries in implementation of DOTS strategies in this region and has a moderate incidence of TB (21 cases per 100'000 population) [2]. However, bordering with high-TB burden countries (Afghanistan and Pakistan) and increasing rates of drug resistance TB, threaten the control strategies [3,4].

Since the discovery of polymorphic DNA, molecular typing of *Mycobacterium tuberculosis* (MTB) strains has become a valuable tool for control of disease. Until now, several genotyping methods for MTB strains exist, of which *IS6110*-restriction fragment length polymorphism (RFLP), spoligotyping and variable number tandem repeat (VNTR) were among the most commonly used [5,6]. Recently, MTB strains were identified by a number of insertion or deletion events in specific genomic regions known as regions of differences (RDs) [7,8]. Among these RDs, deletions in RD1, RD150 and RD181 were mostly associated with drug resistant strains i.e. Beijing lineage strains [9]. Previously, the association between these set of RDs deletions and a family of MTB strains have been reported by several studies [9-12]. However, whether these regions have an epidemiological significance remains to be investigated. Therefore, this study was designed to determine the frequency of RD deletions in a collection of MTB strains in Iran and to examine the potential power of these RDs for genotyping analysis.

### Materials and Methods

#### Setting and study population

This study was conducted over a one year of period (from March 2011 to February 2012) in the only national TB center in Iran. The study was cross-sectional that was performed single blinded. A total of 190 patients with clinical signs and symptoms of TB (107 male and

83 female) were included in the study after a signed informed consent. This survey was approved by the local Ethics Committee.

#### Isolation of MTB

Samples from each patient were decontaminated by Petroff's method and were inoculated into Lowenstein-Jensen (LJ) media (Merck) [13]. The slope cultures were incubated at 37°C and examined for growth once weekly up to 6 weeks. Each isolate was examined regarding morphology, pigmentation and date of growth. Bacterial isolates identified as MTB complex using standard spoligotyping analysis [6,14].

#### Drug susceptibility testing

Drug susceptibility was determined by the proportion method on Lowenstein-Jensen medium using the critical concentrations for RMP (40 µg/ml), INH (0.2 µg/ml), ethambutol (EMB) (2 µg/ml) [15].

#### DNA extraction

QIAGEN DNA extraction kit was used for DNA extraction with the manual procedure recommended in kit [16]. Quality and quantity of DNA was obtained with Picodrop spectrometer.

#### Spoligotyping

Spoligotyping was performed for all MTB isolates as previously

**\*Corresponding author:** Mohammad Javad Nasiri, Mycobacteriology Research Centre, NRITLD, Shahid Beheshti University of Medical Sciences, Tehran, 19556, P.O: 19575/154, Iran, Tel: 98-21-29901; E-mail: [mj.nasiri@sbm.ac.ir](mailto:mj.nasiri@sbm.ac.ir)

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described [6]. Template DNA was amplified by PCR using specific primers as described by Kemerbeek et al. [6]. The PCR product was hybridized to 43 immobilized oligonucleotides derived from the spacer sequences of MTB H37Rv and M.bovis BCG P3. Presence of spacer sequences in the direct repeat region was detected by reversed-line blot hybridization. Hybridized DNA was detected by enhanced chemiluminescence (ECL, Amersham, UK) and by exposing ECL-Hyper film (Amersham). The obtained spoligo patterns were compared with the SPOLB4 Database.

### Determination of RD deletions

Extracted DNA was amplified by PCR using the primers indicated in Table 1. The target DNA was amplified in a 50 µl mixture containing 1× reaction buffer (50 mM KCl, 10 mM Tris-HCl (pH 9.0), 2 mM MgCl<sub>2</sub>), 2.5 mM of dNTPs, 2U of *Taq* DNA-polymerase (mi Taq, Metabion, Martinsried, Germany), and 20 pmol of each primers. Amplification was performed in an automated thermal cycler (Eppendorf, Germany) with the following conditions: Initial denaturation at 95°C for 3min, followed by 40 cycles of denaturation for 30 sec at 94°C, annealing for 1 min at 65°C, and extension for 90 sec at 72°C and an additional extension step at 72°C for 10 min. The PCR products were electrophoresed in 1.5% agarose gels and visualized in a UV Trans illuminator.

### Statistical analysis

Statistical analysis was carried out using SPSS statistical software, version 18 (SPSS Inc., Chicago, IL, USA).

## Results

### Drug susceptibility testing

Of 190 MTB isolates for whom drug susceptibility testing was performed, 30 (16%) were multidrug resistance (MDR), 12 (7%) were isoniazid (INH) resistant, 8 (4%) were ethambutol (EMB) resistant and 5 (3%) were rifampicin (RMP) resistant. The remaining 135 (70%) isolates were pan-susceptible.

### Spoligotyping

According to the spoligotyping patterns, 138 (73%) isolates were classified as shared types according to international database. The remaining 52 (27%) isolates generated unknown spoligopatterns. As shown in Table 2, H4 (37.8%) family was the most prevalent spoligotype

followed by CAS (19.4%), T1 (9.4%) and Beijing (5.7%) strains. Out of 11 Beijing strain lineages analyzed in the current study, 55% were susceptible to the mentioned drugs and 36% were MDR (Table 3).

### RD deletions in different MTB spoligotypes

As shown in Figure 1, RD PCR results showed no discriminative diversity in locus RD1. The genomic allelic diversity in RD181 region was only discriminative in 11 samples which all were Beijing. Results are mentioned in Figure 2. Furthermore no discriminative allelic diversity in genomic region of RD150 was observed. The spoligotypes and distribution of RD deletions in the RD1, RD150 and RD181 regions were determined for all studied strains (Table 4).

## Discussion

RDs have been described in numerous surveys as important markers for molecular epidemiology of TB [9]. The present study was aimed to explore the epidemiological significance of highly polymorphic regions (RD1, RD150 and RD181) among MTB strains.

Based on SIT from SITVIT2, the major identified isolates of MTB were characterized with Haarlem (n=72, 37.8%), CAS (n=37, 19.4%), T (n=18, 9.4%) and Beijing (n=11, 5.7%) lineages (Table 2). These strains have also been described as the most prevalent MTB families in Iran [17-19].

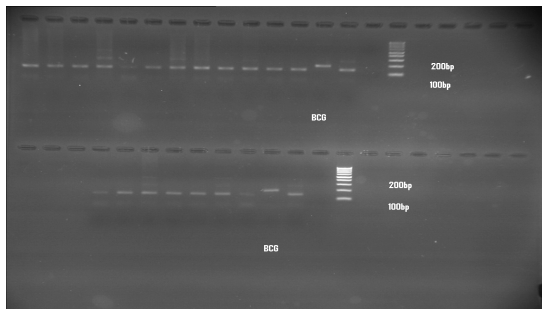
Recently, it was shown that deletions in specific RDs are significantly associated with particular genotypes of MTB. Kanji et al. showed that deletions in the CAS lineages were limited to RD149, RD152 and RD750 [20]. They also suggested that strains with these kinds of deletions are a prominent cause of TB among Asians ethnics. Furthermore, deletion in RD750 appears to have increased the capacity of CAS genotypes to disturb immune responses and contribute to its persistence in host [20,21]. In the case of Beijing lineage, it was reported that patients infected by Beijing subtype with simultaneous deletions of RD150 and RD181 were more likely to develop severe form of TB [22]. Similarly, in the survey conducted by Tsolaki et al., deletion in RD105, RD142, RD150 and RD181 was significantly associated with Beijing genotypes [9]. In our study, only RD181 deletion was found in all the Beijing strains and was highly discriminative (Table 4). Indeed, the lack of association between Beijing strains and other RD regions (i.e. RD 150) could be explained by the small number of Beijing samples analyzed in this study. The Beijing family was first identified in 1995 as

Locus	Primers	PCR Product size	References
RD1	ET1: 5'AAG CGG TTG CCG CCG ACC GAC C 3' ET2: 5'CTG GCT ATA TTC CTG GGC CCG G3' ET3: 5'GAG GCG ATC TGG CGG TTT GGG G3'	1.5 kb	Kanji et al. [20]
RD150	F: 5'TGT GGC GTG GCT CGG CAA ATA G3' R: 5'CGG GAC GGC AAA CGG GTG AT3'	2.5 kb	Tsolaki et al. [9]
RD181	F: 5'CGC AAC GGC CGC GGT GAA CTC T3' R: 5'CGG GCG GCT GCG GGA ACC TT3'	0.7 kb	Tsolaki et al. [9]

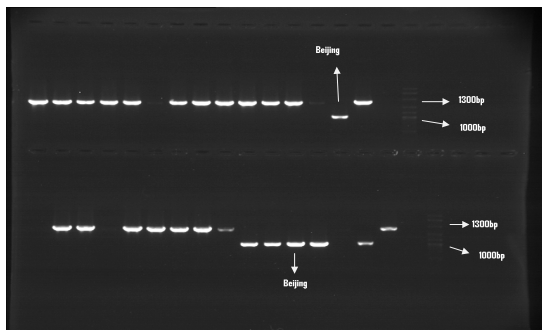
Table 1: Primer Sequences for the detection of RD1, RD150 and RD181.

Frequency	Genetic group	Spoligo pattern	Strain
72 (37.8%)	2,3		H4
37 (19.4%)	1		CAS
18 (9.4%)	2,3		T1
11 (5.7%)	1		Beijing
52 (27.3%)	-	-	Unknown
190 (100%)	-	-	Total

Table 2: The spoligotyping pattern of MTB strains.



**Figure 1:** Electrophoresis fractionation of PCR products in 3% agarose gel regarding existing deletion in RD1. The deletion can well be observed in BCG. Molecular weight marker (100 bp ladder) was used for RD1.



**Figure 2:** Electrophoresis fractionation of PCR products in 1% Agarose gel to detect existing deletion in RD181. The deletion can be diagnosed in the genomic region of RD181 in Beijing strains. PCR products molecular weight was 1000 bp. Molecular weight marker (100 bp ladder) was used.

Families	Susceptible	MDR	INH	ETB	RF	Total
H4	64 (88%)	3 (4.5%)	2 (3%)	-	3 (4.5%)	72 (100)
CAS	29 (76%)	3 (9%)	3 (9%)	1 (3%)	1 (3%)	37 (100)
T1	14 (78.5%)	3 (14%)	1 (7.5%)	-	-	18 (100)
Beijing	6 (55%)	4 (36%)	1 (9%)	-	-	11(100)
Unknown	25 (47.5%)	6 (10.5%)	11 (21%)	5 (10.5%)	5 (10.5%)	52 (100)

**Table 3:** Drug resistant patterns of MTB strain.

Locus	RD1	RD181	RD150
H4	-	-	-
CAS	-	-	-
T1	-	-	-
Beijing	-	100%*	-

\*RD181 deletion was found in all Beijing strains

**Table 4:** RD1, RD150 and RD181 deletions in MTB strains.

a closely related group of tubercle bacilli in China, but soon after they have been described by many countries throughout the world [23-26]. Likewise, the prevalence of Beijing family in Iran showed a significant increase in recent years [17,27]. According to the Farnia et al. [], the Beijing strains were among the major identified super families in Iran [17]. Furthermore, they indicated that more than 50% of the Beijing genotypes were isolated from Afghans and there was a linkage patterns between these cases and Iranian patients. This lineage has also been shown as one of the predominant strain in the Asian countries where TB is endemic, i.e. Afghanistan and Pakistan. These countries shares long geographical borders with Iran and its ethnic groups frequently

move inside the borders of Iran [28]. Consequently, long borders and frequent movement of ethnic groups may be one of the factors to explain the increasing prevalence of the Beijing super family in Iran. In the other hand, Beijing lineage strains have been found to be associated with severe form of disease, global transmission and drug resistance [4,22]. Therefore, early diagnosis of this kind of strains is one of the most important priorities for TB control strategies. Our result indicated that RD181 deletion can be a useful marker for detection of the Beijing lineage strain. In addition, Beijing families can easily be identified by RD assay (a PCR-based method) as compared with spoligotyping.

In conclusion, deletion in RD181 was prominent among the prevalent Beijing strains in Iran. In this regard, this deletion marker can be used in identification, phylogeny, and molecular epidemiology of the Beijing genotype. However, further analysis with large number of strains would be necessary to elucidate the discriminative power of these genetic regions in *M. tuberculosis* genotyping.

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