

# Genetic diversity assessment of the Mycobacterium tuberculosis isolates of patients with tuberculosis in Karaj, Iran

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## Research

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# Abstract

## Background

Tuberculosis (TB) is an old issue that is presently measured as a significant challenge. Molecular characterization of *Mycobacterium tuberculosis* has contributed to TB control. The present survey was aimed to assess the genetic diversity of *M. tuberculosis* strains isolated from patients with TB in Karaj, Iran.

## Methods

Seventeen *M. tuberculosis* isolates from 2012 to 2013 were collected and subjected to an IS6110 restriction fragment length polymorphism (IS6110-RFLP) analysis. Demographically, 6 females and 14 males who had Iranian citizenship were included in this study.

## Results

Sixteen different genetic types were obtained after enzymatic digestion and RFL analysis. Copy numbers of IS6110 in each isolate ranged from 0 to 12. The majority of isolates (66%) harbored copy numbers between 6 and 12. Each isolates harbored 6.9 copies of the IS6110 marker. Nine isolates harbored 10 to 12 copies of the IS6110 marker, 5 isolates harbored 6 to 10 copies, and 2 others harbored copies less than 6. No copy of IS6110 was found among the 4 isolates. No relationship was found between gender and copy numbers.

## Conclusions

The high genetic diversity found amongst the *M. tuberculosis* isolates maybe show different sources of infection and the importance of reemerging of the TB. However, further surveys should perform to assess other molecular epidemiologic aspects of *M. tuberculosis* in Iran.

## Background

Tuberculosis (TB) is a worldwide health threat globally. According to the World Health Organization (WHO) report, there were 10.0 million new TB cases (5.6 million men, 3.2 million women, and 1.2 million children), which resulted in 1.4 million deaths in 2020 [1]. Despite an auspicious decline in the incidence of TB in Iran from 36 cases per 100,000 people in 1990 to 17 cases per 100,000 people in 2010 [2], TB endures being an important public health threat among Iranians [3].

TB is a complicated and polymorphic disease [4, 5]. Various combination therapy is available to treat TB [6]. However, there is an emergence of multidrug-resistant TB (MDR-TB) [7]. Presently, the MDR-TB has a

cumulative incidence rate globally. Additionally, MDR-TB is a significant public health threat in TB control and prevention [8]. Thus, it is essential to use rapid and sensitive TB diagnostic methods and assess the new epidemiological aspects of TB in an area with a high incidence rate [9, 10].

Genotyping of *M. tuberculosis* strains using IS6110 restriction fragment length polymorphism (IS6110-RFLP) is considered a practical approach in TB molecular epidemiology [11, 12]. Such genotyping helps TB control by allowing the unpredicted outbreaks identification and laboratory cross-contamination, and it helps discriminate exogenous reinfection from endogenous reactivation, in that way permitting an understanding of the strain's genetic diversity [13].

According to the high incidence rate of TB among Iranians and uncertain status of the genotyping patterns of isolates in some area of Iran, the present survey was aimed to assess the molecular features of the *M. tuberculosis* isolates from the sputum samples of patients referred to the Alborz tuberculosis center using the RFLP method.

## Materials And Methods

### Ethical issues

Written informed consent was taken from volunteer patients or their parents. The relevant authorities issued ethical approval for this retrospective genotyping work at the Alborz University of Medical Sciences Ethics Committee. The present survey principles were ethically approved by the Department of Tuberculin & Mallein, Razi Vaccine & Serum Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Karaj, Iran.

### Samples and preparation

Twenty sputum samples were taken from randomly selected 20 pulmonary TB-suspected outpatients. These were visiting patients to the university hospitals of Alborz from December 2012 to October 2013. Patients' demographic data were collected, and their consent through questionnaires filled out at the clinic when specimens were taken. Samples were immediately transferred to Razi Vaccine & Serum Research Institute, Karaj, Iran, for further analysis. Samples were subjected to Petroff's protocol for digestion/decontamination.

### *M. tuberculosis* culture

Processed samples were used to inoculate slopes of plain (glycerinated) and Lowenstein–Jensen medium (LJ) supplemented with sodium pyruvate and glycerol (Merck, Germany). Cultures were incubated at 37°C for 12 weeks. The weekly inspection was performed to found any bacterial growth. The acid-fast staining was used for the microscopic assessment of cultures with visual features of *M. tuberculosis* complex (MTC) [14]. All principles were performed according to the instructions of the Department of Tuberculin & Mallein, Razi Vaccine & Serum Research Institute, Karaj, Iran. Identification of

*M. tuberculosis* isolates was performed using the PCR amplification of the 16S rRNA gene and IS6110 marker [15, 16].

## DNA extraction and quality assessment

For DNA extraction, 3 loopful colonies of *M. tuberculosis* were scraped, transferred to a microtube that contained 400µl Tris-ethylenediaminetetraacetic acid (EDTA) buffer (pH 8) inactivated by heating using a water bath (80°C for 30 min). Then, 50µl of 10mg/mL lysozyme was added, vortexed, and incubated overnight (37°C). Sodium dodecyl sulfate (SDS)/proteinase K (70µl of 10% [w/v] SDS and 5µl of 10mg/mL of proteinase K) were added to tubes vortexed and incubated at 65°C for 10 min. One-hundred microliters of 5M NaCl and *N*-cetyl-*N,N,N*-trimethylammonium bromide (CTAB)-NaCl solution (4.1g of NaCl and 10 g of CTAB per 100mL) was added and incubated at 65°C for 10 min. An equal volume of chloroform: isoamyl alcohol (24:1v/v) was added to the mixture. After centrifugation for 5 min, 0.6 volumes of isopropanol were added to the supernatant liquid to precipitate the DNA. After incubation for 20 min at -20°C and centrifugation for 15 min at 12,000 × g and 4°C, the pellet was washed once with 70% (v/v) ethanol, and the air-dried pellet was dissolved in 20µL of Tris-EDTA buffer [17]. Purity (A260/A280) and extracted DNA concentration were then checked (NanoDrop, Thermo Scientific, Waltham, MA, USA). The DNA's truth was assessed on a 2% agarose gel stained with ethidium bromide (0.5 µg/mL) (Thermo Fisher Scientific, St. Leon-Rot, Germany) [18, 19].

## Restriction fragment length polymorphism (RFLP) analysis

DNA fingerprinting was performed by a standardized procedure described previously (). Briefly, chromosomal DNA (2-3.5 µg) was restricted with PvuII at 37°C overnight. The restriction fragments were separated overnight by 1% (w/v) agarose gel electrophoresis in 1× Tris-borate-EDTA buffer. The fragments were transferred onto positively charged nylon membranes using the capillary method. RFLP analysis was performed using an internationally standardized procedure as described previously [20]. The IS6110 fingerprint patterns were analyzed using GelCompar II software version 6.5 (Applied Maths, Sint-Martens-Latem, Belgium).

## Results

Patients of the present study consisted of 6 females and 14 males who had Iranian citizenship. According to the patients' files, all sputum samples were received from the patients.

All *M. tuberculosis* isolates and the standard control (*M. tuberculosis* H37Rv strain) were examined for genomic fingerprinting and polymorphism in Alborz province. Due to technical reasons, including the quality of the genomic material extracted from the sample, DNA extracted from 20 isolates was suitable for this test. In this study, 17 isolates were enzymatically digested by Pvu II enzyme and assessed in the RFLP. Figure 1 shows the RFLP pattern of *M. tuberculosis* isolates using the Pvu II enzyme.

Visual comparison of high molecular weight bands (2027 to 23130 bp) and using Gel Pro software (analysis based on the number and size of bands), from 20 strains digested with Pvu II enzyme, after

RFLP and hybridization with the probe IS6110 and detection, 16 different genetic types were obtained (Fig. 2), which were named HPIS1 to HPIS12 and identified, and the number of unique genotypes in this method was determined to be 16.

The number of copies of IS6110 in each isolate ranged from 0 to 12. However, most isolates (66%) had copy numbers between 6 and 12. On average, each isolate had 6.9 copies of IS6110. 9 strains containing 10 to 12 copies of IS6110 5 strains containing 6 to 10 copies, and 2 strains had copies less than 6. No copy of IS6110 was found among the 4 strains. No relationship was found between gender and copy numbers.

## Discussion

The molecular epidemiology of *M. tuberculosis* was assessed in different parts of Iran [21–23]. Similarly, the molecular epidemiology of *M. tuberculosis* was assessed in other countries, including United States [24], Poland [25], Belgium [26], China [27], Brazil [28], Africa [29], India [30], Saudi Arabia [31], and Russia [32]. They reported some similar genetic diversity according to the applied methods and source of the infection.

The present study showed 17 different genetic types in the *M. tuberculosis* strains isolated from patients in Karaj, Iran, according to the RFLP-IS6110 findings. This result showed the remarkable genetic diversity of this pathogen in Alborz province, Iran. Increasing the number of isolates in future supplementary researches is likely to increase the observed polymorphism even higher than current values. The active cases of tuberculosis in this province are probably due to the infection's reactivation, and the recent transmission has a much lesser role. In the present study, a large percentage of isolates had copy numbers between 6 and 12, which the average number of copies of IS6110 marker was similar to the results of the study of Doroudchi et al. (2000) [33]. In many studies worldwide [34–36], some *M. tuberculosis* isolates did not have the IS6110 component. In the present study, 4 isolates did not have a copy of IS6110 marker. In another study in Vietnam [37], RFLP-IS6110 and Mycobacterial Interspersed Repetitive Unit Variable Number Tandem Repeat (MIRU-VNTR) techniques were used to assess the genetic characters of 2664 isolates. There was a great variety of isolates in both techniques resulting in the recurrence of latent tuberculosis infection in Vietnam. Additionally, many isolates from Vietnam did not contain any copy of the IS6110 marker, which was less resistant to antibiotics, especially streptomycin. Various researches reported that the IS6110 is the most common genetic marker for identifying and genetic identification of *M. tuberculosis* with a high resolution for DNA fingerprinting of isolated strains in the study population [38, 39]. Therefore, this fragment can be the basis for future molecular epidemiological studies on TB. The high genetic diversity of IS6110 has been reported in previous Iranian studies [40–44]. The high genetic diversity of IS6110 in different strains of *M. tuberculosis* probably indicates that most people with different origins have been infected. As a result, the infection's reactivation has played a more significant role in spreading tuberculosis in Iran. Although the disease's activation due to patients' infection in the years before the onset of the disease has been

introduced as an important factor explaining this epidemiological finding, the definitive causes or reasons for this condition remain unknown.

Due to the limited number of samples studied in the present study, it is impossible to comment with certainty on mycobacteria other than the *M. tuberculosis* complex without the IS6110 sequence in Alborz province, Iran. So, in this case, more study is needed. Regarding the diagnosis of atypical mycobacteria in Iran, only one report was published in 2009, which showed that 43 out of 371 samples (11.5%) were diagnosed as non-tuberculous mycobacteria [45].

## Conclusion

In conclusion, high genetic diversity was found amongst the *M. tuberculosis* strains isolated from patients in Alborz province, Iran, according to the IS6110-RFLP technique. A total of 17 strains were identified in RFLP. This molecular technique is acknowledged as a first-line assay in the molecular epidemiology assessment of *M. tuberculosis*. Findings revealed that *M. tuberculosis* is the leading cause of tuberculosis in the region. Given the patients' age and the genetic diversity of the strains, the assumption of hemoplasia is raised. In the end, this research can be considered as a good background for future studies in the region, comparing the obtained results with each other and better analysis of the epidemiological situation of tuberculosis in Alborz province, Iran.

## Declarations

### Ethics approval and consent to participate

Written informed consent was taken from volunteer patients or their parents. The relevant authorities issued ethical approval for this retrospective genotyping work at the Alborz University of Medical Sciences Ethics Committee. The present survey principles were ethically approved by the Department of Tuberculin & Mallein, Razi Vaccine & Serum Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Karaj, Iran.

### Consent for publication

Not applicable.

### Availability of data and materials

All data analyzed during this study are included in this published article.

### Competing interests

The authors declare that they have no competing interests.

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### **Authors' contributions**

MSF, HM and KT performed the molecular analysis, designation of primers, and drafting the manuscript. YK and NM performed the sampling and culture. HM and KT contributed in the designation of research, statistical analysis and writing of the manuscript. All authors read and approved the final manuscript.

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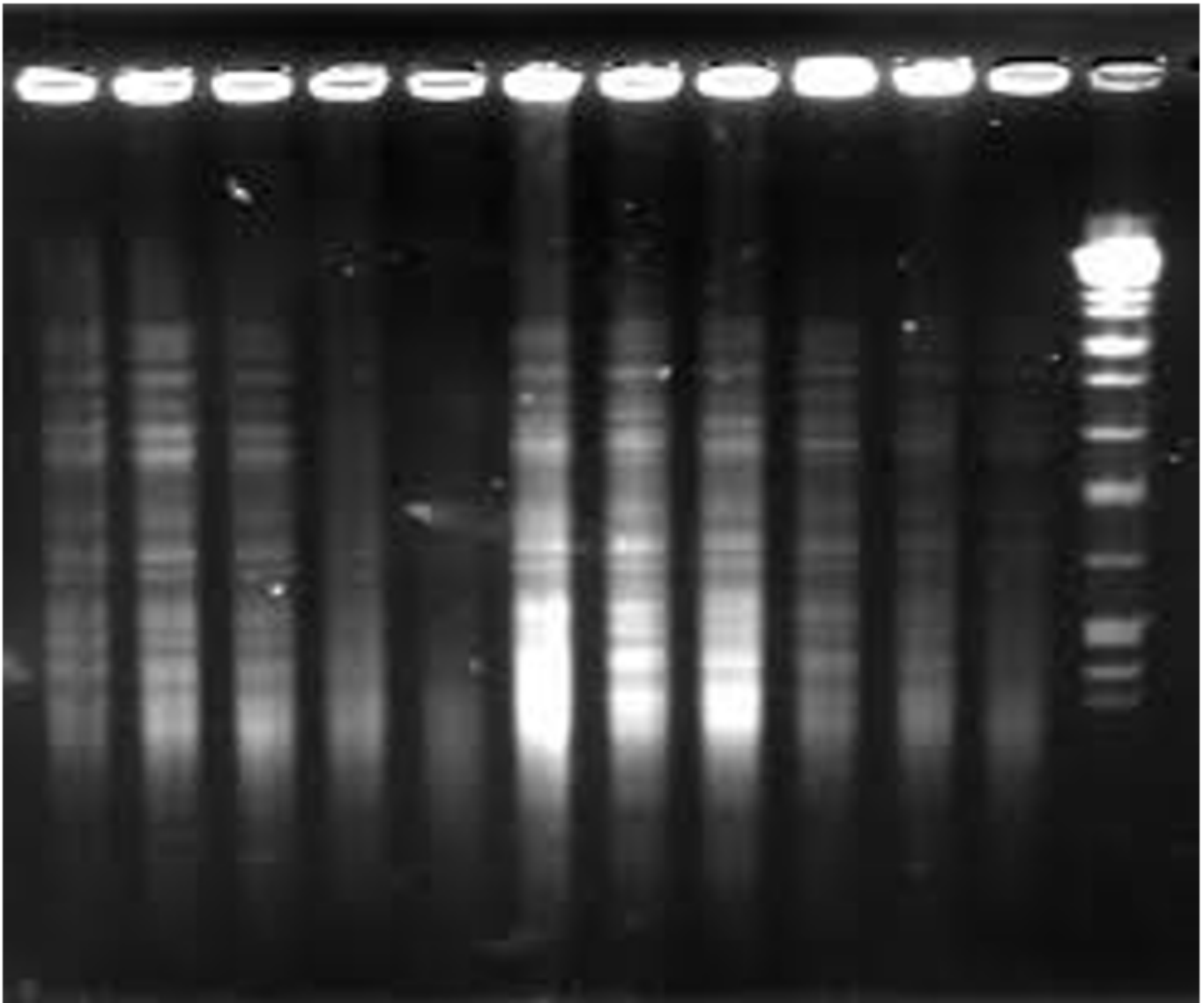
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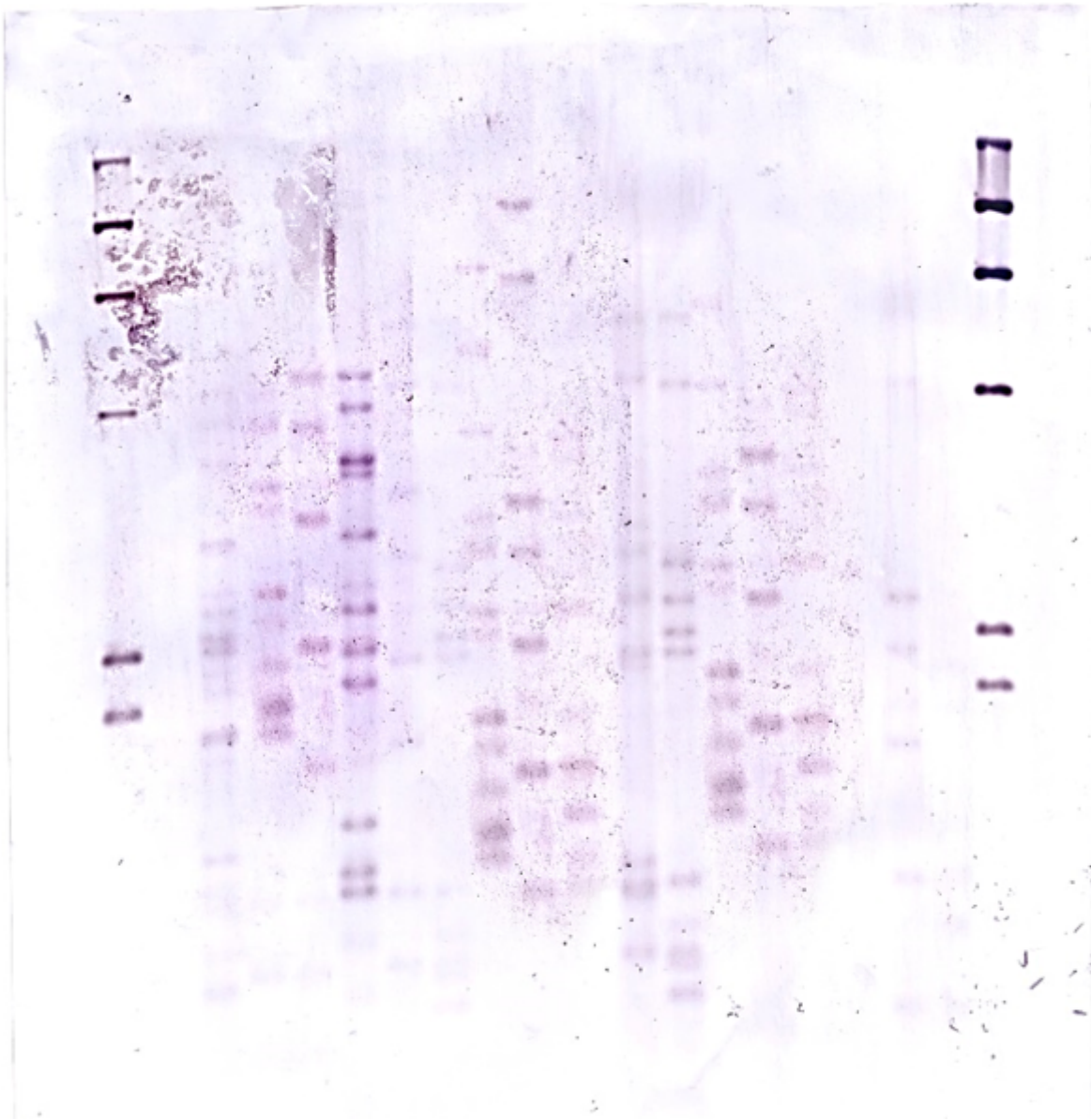
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## Figures



**Figure 1**

RFLP pattern of *M. tuberculosis* isolates using the Pvu II enzyme.



**Figure 2**

Findings of the RFLP test based on IS6110 markers and Pvu II enzyme.