Original Article

Genetic Diversity of Drug-resistant *Mycobacterium tuberculosis* Isolates in Isfahan Province of Iran

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Abstract

Background: Increasing drug resistance is an important factor in the complexity of tuberculosis (TB) control. The identification of disease transmission type, recurrence of a previous infection, or new transmission of the disease is the key factor in the control of TB. In this study, we aimed to identify the genetic diversity of drug-resistant *Mycobacterium tuberculosis* isolates in Isfahan province of Iran through the mycobacterial interspersed repetitive unit-variable number tandem repeat (MIRU-VNTR) typing method based on 24 loci. **Materials and Methods:** Of 300 isolates obtained from a variety of clinical specimens, 18 drug-resistance *M. tuberculosis* clinical isolates (resistant to a single drug to more than one drug) were collected between 2013 and 2015 from regional TB reference laboratory in Isfahan. All drug-resistance *M. tuberculosis* isolates were typed by 24-locus MIRU-VNTR typing. **Results:** The highest percentage of isolates, 38.8%, belonged to the East-Asian lineage (lineage 2), while the lineages Indo-Oceanic (lineage 1), East-African–Indian (lineage 3), and Euro-American (lineage 4) represented 5.5%, 22.2%, and 33.3%, respectively. Among the 33.3% (6/18) Euro-American strains, the Latin American– Mediterranean and Ural sub-lineage were 22.2% (4/18) and 11.1% (2/18), respectively. **Conclusion:** The results of this study show that the lineages of drug-resistant *M. tuberculosis* isolates in Isfahan province of Iran are similar to those reported in the Eastern Mediterranean region (indicative of the epidemiological relationship between the countries in the region). Continued molecular monitoring is important as it has been proposed that the genetics and evolutionary backgrounds of drug-resistant *M. tuberculosis* strains may have an impact on the transmissibility profile.

Keywords: *Iran, mycobacterial interspersed repetitive unit-variable number tandem repeat, Mycobacterium tuberculosis, typing*

Introduction

Tuberculosis (TB) is a major global public health threat with 9.6 million new active cases and 1.5 million TB deaths in 2014[1] in spite of anti-TB drugs. Increasing drug resistance is an important factor in the complexity of TB control.[2] In terms of the prevalence of TB and multidrug-resistant TB (MDR-TB), Iran is ranked moderate, but being bordered by countries with a high prevalence of TB, including Afghanistan and Pakistan to the East, Iraq to the West, and the high prevalence of TB and MDR-TB in the Republic of Azerbaijan to the Northwest, makes it required to follow the transmission path of TB and control its prevalence. The identification of disease transmission type, recurrence of a previous infection (endogenous), or new transmission of the disease (exogenous) are the key factors in the control of TB. A key factor in controlling TB both at an international and national level is disruption of transmission chains.[3] Molecular typing of *Mycobacterium tuberculosis* complex is an important adjunct to TB control, for example, to confirm or detect outbreaks and to monitor the disease transmission. Most recently, there have been important advances in developing the molecular tools required for the rapid diagnosis of TB.[3] Analysis of mycobacterial interspersed repetitive unit-variable number tandem repeat (MIRU-VNTR) sequences for genotyping strains of the *M. tuberculosis* complex has emerged as a valuable marker.[4] MIRU-VNTR typing is compared with IS6110 restriction fragment length polymorphism typing which has been shown to have similar discriminatory power.[4,5] The optimized 15- and 24-locus MIRU-VNTR typing system has been proposed for international standardization that provides reproducible and rapid results that are displayed as a 24- or 15-digit numerical code which allows for easy exchange of

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data.[3,6] More recently, MIRU-VNTR typing has been successfully used directly to smear-positive specimens and can be used to early mycobacterial cultures.[4,7] Previous studies have shown this method to be useful in comparing strains (i) among household contacts, (ii) at national and international levels, (iii) to determine the evolutionary pathway of TB, and (iv) associated with drug resistance.[4,5,8,9]

The objective of our study was to determine the genetic diversity of drug-resistant *M. tuberculosis* isolates by 24-locus MIRU-VNTR typing analysis in Isfahan province of Iran.

Materials and Methods

Bacterial isolates and drug susceptibility testing

In the present study, 300 isolates were obtained from a variety of clinical specimens at the regional TB reference laboratory in Isfahan from October 2013 to December 2015. All the isolates were confirmed by microscopic examination, culture, and nitrate and niacin tests. The drug susceptibility testing was performed on Lowenstein–Jensen (LJ) medium using a proportion method for rifampin (1 μg/mL), isoniazid (0/2 μg/mL), streptomycin (STM) (2 μg/mL), and ethambutol (5 μg/mL), according to the WHO guidelines.[10] *M. tuberculosis* H37Rv was used as control. MDR-TB was defined as *M. tuberculosis* isolate resistant to at least rifampin and isoniazid.

Genomic DNA extraction

M. tuberculosis genomic DNA was extracted through the cetyltrimethylammonium bromide (CTAB) method. One loopful of mycobacterial colonies subcultured on LJ medium was transferred to a microtube containing 400 μL of Tris-ethylenediaminetetraacetic acid (EDTA) buffer (10 mM Tris-Cl, 1 mM EDTA). The bacteria were inactivated by heating at 80°C for 30 min. Lysozyme 50 mg/mL (30 μL) was added, and the microtubes were incubated at 37°C overnight, after which 70 μl 10% sodium dodecyl sulfate and 10 μl proteinase K (20 mg/mL) were added to each microtube. The microtubes were vortexed and incubated for 15 min at 65°C. After adding 100 μl 5M NaCl and 100 μl CTAB/NaCl which were prewarmed at 65°C, the tubes were vortexed and incubated for 10 min at 65°C. Seven hundred microliter phenol/chloroform was added and the microtubes were inverted and centrifuged at 11,000 g, for 15 min. The upper phase was transferred to a new microtube. Five hundred microliter of isopropanol was added and the microtube was incubated at −20°C overnight. Then, the microtubes were centrifuged at 11,000 g, 30 min. The supernatant was discarded, and after evaporation of the alcohol, the pellet was resuspended in 50 μL of Tris-EDTA buffer. Concentrations and purity of extracted DNA were determined using ultraviolet-photometer (Biometra, Germany) at 260 and 280 nm. Purified DNA was stored at −70°C until polymerase chain reaction (PCR) experiments.

Molecular identification

The isolates were confirmed as *M. tuberculosis* by amplifying a 245-base-pair sequence of IS6110 using the primers INS-1 (5′CGTGAGGGCATCGAGGTGGC3′) and INS-2 (5′GCGTAGGCGTCGGTGACAAA3′) as described previously.[11] The reaction was carried out under this program: 94°C, 5 min; (94°C, 30 s; 65°C, 30 s; 72°C, 45 s) × 33; 72°C, 10 min; 4°C forever.

24-locus mycobacterial interspersed repetitive unit-variable number tandem repeat typing

The drug-resistance *M. tuberculosis* isolates were genotyped by PCR amplification of the original 24 MIRU-VNTR loci (Qub 26, Mtub 04, Mtub 34, Mtub 21, Mtub 30, MIRU 26, MIRU 10, ETRA, ETRB, MIRU 23, MIRU 31, MIRU 16, MIRU 40, Qub 11b, Mtub 39, MIRU 02, MIRU 39, ETRC, Qub 4156, MIRU 20, MIRU 04, Mtub 29, MIRU 24, and MIRU 27).

The primers and the conditions for their amplification are described previously.[4]

When each locus is amplified separately, the amplified fragments were analyzed by electrophoresis using 1.5%–2% agarose gels. By using the position of the size standard marker, the size of the amplified fragment was determined. Copy numbers were obtained by comparing the band sizes with an allele naming table for each tandem repeat locus made available in Microsoft Excel format on the MIRU-VNTR plus website (http://www.miru-vntrplus.org). The H37Rv was used as control for allele assignation.

Molecular data analysis

The final result of 24-locus MIRU-VNTR typing (MIRU-VNTR data) is a numerical code, corresponding to the repeat number in each VNTR locus. MIRU-VNTR data were analyzed using the tools available on the MIRU-VNTR plus website. The MIRU-VNTR patterns were compared with the MIRU-VNTR plus Database to discover *M. tuberculosis* lineages and relatedness.

The allelic diversity for each locus was determined using the Hunter and Gaston discriminatory, calculated using Simpson's index of diversity formula, as follows:[12]

$$D = 1 - \frac{1}{N\,(N-1)} \sum_{j=1}^{s} x_j\,(x_j - 1)$$

where, D is the index of discriminatory power, N is the number of unrelated strains tested, S correlates with the number of different types, and x_j corresponds to the number of strains belonging to the j^{th} type, considering that strains will be categorized into mutually exclusive groups.

Based on this index, the loci were designated as poorly discriminatory (Hunter–Gaston discriminatory index [HGDI] <0.3), moderately (HGDI 0.3–0.6), and highly (HGDI >0.6), as described by Sola *et al*.[13]

Results

Bacterial isolates and drug susceptibility testing

A total of 18 isolates resistant to one or more first-line drugs (i.e., isoniazid, rifampicin, ethambutol, and STM) were enrolled in this study. The age of drug-resistance *M. tuberculosis* positive patients ranged from 25 to 81 years (mean: 51 years). Gender information was available for all cases: males represented 77.8% of the cases. Clinical status was available for all TB patients: 61.1% (11/18) were previously treated and 38.9% (7/18) were new cases. All of them had pulmonary TB. Based on patient information, one of the previously treated cases had a history of imprisonment, and another was HIV positive.

All isolates showed resistance to one or more antimycobacterial drugs. Monoresistance to isoniazid, STM, and ethambutol was found in 22.2%, 22.2%, and 11.1% of the tested strains, respectively, as opposed to none for rifampin. MDR isolates represented five of seven new TB cases included in the study. In contrast, the three remaining MDR isolates were retreatment cases.

24-locus mycobacterial interspersed repetitive unit-variable number tandem repeat typing and Molecular data analysis

Eighteen isolates were typed by MIRU-VNTR typing method and 18 patterns were found. All patterns were unique [Figure 1].

The MIRU-VNTR patterns were compared with the MIRU-VNTR plus database to discover *M. tuberculosis* lineages and relatedness. The similarity option and tree-based analysis of the MIRU-VNTR plus website was performed, and the following four global lineages were observed: 38.8% belonged to the East-Asian lineage (lineage 2), while the lineages Indo-Oceanic (lineage 1), East African–Indian (EAI) (lineage 3), and Euro-American (lineage 4) represented 5.5%, 22.2%, and 33.3%, respectively. Among the 33.3% (6/18) Euro-American strains, the Latin American Mediterranean (LAM and Ural sub-lineage were 22.2% (4/18) and 11.1% (2/18), respectively [Table 1].

The sub-lineages of the MDR isolates belonged to Beijing (n = 5), LAM (n = 2), and Delhi/Central Asian Strain (CAS) (n = 1) [Figure 1].

Based on personal history and clinical examination, 11 (61.1%) patients had been previously treated (one had HIV infection and another had a history of imprisonment) and the remaining 7 (38.9%) were new cases. The sub-lineages of the *M. tuberculosis* isolates of the previously treated non-HIV and nonhistory of imprisonment patients belonged to Beijing (n = 3), LAM (n = 3), Ural (n = 2), and EAI (n = 1); the sub-lineage of the *M. tuberculosis* isolates of the HIV-positive patient and the patient who had a history of imprisonment belonged to Delhi/CAS [Figure 2].

The allelic diversity was calculated for each MIRU-VNTR locus, and according to this index, each was classified as highly (HGDI >0.6), moderately (0.6 > HGDI <0.3), or poorly discriminatory (HGDI ≤0.3),[13] as summarized in Table 2. The highest allelic diversity indexes were for MIRU 10, Qub 26, Mtub 04, Mtub 21, MIRU 31, MIRU 26, Mtub 30, ETRA, ETRB, MIRU 23, Mtub 34, and MIRU 16. The allelic diversity index was low (h ≤0.3) for MIRU 04, MIRU 20, Mtub 29, MIRU 24, and MIRU 27.

Discussion

TB is a great danger to public health and remains a major infectious disease globally. Of the 480,000 cases of MDR-TB estimated to have occurred in 2014, only about a quarter of these –123,000 were detected and reported.[1]

Iran, one of the Eastern Mediterranean countries, is located between the high MDR-TB (Azerbaijan and Armenia) and high-TB burden (Afghanistan and Pakistan) countries in the region.

Based on the latest report, the incidence rate of TB in Iran was 22 cases per 100,000 population that has a lower incidence of TB than all of the neighboring countries (Afghanistan: 189, Pakistan: 270, Iraq: 43, Azerbaijan: 77, and Armenia: 45/100,000),[1] but extended borders with countries harboring endemic TB and immigration to Iran have influenced TB distribution

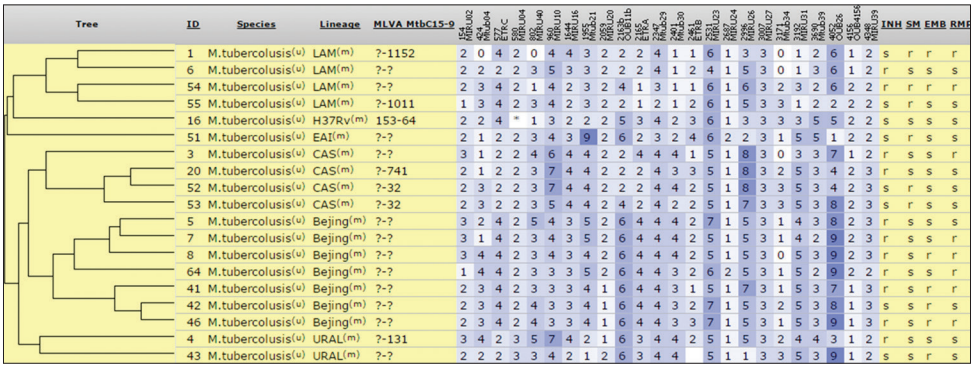

Figure 1: Genetic tree based on 24-locus mycobacterial interspersed repetitive unit-variable number tandem repeat data of 18 drug-resistant *Mycobacterium tuberculosis* isolates from Isfahan province of Iran. A dendrogram was generated using the Unweighted Pair Group Method with Arithmetic Mean algorithm using tools available from the mycobacterial interspersed repetitive unit-variable number tandem repeat plus identification database

Table 1: Distribution of lineages among drug-resistant *Mycobacterium tuberculosis* isolates (*n*=49)

Global lineage	Sub-lineage	Number of isolates	Percentage of isolates
Indo-Oceanic	East African Indian	1	5.5
East Asian	Beijing	7	38.8
East African Indian	Delhi/Central Asian	4	22.2
Euro-American	Lineage 4 total	6	33.3
	Latin American Mediterranean	4	
	Ural	2	
Total		49	100

Table 2: Allelic diversity of each mycobacterial interspersed repetitive unit-variable number tandem repeat locus and their discriminatory power

Locus	Allelic diversity (h)	Alleles quantity	Discriminatory power
Qub 26	0.84	9	High
Mtub 04	0.73	5	High
Mtub 34	0.73	4	High
Mtub 21	0.72	6	High
Mtub 30	0.71	4	High
MIRU 26	0.7	7	High
MIRU 10	0.7	5	High
ETRA	0.67	4	High
ETRB	0.63	4	High
MIRU 23	0.63	4	High
MIRU 31	0.63	4	High
MIRU 16	0.61	3	High
MIRU 40	0.59	5	Moderate
Qub 11b	0.59	4	Moderate
Mtub 39	0.56	4	Moderate
MIRU 02	0.49	3	Moderate
MIRU 39	0.47	2	Moderate
ETRC	0.46	2	Moderate
Qub 4156	0.46	2	Moderate
MIRU 20	0.3	2	Low
MIRU 04	0.24	2	Low
Mtub 29	0.24	3	Low
MIRU 24	0.14	2	Low
MIRU 27	0	1	Low

threatening control strategies. The prevalence of TB in Afghan immigrants in Kerman (a province of Iran) is 12%.[14]

Drug resistance to STM (22.2) and isoniazid (22.2%) was found to be the highest proportion among anti-TB drugs, which is consistent with the previous reports from Iran.[15-18] The cause of the high rates of primary drug resistance to STM and isoniazid in *M. tuberculosis* isolates from new

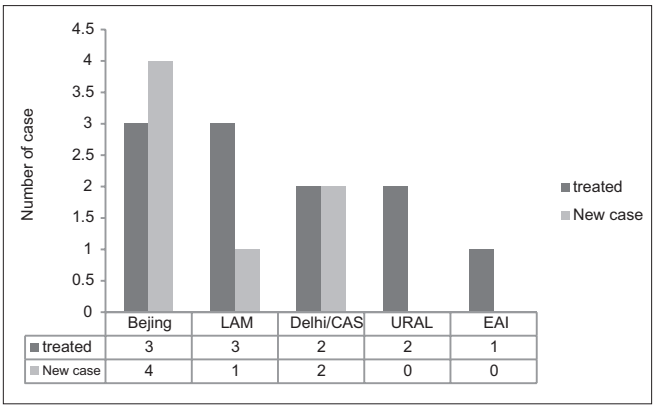

Figure 2: Distribution of lineages of drug-resistant *Mycobacterium tuberculosis* isolates among new case and treated patients

cases is difficult to explain. The high prevalence of STM resistance isolates may be due to widespread use of STM in the past for treatment of the brucellosis, a disease that is endemic in Iran.[16]

Molecular epidemiology studies can monitor TB spread and help prevent its transmission. In this study, the MIRU-VNTR technique which is a rapid PCR-based identification system was used for the differentiation of clinical *M. tuberculosis* isolates. Identification genetic lineages of *M. tuberculosis* isolates in Iran can be used in subsequent studies to determine the transmission chain of *M. tuberculosis*; this is a key factor in controlling TB not only in Iran but worldwide.

Lineage identification is facilitated by the MIRU-VNTR plus database. This database includes the genotyping data of 186 reference isolates representing the main *M. tuberculosis* lineages/sub-lineages. This collection includes: EAI, Beijing, Delhi/CAS, LAM, Haarlem, Cameroon, Ural, NEW-1, Uganda I and II, Turkish, S, Ghana, and X.[19]

In the present study, 18 drug-resistance *M. tuberculosis* clinical isolates (resistant to a single drug to more than one drug) were collected. The major identified isolates were those belonging to the Beijing (*n* = 7, 38.8%) sub-lineage. The Beijing sub-lineage is the cause of major concern worldwide. The Beijing genotype frequently becomes drug resistant and induces more severe forms of TB with higher probability of treatment failure.[20] This sub-lineage was first described in China and was already highly prevalent in 17 different areas around Beijing from 1956 to 1960.[21] Ritacco *et al*. showed that transmission of *M. tuberculosis* strains of the Beijing sub-lineage is not frequent in Latin America; they determined the frequency of the Beijing genotype of *M. tuberculosis* in seven countries in South America; including 5.9% of the patients from Peru, 1.0% of the patients from Argentina, 0.8% of the Brazilian cases, 0.6% of the patients from Paraguay, and none of the samples obtained from Colombia, Ecuador, and Chile. In total, 1.6% of the TB cases carried Beijing isolates. Furthermore, in this study, no association was found

between carrying a strain with the Beijing sub-lineage and having drug-resistant disease.[22] America is far from the origin of emergence Beijing sub-lineage, so it is logical that the low prevalence of this genotype is predictable. Strains of the Beijing genotype have also been detected in South Africa.[23-25] This genotype is more prevalent in Asia (44.7%) and is lowest in America (8.9%).[20,26] In South East Asia (Western Pacific), the Beijing family with 30% of all isolates in Cambodia and 32.5% in Vietnam has the most highly reported incidence.[27,28] The Beijing family isolation rate in many Asian countries has been reported to be >50%.[29] Ramazanzadeh *et al*. showed that 6.4% of the MDR-TB strains in Iran belonged to the Beijing family through the spoligotyping method.[30] Similar to our study, Haeili *et al*. showed that MDR was significantly associated with Beijing strains.[31] Molecular types of *M. tuberculosis* in Isfahan are unknown because *M. tuberculosis* isolates have not been routinely genotyped. Only a few studies have investigated *M. tuberculosis* genetic diversity in Isfahan. Hashemi *et al*. used the random amplification of polymorphic DNA analysis to find the heterogeneity of strains of *M. tuberculosis* collected from clinical specimens of patients in Isfahan and Tehran.[32]

Our study also identified the Delhi/CAS sub-lineage of *M. tuberculosis* strains. This sub-lineage has been reported in many countries; however, the "high incidence" areas of the CAS family are the Middle East and Central and Southern Asia.[33] The Delhi/CAS sub-lineage is exclusively detected in the Xinjiang Uygur Autonomous Region in China.[34] This region is adjacent to several Central Asian countries. Historically, those countries, along with Iraq, are all located along what is famously known as the "Silk Road," the backbone route of trade between the Far and Middle East in history.[35] The travels of merchants and other people along this route may have played a major role in the transmission of this strain type. In New Delhi, Mumbai, Afghanistan, and Pakistan, the Delhi/CAS sub-lineage is recognized as a predominant strain.[36] Iran is neighbor to Afghanistan and Pakistan. Consequently, one of the factors affecting the prevalence of the CAS family in Iran is the frequent migrations, especially from these countries to Iran. Similar to our results, Mustafa Ali *et al*. showed that the CAS1-Delhi harbored the most common strain type in Baghdad.[37] Torkaman *et al*. reported that the major identified isolates of *M. tuberculosis* isolates in Iran were the Haarlem (29.4%) and CAS (25.4%) sub-lineages.[38]

Conclusion

In summary, this study has presented insights into the genetic diversity of drug-resistant *M. tuberculosis* isolates in Isfahan province of Iran. According to our results, the isolates of lineage 2 constitute the majority of the drug-resistant *M. tuberculosis* strains. These lineages are similar to those reported in the Eastern Mediterranean region; this shows the epidemiological relationship between the countries in the region. The level of difference among drug-resistant *M. tuberculosis* strains was unexpected. Continued molecular monitoring is important as it has been proposed that the genetics and evolutionary backgrounds of drug-resistant *M. tuberculosis* strains may have an impact on the transmissibility profile. This knowledge will give new insights into drug-resistant *M. tuberculosis* population, its structure, and how it associates with the epidemiology of drug-resistant *M. tuberculosis* in Iran and beyond.

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Conflicts of interest

There are no conflicts of interest.

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