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

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**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 Picocheck spectrometer.

**Spoligotyping**

Spoligotyping was performed for all MTB isolates as previously described.

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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-Hyperfilm (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 MgCl2, 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 3 min, 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 resistant (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 Beijing family was first identified in 1995 as (i.e. RD 150) could be explained by the small number of Beijing samples and the lack of association between Beijing strains and other RD regions 

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 RD165, 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

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

| Locus | Primers | PCR Product size | References |
|-------|---------|------------------|------------|
| RD1   | ET1: 5’AAG CGG TTG CCG CGG ACC GAC C3’ ET2: 5’CTG GCT ATA TTC GTG GCC CGG G3’ ET3: 5’GAG GCG GAC ATC TGG CGG TTT GGG G3’ | 1.5 kb | Kanji et al. [20] |
| RD150 | F: 5’TGT GGC GTG GCT GCG CAA ATA G3’ R: 5’CGG GAC GGC ACC AAA CGG GTG AT3’ | 2.5 kb | Tsolaki et al. [9] |
| RD181 | F: 5’CGG AAC GGC CGG GGT GAA CTC T3’ R: 5’CGG CGG GCT GCG GGA ACC TT3’ | 0.7 kb | Tsolaki et al. [9] |

**Table 2:** The spoligotyping pattern of MTB strains.
RD181 deletion was found in all Beijing strains. Long geographical borders with Iran and its ethnic groups frequently shown as one of the predominant strain in the Asian countries where between these cases and Iranian patients. This lineage has also been genotypes were isolated from Afghans and there was a linkage patterns [17]. Furthermore, they indicated that more than 50% of the Beijing Beijing strains were among the major identified super families in Iran. Likewise, the prevalence of Beijing family in Iran showed a significant have been described by many countries throughout the world [23-26].

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

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

| Strain | RD181 | RD150 |
|--------|-------|-------|
| H4     | -     | -     |
| CAS    | -     | -     |
| T1     | -     | -     |
| Beijing| 100%* | -     |

*RD181 deletion was found in all Beijing strains.

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