ORIGINAL ARTICLE

# Genotyping and drug susceptibility testing of *Mycobacterium tuberculosis* in Iran: a multi-centre study

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## Abstract

Tuberculosis (TB) is a deadly infection and caused 1.4 million deaths in 2018. Assessing the geographic distribution of major lineages of *Mycobacterium tuberculosis* can contribute greatly to TB control. Mycobacterial interspersed repetitive unit variable number tandem repeat (MIRU-VNTR) typing is commonly used to differentiate various lineages of *M. tuberculosis*. A total of 2747 clinical specimens were collected consecutively from October 2018 through June 2019. Clinical isolates were identified as *M. tuberculosis* using standard biochemical tests. The standard 15-locus MIRU-VNTR typing was used for the genotyping of clinical isolates. Drug susceptibility testing was performed using the conventional proportion method. From the collected specimens, 100 were culture positive for *M. tuberculosis*. Using MIRU-VNTR, 99 different patterns were detected among the 100 isolates. They were distributed in one cluster comprising two strains and 98 unique patterns. Most of our isolates were similar to New-1 and Delhi/CAS strains. Of the *M. tuberculosis* isolates, 83 (83.0%) were pan-susceptible and 17 (17.0%) were resistant to at least one drug. Our study showed that MIRU-VNTR is a useful method for studying the genetic diversity of *M. tuberculosis* isolates in different regional settings and will help the health authorities to construct a preventive programme for TB.



**Keywords:** Iran, MIRU-VNTR, mycobacterial interspersed repetitive unit variable number tandem repeat, tuberculosis, typing
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## Introduction

Tuberculosis (TB) is the major cause of morbidity and mortality worldwide, especially in developing countries. In 2018, the death rates from TB among human immunodeficiency virus-negative and -positive individuals were 1.2 million and 251 000, respectively [1]. Based on the WHO report in 2018, the TB incidence rate in Iran was estimated at 14 per 100 000 population [1]. All neighbouring countries, including Turkey, Iraq, Turkmenistan, Pakistan and Afghanistan, showed higher TB incidence rates than Iran [1]. An important threat of TB is the emergence of drug-resistant strains, especially multidrug-resistant TB [2,3].

Genotyping methods have increasingly been applied to evaluate the epidemiology and transmission dynamics of *Mycobacterium tuberculosis* [4–7]. In addition, these methods could be useful in long-term supervision, outbreak studies and tracing of laboratory contaminations [8–11]. These techniques can also distinguish between new and old *M. tuberculosis* infections and can detect the source of infection [12,13].

There are various types of methods for genotyping studies including *IS6110*-restriction fragment length polymorphism, spoligotyping and single nucleotide polymorphisms [14]. One of the commonest genotyping techniques is mycobacterial interspersed repetitive unit variable number tandem repeat (MIRU-VNTR), which has been widely used for epidemiological studies



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[5,15]. Recent TB research and programmes around the world have provided a large database of various *M. tuberculosis* isolates using MIRU-VNTR typing [16]. In addition, as a result of its high discriminatory power, reproducibility and affordable cost, MIRU-VNTR is used routinely for genotyping of *M. tuberculosis* strains in many settings [17,18]. The aim of this study was to analyse the geographical distribution and study the phylogenetic relationship of *M. tuberculosis* strains collected at the Tehran regional TB reference laboratory (TRTB-RL).

## Materials and methods

### Setting and samples

This cross-sectional study was performed at the TRTB-RL from October 2018 through June 2019. TRTB-RL is well-equipped with biosafety level III facilities for drug susceptibility testing. TRTB-RL acts as a regional centre for TB laboratories, and clinical samples from different provinces of Iran transfer their samples to this laboratory for further identification and drug susceptibility testing. A total of 2747 clinical specimens were consecutively collected from individuals with suspected TB who were referred to the TRTB-RL from different provinces of Iran. These individuals had clinical signs and symptoms of TB and were undergoing examination for possible active TB. The study was approved by the ethics committee of Shahid Beheshti University of Medical Sciences, Tehran, Iran (IR.SBMU.MSP.REC.1398.285).

### Microscopy examination and identification of *M. tuberculosis*

Clinical specimens were processed by the standard sodium hydroxide method and smears were prepared by the Ziehl–Neelsen staining method [19]. After decontamination, specimens were inoculated to the Löwenstein–Jensen medium. For the identification of mycobacteria, the slope cultures were incubated at 37°C and examined for growth once weekly up to 6 weeks. Bacterial isolates were identified as *M. tuberculosis* using standard biochemical tests (i.e. production of niacin, nitrate reduction, catalase) [19,20].

### Drug susceptibility testing

Drug susceptibility testing for isoniazid, rifampicin and ethambutol was performed using the conventional proportion method on Löwenstein–Jensen medium [19]. For pyrazinamide, a modified proportion method was used as described previously by Heifets et al. [21]. In this modified proportion method we used Middlebrook 7H10 agar-based medium (Becton Dickinson and Co., Cockeysville, MD, USA) for preparing the growth conditions.

### VNTR typing

The DNA was extracted using a previously described method [22]. Molecular typing was performed using the MIRU-VNTR typing method. The standard 15-locus MIRU-VNTR typing method was performed as described by Supply et al. [23].

### Analysis of VNTR allelic diversity and genetic relationships

All results were entered into Excel® spreadsheets and analysed by MIRU-VNTRplus software [24]. The allelic diversity of each VNTR locus was calculated by the Hunter–Gaston discriminatory index, and genetic relationships among the isolates were estimated using the categorical coefficient and UPGMA [24]. Cluster definition was based on a distance cut-off of 0 and similar patterns in 15-locus typing [25].

### Database comparison

The 15-locus MIRU-VNTR patterns were compared with the patterns from the MIRU-VNTRplus database to determine *M. tuberculosis* strain lineages and relatedness [26]. Distribution of identified lineage was done by similarity search with reference strains.

## Results

### Mycobacterial isolates

During the study period, a total of 2747 specimens from suspected TB cases were collected, of which 1734 (63.1%) were pulmonary (bronchoalveolar lavage fluid, tracheal, sputum) and 1013 (36.9%) were extrapulmonary (urine, abscess, osteoarticular, biopsy, pericardium, cerebrospinal fluid, gastric lavage, blood and ascites). From the collected specimens, 100 were culture positive for *M. tuberculosis*. More than 80.0% of the isolates were obtained from newly individuals with diagnosed TB. All the isolates were defined as *M. tuberculosis* according to growth rates, pigmentation properties of colonies, production of niacin, nitrate reduction and catalase.

### Drug susceptibility testing

Of 100 *M. tuberculosis* isolates, 83 (83.0%) were pan-susceptible and 17 (17.0%) were resistant to at least one drug. The majority

**TABLE 1. The drug-resistant patterns of the collected isolates**

| Type of resistance | No. of resistant isolates |
|---|---|
| Pan-susceptible | 83 |
| Mono-resistance (isoniazid-rifampicin-ethambutol-pyrazinamide) | 12 (8-1-3-0) |
| Multidrug resistance | 5 |


FIG. 1. Genetic relationships of isolates based on the UPGMA tree.

of isolates were isoniazid mono-resistant (8.0%) followed by ethambutol mono-resistant (3.0%) and rifampicin mono-resistant (1%). Multidrug-resistant TB was observed in five (5.0%) of the investigated isolates, respectively. The antibiotic-resistance patterns of the collected isolates are presented in Table 1.

### VNTR typing

Using MIRU-VNTR, 99 different patterns were detected among the 100 isolates. They were distributed in one cluster comprising two strains and 98 unique patterns. A comparison of the 15-locus MIRU patterns obtained with the international MIRU-VNTRplus database showed that most of our isolates were similar to New-1 and Delhi/CAS strains. The discriminatory power of MIRU-VNTR typing for all isolates was high (Hunter–Gaston discriminatory index 0.98). The dendrogram generated using the UPGMA algorithm based on the MIRU-VNTRplus database describes the genetic relationships of the 100 isolates (Fig. 1). As shown in the minimum spanning tree (Fig. 2), there were nine clonal complexes including 25 isolates.

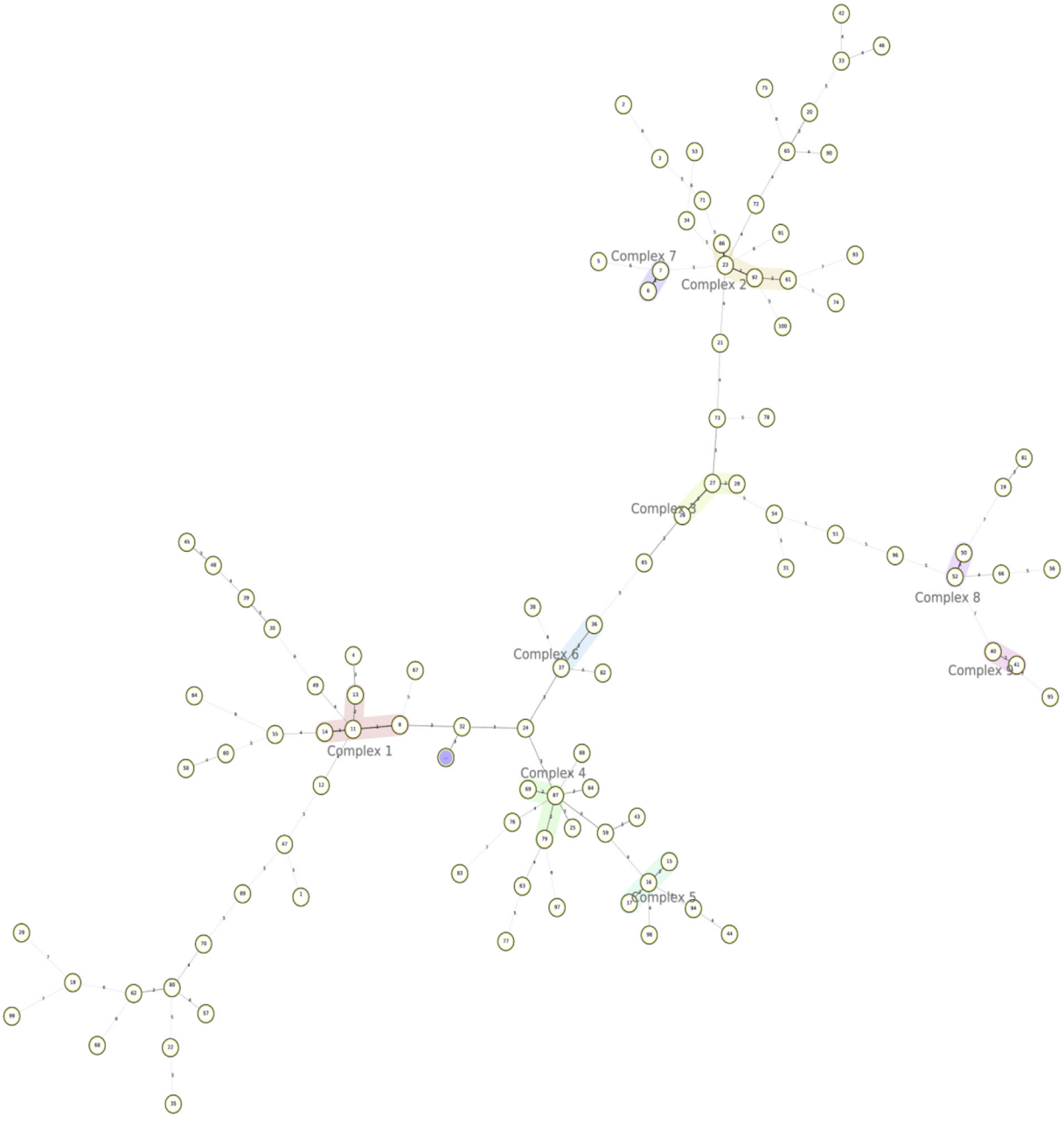


FIG. 2. The minimum spanning tree showed the nine clonal complexes.

These 25 isolates had different lineages and most of the isolates were from lineages NEW-1 (12 isolates) and Delhi/CAS (eight isolates).

## Discussion

A major problem in TB control programmes is the emergence of drug-resistant TB. Genotyping methods are useful tools for studying the genetic diversity of *M. tuberculosis* isolates and will help the health authorities to construct a preventive programme for TB.

The results of this study showed that the genetic population structure of the included isolates was heterogeneous, like other low-incidence countries, and 99 different patterns were detected among the 100 isolates [4]. Likewise, these results were compatible with previous studies about the genetic population structure of *M. tuberculosis* in Iran [27–29].

In our study 17 (17.0%) isolates were resistant to at least one drug and most of the included patients were newly infected individuals. Most of the resistant isolates (7 out of 17) belonged to NEW-1 lineage, which may be a result of a higher potential of mutation or rapid dissemination of this lineage.

It was noteworthy that the incidence rate of TB in Iran was moderate, whereas in neighbouring countries, including Pakistan, Afghanistan, Turkey, Iraq, Azerbaijan and Armenia, the rates are two to three times higher than in Iran. Multidrug-resistant TB rates are also higher in these countries [19,29,30]. Therefore, the genotypic diversity of *M. tuberculosis* in Iran might be a result of communication and trading with neighbouring countries. Tehran is the most populous city in Iran and it is the preferred destination for travellers and migrants from neighbouring countries, which could be the cause of the genetic diversity of TB in this study [29].

Analysing the geographical distribution of *M. tuberculosis* around the world showed that some lineages are more common in specific areas as a result of adaptation to specific human populations [31]. The TRTB-RL received clinical samples from nine provinces of Iran and our study showed that most of the *M. tuberculosis* isolates were similar to some of the major *M. tuberculosis* lineages, including New-1 and Delhi/CAS. Based on previous studies in Iran, most of the *M. tuberculosis* lineages were Ural, NEW-1, West African, Delhi/CAS, T and Latin American-Mediterranean (or LAM) [29,32]. These results indicate that lineages of New-1 and Delhi/CAS are more adapted to the Iranian population.

There were some limitations to our study. First, our isolates were not representative of the whole Iranian TB population. Second, as MIRU-VNTR requires isolation of *M. tuberculosis* by culture, some isolates were excluded from the study because of contamination in the culture media. Third, as a result of the limited demographic information, the epidemiological links between patients with the same and similar strains could not be determined.

In conclusion, our study showed that the genetic population structure of the included isolates was heterogeneous at the TRTB-RL. It also indicated that MIRU-VNTR is a useful method for studying the genetic diversity of *M. tuberculosis* isolates in different regional settings and can help the health authorities to construct a preventive programme for TB.

## Author contributions

MJN and SA conceived and designed the study; SMJM and SA performed the experiments; SMJM, MJN, GH and HG analysed the data; MJN, SMJM wrote the main manuscript text and MJN, MM and HD revised the manuscript.

## Conflict of interest

The authors declare no competing interests.

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