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Original Article

Genotypic Characterization of Drug-Susceptible and Drug-Resistant *Mycobacterium tuberculosis* Complex Isolates in Central Iran Using 24-Locus MIRU-VNTR Analysis

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Abstract

Background: Identifying the genotypes of *Mycobacterium tuberculosis* (MTB) enables the classification of tuberculosis (TB) isolates at the strain level and facilitates phylogenetic analysis of the bacterium. This study aimed to determine and evaluate the genetic diversity and phylogenetic relationships of both drug-susceptible and drug-resistant *Mycobacterium tuberculosis* complex (MTBC) isolates with respect to first- and second-line anti-tuberculosis drugs using the 24-locus Mycobacterial interspersed repetitive units-variable number of tandem repeats (MIRU-VNTR) genotyping approach.

Methods: A total of 81 MTBC isolates, including both drug-susceptible and drug-resistant strains, were collected from patients with pulmonary and extrapulmonary tuberculosis. All collected isolates were then subjected to genotyping using the 24-locus MIRU-VNTR method.

Results: Among the 81 MTBC isolates, 72 distinct MIRU-VNTR patterns were identified. Of these, six were clustered together, while 66 isolates exhibited unique patterns. The identified genotypes included Delhi/CAS (35 isolates), NEW-1 (25 isolates), LAM (eight isolates), H37Rv (four isolates), Beijing (three isolates), *Mycobacterium bovis* (three isolates), URAL (two isolates), and UgandaI (one isolate).

Conclusion: The Delhi/CAS genotype was the most common, while the UgandaI genotype was the least common among the studied isolates. Additionally, isoniazid-resistant isolates were more frequently observed within the Delhi/CAS genotype compared to others; however, no specific genotype was linked to drug resistance.

Keywords: MIRU-VNTR, *Mycobacterium tuberculosis*, Iran, Genetic diversity

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Introduction

Tuberculosis (TB) is a complex chronic infectious disease caused by the *Mycobacterium tuberculosis* (MTB) bacterium. TB primarily affects the lymph nodes and lungs but can spread to other organs and tissues of the body (1). Globally, annual TB-related mortality declined between 2010 and 2019, but this decline was reversed in 2020 and 2021. The estimated rise in TB-related deaths during this period was largely attributed to disruptions in TB diagnosis and management caused by the recent pandemic caused by the COVID-19 pandemic. Between 2019 and 2021, reported new TB cases fell from 7.1 million to 5.8 million in 2020, subsequently rose to 6.4 million in 2021, suggesting a marked increase in undiagnosed and untreated cases. The estimated 6.4% decline in TB deaths in 2022 compared to 2021 reflects a significant global recovery in TB case detection and management (2).

Since the early 1990s, various genotyping methods have been developed to investigate genetic variations of *Mycobacterium tuberculosis* complex (MTBC) isolates. Genotyping of MTBC offers several benefits, including the ability to differentiate between recent transmission and reactivation, as well as to identify the most prevalent circulating (3). Mycobacterial interspersed repetitive units-variable number of tandem repeats (MIRU-VNTR) genotyping is one of the most commonly utilized methods by researchers. This method relies on the tandem repeat elements located in the intergenic regions of the MTBC genome and the variation in their copy numbers (3). It is now the current reference genotyping method due to its high discriminatory power and reproducibility (4).

The lineages and sub-lineages of MTB are determined based on large sequence polymorphisms (LSPs) and single-nucleotide polymorphisms (SNPs), which together provide a strong phylogenetic classification system (5, 6). To date, seven major MTB lineages have been described using unique-event polymorphism (UEP), SNPs, or genomic deletion. Geographically, most TB lineages are found in particular regions and are therefore named according to their primary geographic distribution. These include Lineage 1 (Indo-Oceanic), Lineage 2 (East Asian, including the Beijing sub-lineage), Lineage 3 (CAS/Delhi), Lineage 4 (Euro-American), Lineage 5 (West African 1), Lineage 6 (West African 2), and Lineage 7 (Ethiopian) (7-9). The global distribution of these lineages provides important insight into the evolutionary adaptation of MTB to human populations (9).

This study focused on assessing the genetic diversity and phylogenetic relationships among clinical MTBC isolates, which exhibit varying susceptibility to first-line anti-tuberculosis drugs, including rifampin (RIF), ethambutol (EMB), and isoniazid (INH), as well as second-line drugs (SLIDs) such as amikacin (AMK), ofloxacin (OFX), capreomycin (CAP), and kanamycin (KAN). Genotyping was performed using the 24-locus MIRU-VNTR typing method on isolates collected in central Iran.

Materials and Methods

Clinical Isolates

In this cross-sectional study, 81 clinical isolates, both drug-resistant and drug-susceptible to first-line and second-line anti-tuberculosis drugs, were collected from Iranian and Afghan patients with pulmonary tuberculosis (PTB) and extrapulmonary tuberculosis (EPTB) by convenience sampling. The patients were referred to the Molla Haadi Sabzevari Healthcare Center (Reference Laboratory) in Isfahan province, central Iran, between 2014 and 2017. Patients originated from several regions of central Iran, including Isfahan, Tehran, Qom, Arak, Yazd, Shahrekord, and Kashan. The PTB samples were sputum and trachea, while the EPTB samples consisted of gastric lavage, abscess, cerebrospinal fluid, pleural aspiration, menstrual blood, and urine (Table S1, Supplementary file 1).

Inclusion Criteria

All isolates resistant to the first-line anti-tuberculosis drugs during the study period were included, while the susceptible isolates were randomly selected.

Exclusion Criteria

Nontuberculous mycobacteria isolates were excluded.

Drug Susceptibility Testing

Drug susceptibility testing (DST) for first-line and second-line anti-tuberculosis drugs had been previously conducted on these isolates and published elsewhere (10). According to the World Health Organization (WHO) 2014 guidelines, the proportion method on Lowenstein-Jensen (LJ) medium was used to assess susceptibility to both first-line drugs and second-line injectable drugs (SLIDs) (11). The evaluation of SLIDs was also conducted on rifampin-resistant (RR) and multidrug-resistant (MDR) isolates. The critical concentrations employed on LJ medium included: INH (0.2 µg/mL), EMB (2 µg/mL), AMK (30 µg/mL), RIF (40 µg/mL), OFX (4 µg/mL), CAP (40 µg/mL), and KAN (30 µg/mL) (12) (Table S1, Supplementary file 1).

DNA Extraction

Genomic DNA was extracted from all isolates using the cetyltrimethylammonium bromide (CTAB) extraction protocol (13).

24-Locus MIRU-VNTR Genotyping

Standardized primers targeting the flanking regions of the 24-locus MIRU-VNTR were used for genotyping, as previously described (13). Briefly, the MIRU-VNTR-24 loci were amplified in a series of separate polymerase chain reactions (PCRs) using the relevant primers (Table S2, Supplementary file 1). PCR was conducted in a final reaction volume of 20 µL containing 0.8 µL of each forward and reverse primer (10 pmol), 10 µL of 2x PCR master mix (Ampliqon, Odense, Denmark; containing1.5-2 mM MgCl2), 5 µL of 5x Q-solution (Qiagen, Germany), 1 µL of template DNA, and 2.4 µL of DNase-free distilled H2O. For certain loci, a higher MgCl2 concentration (2.5-3 mM MgCl2) was required, which was added manually.

The MTB H37Rv strain was utilized in each PCR run as a positive control, while DNAase-free distilled water served as a non-template negative control. PCR amplification involved an initial denaturation at 95 ˚C for 15 minutes, followed by 40 cycles of denaturation at 94 ˚C for one minute, annealing at 65 ˚C, 66 ˚C, or 68 ˚C for one minute (depending on the locus), extension at 72˚C for one minute, and a final extension step at 72 ˚C for 10 minutes. The amplified products were subsequently visualized using electrophoresis on 1.5-2% agarose gels, and amplicon sizes were estimated using 50 bp and 100 bp DNA ladders.

24-Locus MIRU-VNTR Genotyping Analysis

To determine MTB lineages and sublineages and to assess phylogenetic relationships among isolates, MIRU-VNTR patterns were aligned and compared using the MIRU-VNTRplus database (http://www.miru-vntrplus.org).

Strain Identification

Strain identification was conducted using the phylogenetic tree and the Similarity Search tool available on the MIRU-VNTRplus website. The Similarity Search tool displays the closest matching reference strains based on selected distance measures and genotyping methods, enabling the determination of the species and lineage of user-submitted strains. Furthermore, the phylogenetic tree compares all user strains with all reference database strains to establish their relative genetic positions.

Hunter-Gaston Discriminatory Index and Allelic Diversity

The Hunter-Gaston Discriminatory Index (HGDI) was utilized to calculate the discriminatory power of the MIRU-VNTR genotyping method, based on the following equation (14):

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

The allelic diversity (h) is a valuable index indicating the variability at each MIRU locus (15) using the following equation:

$$h = 1 - \Sigma\, x_i^{\,2}\, [n/\,(n\text{-}1)]$$

Based on allelic diversity values, MIRU loci had high discriminatory power ($h > 0.6$), moderate discriminatory power ($0.3 \le h \le 0.6$), or poor discriminatory power ($h < 0.3$) (16).

Clustering Rate and Recent Transmission

A cluster can be defined as two or more patients infected with MTB strains exhibiting identical DNA fingerprint patterns (17-21). The clustering index was calculated by dividing the total number of clustered isolates in each group by the total number of isolates in that group (22). The clustering rate (R) was assessed using the following formula:

$$R = n_c/n$$

where n_c represents the number of clustered isolates, n represents the total number of isolates in the sample, and higher values indicate a greater degree of clustering (23). The following equation is used to calculate the recent transmission rate:

$$(nc\text{-}\ c)/n$$

where n_c is the number of clustered isolates, c is the number of clusters, and n represents the total number of analyzed isolates in the study population. Based on this index, TB patients were categorized into two groups: clustered and non-clustered. Patients were regarded as clustered when TB had been transmitted recently, whereas non-clustered cases were considered to result from reactivation of latent infection (9,23).

Minimum Spanning Tree

The minimum spanning tree (MST) is a method used to illustrate relationships among strains in a visually intuitive graphical format. It can be generated based on strain genotypes and utilizes the number of locus variants rather than distances. Genotypes were grouped into clonal complexes (CCs) according to differences in one or more loci compared with other genotypes. The maximum locus difference within a CC can be defined, and the minimum size of a CC is two genotypes.

Statistical Analysis

The resulting data were gathered and analyzed using statistical software SPSS version 16 (SPSS Inc., Chicago, IL, United States) with appropriate statistical tests.

Results

Clinical Isolates and Drug Susceptibility Testing

Of the 81 patients, 30 (37.03%) were females, and 51 (62.9%) were males. Among them, 11 (13.5%) were Afghan, and 70 (86.4%) were Iranian. Ten (12.3%) isolates, three of which were obtained from IFNγ-deficient infants, were associated with EPTB, while 71 (87.6%) isolates, including 8 (9.8%) HIV-positive cases, were obtained from PTB patients. Overall, eight (9.8%) patients were co-infected with HIV.

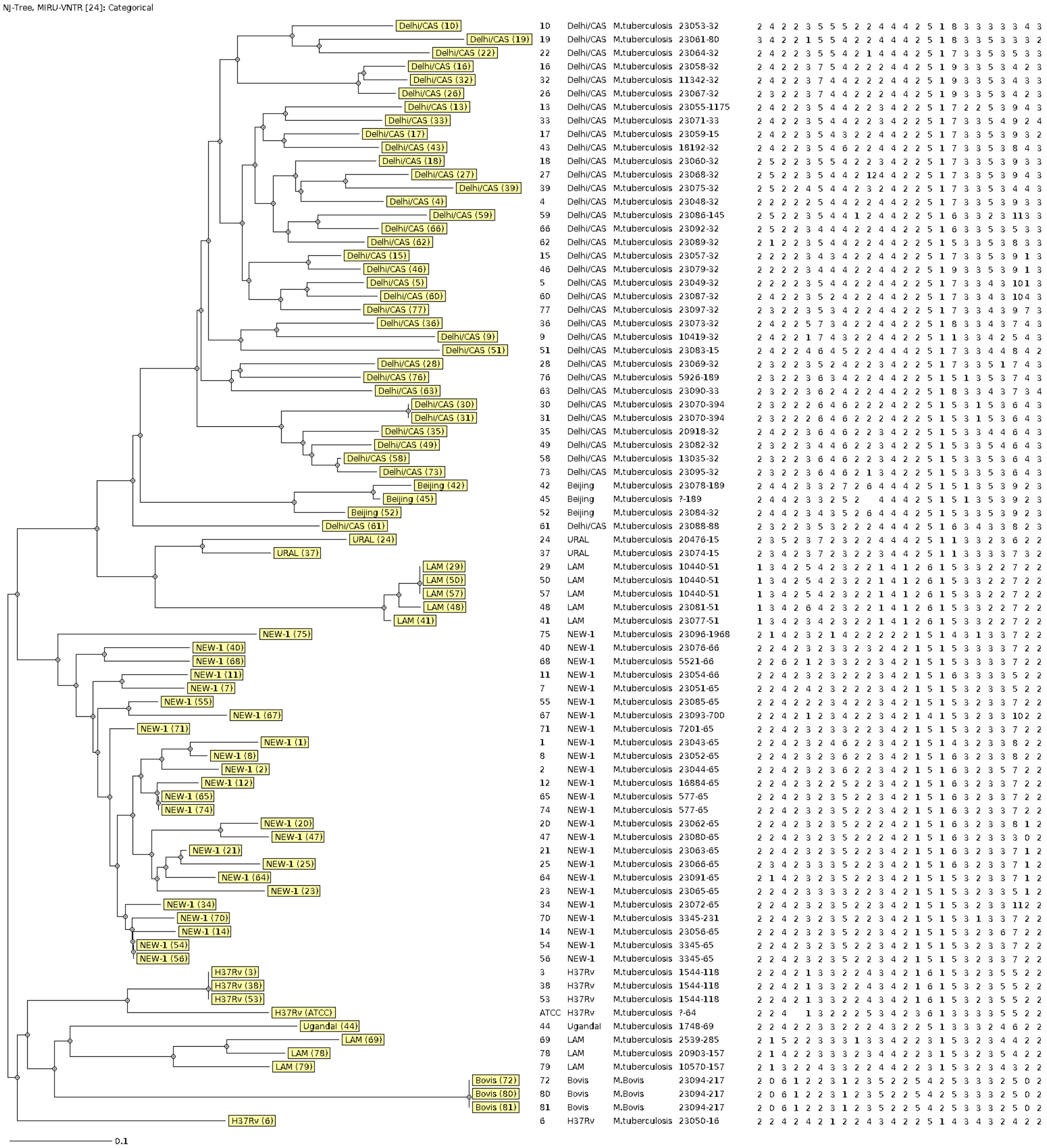
All 10 EPTB isolates (100%) were susceptible to the first-line anti-tuberculosis drugs, including EMB, RIF, and INH. Among the 71 PTB isolates, 32 (45.1%) showed resistance to at least one first-line drug, and 13 (40.6%) of these were also resistant to SLIDs. In contrast, 39 (54.9%) isolates were susceptible to first-line drugs.

Among the 32 first-line drug-resistant isolates, 23 (71.8%) were resistant to INH, 13 (40.6%) to EMB, and 3 (9.3%) to RIF, respectively. Moreover, six (18.75%) of these 32 first-line drug-resistant isolates were classified as MDR or pre-extensively drug-resistant (Pre-XDR). Considering SLIDs, all 13 MDR and RR isolates were resistant to KAN and CAP, six (46.1%) were resistant to AMK, and only one isolate (7.6%) was resistant to OFX. Among the eight HIV-TB co-infected patients, five (62.5%) isolates were resistant to INH (Table S1, Supplementary file 1).

MIRU-VNTR (24-locus) Genotyping and Molecular Data Analysis

Strain Identification

A genetic similarity search was performed, and a phylogenetic tree was constructed using the MIRU-VNTRplus website. Analysis of the 81 MTBC isolates revealed the presence of three major lineages (Lineages 2, 3, and 4) out of the seven globally recognized lineages, in addition to the *Mycobacterium bovis* lineage (Table S1, Supplementary file 1). The identified lineages and sub-lineages were as follows: Lineage 2 (East Asian lineage), represented by the Beijing sub-lineage (n = 3; 3.7%); Lineage 3 (East-African Indian lineage), represented by Delhi/CAS sub-lineage (n = 35; 43.2%); and Lineage 4 (Euro-American lineage), including the NEW-1 (n = 25; 30.8%), LAM (n = 8; 9.8%), H37Rv (n = 4; 4.9%), URAL (n = 2; 2.4%), and UgandaI (n = 1; 1.2%) sub-lineages. In addition, three isolates (3.7%) were identified as *M.*



10	Delhi/CAS	M.tuberculosis	23053-32	2	4	2	2	3	5	5	5	2	2	4	4	4	2	5	1	8	3	3	3	3	3	4	3
19	Delhi/CAS	M.tuberculosis	23061-80	3	4	2	2	1	5	5	4	2	2	4	4	4	2	5	1	8	3	3	5	3	3	3	2
22	Delhi/CAS	M.tuberculosis	23064-32	2	4	2	2	3	5	5	4	2	1	4	4	4	2	5	1	7	3	3	5	3	5	3	3
16	Delhi/CAS	M.tuberculosis	23058-32	2	4	2	2	3	7	5	4	2	2	2	4	4	2	5	1	9	3	3	5	3	4	2	3
32	Delhi/CAS	M.tuberculosis	11342-32	2	4	2	2	3	7	4	4	2	2	2	4	4	2	5	1	9	3	3	5	3	4	3	3
26	Delhi/CAS	M.tuberculosis	23067-32	2	3	2	2	3	7	4	4	2	2	2	4	4	2	5	1	9	3	3	5	3	4	2	3
13	Delhi/CAS	M.tuberculosis	23055-1175	2	4	2	2	3	5	4	4	2	2	3	4	2	2	5	1	7	2	2	5	3	9	4	3
33	Delhi/CAS	M.tuberculosis	23071-33	2	4	2	2	3	5	4	4	2	2	3	4	2	2	5	1	7	3	3	5	4	9	2	4
17	Delhi/CAS	M.tuberculosis	23059-15	2	4	2	2	3	5	4	3	2	2	4	4	2	2	5	1	7	3	3	5	3	9	3	2
43	Delhi/CAS	M.tuberculosis	18192-32	2	4	2	2	3	5	4	6	2	2	4	4	2	2	5	1	7	3	3	5	3	8	4	3
18	Delhi/CAS	M.tuberculosis	23060-32	2	5	2	2	3	5	5	4	2	2	3	4	2	2	5	1	7	3	3	5	3	9	3	3
27	Delhi/CAS	M.tuberculosis	23068-32	2	5	2	2	3	5	4	4	2	12	4	4	2	2	5	1	7	3	3	5	3	9	4	3
39	Delhi/CAS	M.tuberculosis	23075-32	2	5	2	2	4	5	4	4	2	3	2	4	2	2	5	1	7	3	3	5	3	4	4	3
4	Delhi/CAS	M.tuberculosis	23048-32	2	2	2	2	2	5	4	4	2	2	4	4	2	2	5	1	7	3	3	5	3	9	3	3
59	Delhi/CAS	M.tuberculosis	23086-145	2	5	2	2	3	5	4	4	1	2	4	4	2	2	5	1	6	3	3	2	3	11	3	3
66	Delhi/CAS	M.tuberculosis	23092-32	2	5	2	2	3	4	4	4	2	2	4	4	2	2	5	1	6	3	3	5	3	5	3	3
62	Delhi/CAS	M.tuberculosis	23089-32	2	1	2	2	3	5	4	4	2	2	4	4	2	2	5	1	5	3	3	5	3	8	3	3
15	Delhi/CAS	M.tuberculosis	23057-32	2	2	2	2	3	4	3	4	2	2	4	4	2	2	5	1	7	3	3	5	3	9	1	3
46	Delhi/CAS	M.tuberculosis	23079-32	2	2	2	2	3	4	4	4	2	2	4	4	2	2	5	1	9	3	3	5	3	9	1	3
5	Delhi/CAS	M.tuberculosis	23049-32	2	2	2	2	3	5	4	4	2	2	4	4	2	2	5	1	7	3	3	4	3	10	1	3
60	Delhi/CAS	M.tuberculosis	23087-32	2	4	2	2	3	5	2	4	2	2	4	4	2	2	5	1	7	3	3	4	3	10	4	3
77	Delhi/CAS	M.tuberculosis	23097-32	2	3	2	2	3	5	3	4	2	2	4	4	2	2	5	1	7	3	3	4	3	9	7	3
36	Delhi/CAS	M.tuberculosis	23073-32	2	4	2	2	5	7	3	4	2	2	4	4	2	2	5	1	8	3	3	4	3	7	4	3
9	Delhi/CAS	M.tuberculosis	10419-32	2	4	2	2	1	7	4	3	2	2	4	4	2	2	5	1	1	3	3	4	2	5	4	3
51	Delhi/CAS	M.tuberculosis	23083-15	2	4	2	2	4	6	4	5	2	2	4	4	4	2	5	1	7	3	3	4	4	8	4	2
28	Delhi/CAS	M.tuberculosis	23069-32	2	3	2	2	3	5	2	4	2	2	3	4	2	2	5	1	7	3	3	5	1	7	4	3
76	Delhi/CAS	M.tuberculosis	5926-189	2	3	2	2	3	6	3	4	2	2	4	4	2	2	5	1	5	1	3	5	3	7	4	3
63	Delhi/CAS	M.tuberculosis	23090-33	2	3	2	2	3	6	2	4	2	2	4	4	2	2	5	1	8	3	3	4	3	7	3	4
30	Delhi/CAS	M.tuberculosis	23070-394	2	3	2	2	2	6	4	6	2	2	2	4	2	2	5	1	5	3	1	5	3	6	4	3
31	Delhi/CAS	M.tuberculosis	23070-394	2	3	2	2	2	6	4	6	2	2	2	4	2	2	5	1	5	3	1	5	3	6	4	3
35	Delhi/CAS	M.tuberculosis	20918-32	2	4	2	2	3	6	4	6	2	2	3	4	2	2	5	1	5	3	3	4	4	6	4	3
49	Delhi/CAS	M.tuberculosis	23082-32	2	3	2	2	3	4	4	6	2	2	3	4	2	2	5	1	5	3	3	5	4	6	4	3
58	Delhi/CAS	M.tuberculosis	13035-32	2	3	2	2	3	6	4	6	2	2	3	4	2	2	5	1	5	3	3	5	3	6	4	3
73	Delhi/CAS	M.tuberculosis	23095-32	2	3	2	2	3	6	4	6	2	1	3	4	2	2	5	1	5	3	3	5	3	6	4	3
42	Beijing	M.tuberculosis	23078-189	2	4	4	2	3	3	2	7	2	6	4	4	4	2	5	1	5	1	3	5	3	9	2	3
45	Beijing	M.tuberculosis	?-189	2	4	4	2	3	3	2	5	2		4	4	4	2	5	1	5	1	3	5	3	9	2	3
52	Beijing	M.tuberculosis	23084-32	2	4	4	2	3	4	3	5	2	6	4	4	4	2	5	1	5	3	3	5	3	9	2	3
61	Delhi/CAS	M.tuberculosis	23088-88	2	3	2	2	3	5	3	2	2	2	4	4	4	2	5	1	6	3	4	3	3	8	2	3
24	URAL	M.tuberculosis	20476-15	2	3	5	2	3	7	2	3	2	2	4	4	4	2	5	1	1	3	3	2	3	6	2	2
37	URAL	M.tuberculosis	23074-15	2	3	4	2	3	7	2	3	2	2	3	4	4	2	5	1	1	3	3	3	3	7	3	2
29	LAM	M.tuberculosis	10440-51	1	3	4	2	5	4	2	3	2	2	1	4	1	2	6	1	5	3	3	2	2	7	2	2
50	LAM	M.tuberculosis	10440-51	1	3	4	2	5	4	2	3	2	2	1	4	1	2	6	1	5	3	3	2	2	7	2	2
57	LAM	M.tuberculosis	10440-51	1	3	4	2	5	4	2	3	2	2	1	4	1	2	6	1	5	3	3	2	2	7	2	2
48	LAM	M.tuberculosis	23081-51	1	3	4	2	6	4	2	3	2	2	1	4	1	2	6	1	5	3	3	2	2	7	2	2
41	LAM	M.tuberculosis	23077-51	1	3	4	2	3	4	2	3	2	2	1	4	1	2	6	1	5	3	3	2	2	7	2	2
75	NEW-1	M.tuberculosis	23096-1968	2	1	4	2	3	2	1	4	2	2	2	2	2	1	5	1	4	3	1	3	3	7	2	2
40	NEW-1	M.tuberculosis	23076-66	2	2	4	2	3	2	3	3	2	3	2	4	2	1	5	1	5	3	3	3	3	7	2	2
68	NEW-1	M.tuberculosis	5521-66	2	2	6	2	1	2	3	3	2	2	3	4	2	1	5	1	5	3	3	3	3	7	2	2
11	NEW-1	M.tuberculosis	23054-66	2	2	4	2	3	2	3	2	2	2	3	4	2	1	5	1	6	3	3	3	3	5	2	2
7	NEW-1	M.tuberculosis	23051-65	2	2	4	2	4	2	3	2	2	2	3	4	2	1	5	1	5	3	2	3	3	5	2	2
55	NEW-1	M.tuberculosis	23085-65	2	2	4	2	2	2	3	4	2	2	3	4	2	1	5	1	5	3	2	3	3	7	2	2
67	NEW-1	M.tuberculosis	23093-700	2	2	4	2	1	2	3	4	2	2	3	4	2	1	4	1	5	3	2	3	3	10	2	2
71	NEW-1	M.tuberculosis	7201-65	2	2	4	2	3	2	3	3	2	2	3	4	2	1	5	1	5	3	2	3	3	7	2	2
1	NEW-1	M.tuberculosis	23043-65	2	2	4	2	3	2	4	6	2	2	3	4	2	1	5	1	4	3	2	3	3	8	2	2
8	NEW-1	M.tuberculosis	23052-65	2	2	4	2	3	2	3	6	2	2	3	4	2	1	5	1	6	3	2	3	3	8	2	2
2	NEW-1	M.tuberculosis	23044-65	2	2	4	2	3	2	3	6	2	2	3	4	2	1	5	1	6	3	2	3	5	7	2	2
12	NEW-1	M.tuberculosis	16884-65	2	2	4	2	3	2	2	5	2	2	3	4	2	1	5	1	6	3	2	3	3	7	2	2
65	NEW-1	M.tuberculosis	577-65	2	2	4	2	3	2	3	5	2	2	3	4	2	1	5	1	6	3	2	3	3	7	2	2
74	NEW-1	M.tuberculosis	577-65	2	2	4	2	3	2	3	5	2	2	3	4	2	1	5	1	6	3	2	3	3	7	2	2
20	NEW-1	M.tuberculosis	23062-65	2	2	4	2	3	2	3	5	2	2	2	4	2	1	5	1	6	3	2	3	3	8	1	2
47	NEW-1	M.tuberculosis	23080-65	2	2	4	2	3	2	3	5	2	2	2	4	2	1	5	1	6	3	2	3	3	3	0	2
21	NEW-1	M.tuberculosis	23063-65	2	2	4	2	3	2	3	5	2	2	3	4	2	1	5	1	6	3	2	3	3	7	1	2
25	NEW-1	M.tuberculosis	23066-65	2	3	4	2	3	3	3	5	2	2	3	4	2	1	5	1	6	3	2	3	3	7	1	2
64	NEW-1	M.tuberculosis	23091-65	2	1	4	2	3	2	3	5	2	2	3	4	2	1	5	1	5	3	2	3	3	7	1	2
23	NEW-1	M.tuberculosis	23065-65	2	2	4	2	3	3	3	3	2	2	3	4	2	1	5	1	5	3	2	3	3	5	1	2
34	NEW-1	M.tuberculosis	23072-65	2	2	4	2	3	2	3	5	2	2	3	4	2	1	5	1	5	3	2	3	3	11	2	2
70	NEW-1	M.tuberculosis	3345-231	2	2	4	2	3	2	3	5	2	2	3	4	2	1	5	1	5	3	1	3	3	7	2	2
14	NEW-1	M.tuberculosis	23056-65	2	2	4	2	3	2	3	5	2	2	3	4	2	1	5	1	5	3	2	3	6	7	2	2
54	NEW-1	M.tuberculosis	3345-65	2	2	4	2	3	2	3	5	2	2	3	4	2	1	5	1	5	3	2	3	3	7	2	2
56	NEW-1	M.tuberculosis	3345-65	2	2	4	2	3	2	3	5	2	2	3	4	2	1	5	1	5	3	2	3	3	7	2	2
3	H37Rv	M.tuberculosis	1544-118	2	2	4	2	1	3	3	2	2	4	3	4	2	1	6	1	5	3	2	3	5	5	2	2
38	H37Rv	M.tuberculosis	1544-118	2	2	4	2	1	3	3	2	2	4	3	4	2	1	6	1	5	3	2	3	5	5	2	2
53	H37Rv	M.tuberculosis	1544-118	2	2	4	2	1	3	3	2	2	4	3	4	2	1	6	1	5	3	2	3	5	5	2	2
ATCC	H37Rv	M.tuberculosis	?-64	2	2	4		1	3	2	2	2	5	3	4	2	3	6	1	3	3	3	3	5	5	2	2
44	Ugandal	M.tuberculosis	1748-69	2	2	4	2	2	3	3	2	2	2	4	3	2	2	5	1	3	3	3	2	4	6	2	2
69	LAM	M.tuberculosis	2539-285	2	1	5	2	2	3	3	3	1	3	3	4	2	2	3	1	5	3	2	3	4	4	2	2
78	LAM	M.tuberculosis	20903-157	2	1	4	2	2	3	3	3	2	3	4	4	2	2	3	1	5	3	2	3	5	4	2	2
79	LAM	M.tuberculosis	10570-157	2	1	3	2	2	4	3	3	2	2	4	4	2	2	3	1	5	3	2	3	4	3	2	2
72	Bovis	M.Bovis	23094-217	2	0	6	1	2	2	3	1	2	3	5	2	2	5	4	2	5	3	3	3	2	5	0	2
80	Bovis	M.Bovis	23094-217	2	0	6	1	2	2	3	1	2	3	5	2	2	5	4	2	5	3	3	3	2	5	0	2
81	Bovis	M.Bovis	23094-217	2	0	6	1	2	2	3	1	2	3	5	2	2	5	4	2	5	3	3	3	2	5	0	2
6	H37Rv	M.tuberculosis	23050-16	2	2	4	2	4	2	1	2	2	4	3	4	2	2	5	1	4	3	4	3	2	4	2	2

Figure 1. Phylogenetic Tree of 81 Mycobacterium Tuberculosis Complex Isolates Genotyped Using the 24-locus 471 MIRU-VNTR Method. *Note*. MIRU-VNTR: Mycobacterial interspersed repetitive units-Variable number tandem repeat

bovis (Figure 1). Among the isolates resistant to first-line anti-tuberculosis drugs, the majority belonged to Lineage 3, Delhi/CAS sub-lineage (53.1%; n = 17), followed by Lineage 4 sub-lineages, including NEW-1 (34.3%; n = 11), H37Rv (6.2%; n = 2), LAM (3.1%; n = 1), and URAL (3.1%; n = 1) (Tables 1 and 2).

In contrast, second-line drug-resistant isolates showed the presence of NEW-1 (46.1%; n = 6), Delhi/CAS (38.4%; n = 5), and H37Rv (15.3%; n = 2) sub-lineages (Table 3).

Among susceptible isolates, the following lineages and sub-lineages were observed: Lineage 4, including NEW-1 (n = 14; 17.2%), LAM (n = 7; 8.6%), H37Rv (n = 2; 2.4%), URAL (n = 1; 1.2%), and UgandaI (n = 1; 1.2%) sub-lineages; Lineage 3, including Delhi/CAS (n = 18; 22.2%) sub-lineage; and Lineage 2, including the Beijing sub-lineage (n = 3; 3.7%) and *M. bovis* lineage (n = 3; 3.7%). Among the eleven Afghan patients, all identified lineages were noted: Lineage 2 (Beijing sub-lineage, n = 2; 18.1%), Lineage 3 (Delhi/CAS sub-lineage, n = 5; 45.4%), and Lineage 4 (NEW-1, n = 3; 27.2%, and LAM, n = 1; 9%). Furthermore, among the 10 EPTB patients, Lineage 3 (Delhi/CAS sub-lineage, n = 3; 30%), Lineage 4 (LAM

Table 1. Genotyping of MTBC Isolates Using 24-locus MIRU-VNTR in PTB and EPTB Patients

Genotypes		PTB patients First-line drugs					EPTB patients First-line drugs		Total
Lineages	Sub-lineages	Resistant				Susceptible	Resistant	Susceptible	
		INH	RIF	EMB	MDR-TB				
Lineage3	Delhi/CAS	12	1	-	4	15	-	3	35
Lineage4	NEW-1	4	4	1	2	14	-	-	25
Lineage4	LAM	1	-	-	-	5	-	2	8
Lineage4	URAL	-	-	1	-	1	-	-	2
Lineage4	H37Rv-like	-	2	-	-	2	-	-	4
Lineage4	Ugandal	-	-	-	-	1	-	-	1
Lineage2	Beijing	-	-	-	-	3	-	-	3
M. bovis	*M. bovis*	-	-	-	-	-	-	3	3
Total		17	7	2	6	41	-	8	81
		32							

Note. MTBC: *Mycobacterium tuberculosis* complex; MIRU-VNTR: Mycobacterial interspersed repetitive units–Variable number tandem repeats; PTB: Pulmonary tuberculosis; EPTB: Extra-pulmonary tuberculosis; EMB: Ethambutol; RIF: Rifampin; INH: Isoniazid; MDR: Multi-drug resistant; LAM: Latin American-Mediterranean; H37Rv: Reference strain of *Mycobacterium tuberculosis*; NEW-1: New-1 genotype of lineage 4; CAS: Central Asian strain; *M. bovis*: *Mycobacterium bovis*.

Table 2. Classification of 24-locus MIRU-VNTR Genotyping of Resistant and Susceptible Isolates to First-Line Drugs

Lineages	Sub-lineages	Resistant Isolates (PTB patients)	Susceptible Isolates (PTB & EPTB patients)	Total
Lineage3	Delhi/CAS	17	18	35
Lineage4	NEW-1	11	14	25
Lineage4	LAM	1	7	8
Lineage4	H37Rv	2	2	4
Lineage2	Beijing	-	3	3
M. bovis	*M. bovis*	-	3	3
Lineage4	URAL	1	1	2
Lineage4	Ugandal	-	1	1
Total		32	49	81

Note. PTB: Pulmonary tuberculosis; EPTB: Extrapulmonary tuberculosis; MIRU-VNTR: Mycobacterial interspersed repetitive units–Variable number tandem repeats; LAM: Latin American-Mediterranean; NEW-1: New-1 genotype of lineage 4; H37Rv: Reference strain of *Mycobacterium tuberculosis;* CAS: Central Asian strain; *M. bovis*: *Mycobacterium bovis*.

sub-lineage, n = 2; 20%, and NEW-1 sub-lineage, n = 2; 20%), and *M. bovis* lineage (n = 3; 30%) were observed. Eight of the 81 TB patients were co-infected with HIV, representing Lineage 3 (Delhi/CAS sub-lineage, n = 4; 50%), Lineage 4 (NEW-1, n = 1; 12.5%, LAM, n = 1; 12.5%, and H37Rv, n = 1; 12.5%), and Lineage 2 (Beijing sub-lineage, n = 1; 12.5%). Lineages and sub-lineages related to isolates resistant to first-line and SLIDs, as well as susceptible isolates from PTB and EPTB patients categorized by city, are listed separately in Tables 1-4.

Statistical Analysis

No statistically significant differences were observed among the studied MTBC lineages and between ethnic groups (Iranian vs. Afghan patients) regarding RIF resistance, ethambutol resistance, or MDR. However, the frequency of isoniazid-resistant isolates was significantly greater within the Delhi/CAS sub-lineage compared with other genotypes ($P = 0.002$).

Allelic Diversity, Clustering Rate, and Discriminatory Power

Analysis of strains using the MIRU-VNTRplus database yielded clustering rate (R) and allelic diversity (h) values for each MIRU-VNTR locus (Table S3, Supplementary file 1). Loci with the highest allelic diversity indices ($h \geq 0.6$) included QUB26, MIRU10, Mtub21, Mtub04, MIRU26, QUB4156, ETRA, MIRU16, and MIRU31. In contrast, low allelic diversity indices ($h \leq 0.3$) were observed for MIRU27, MIRU24, MIRU04, MIRU20, Mtub29, and MIRU02. The overall clustering rate for the 24-locus MIRU-VNTR analysis, recent transmission rate, and discriminatory power (D) were recorded at 18.5% (nc/n), 11% (nc-c/n), and 71.1%, respectively.

Minimum Spanning Tree

The MST was constructed using the MIRU-VNTRplus website. Six CCs were identified (n = 33; 40.7%), including 30 Iranian patients (37%), three Afghan patients (3.7%), and 48 singletons (59.2%). The observed CCs were CC1 (n = 18; 22.2%) comprising NEW-1 sub-lineages (Lineage 4), CC2 (n = 5; 6.1%) comprising LAM sub-lineage (Lineage 4), CC3 (n = 3; 3.7%) comprising Delhi/CAS sub-lineage (Lineage 3), CC4 (n = 3; 3.7%) containing Delhi/CAS sub-lineage (Lineage 3), and CC5 (n = 2; 2.4%) comprising Delhi/CAS sub-lineage (Lineage 3), and CC6 (n = 2; 2.4%) comprising Beijing sub-lineage (Lineage 2).

Out of eleven Afghan patients, four isolates were distributed across CC6 (n = 2), CC5 (n = 1), and CC1 (n = 1), as shown in Figure 2. No Iranian patients were identified in CC6. CC1 (n = 8; 9.8%), CC3 (n = 3; 3.7%), and CC5 (n = 1; 1.2%) consisted of first-line drug-resistant isolates. CC1 comprised four INH-resistant, three RIF mono-resistant, and one MDR isolate. Three INH-resistant isolates were located in CC3, and one isolate was identified in CC5. Isolates from HIV-positive patients were identified in CC3 (n = 2; 2.4%), CC2 (n = 1; 1.2%), and CC1 (n = 1; 1.2%). Second-line drug-resistant isolates

Table 3. 24-Locus MIRU-VNTR Genotyping of MTBC Isolates Susceptible and Resistant to Second-line Drugs

Lineages	Sub-lineages	OFX		KAN		CAP		AMK	
		R	S	R	S	R	S	R	S
Lineage4	NEW-1	1	5	6	-	6	-	3	3
Lineage3	Delhi/CAS	-	5	5	-	5	-	2	3
Lineage4	H37Rv-like	-	2	2	-	2	-	-	2
Lineage4	LAM	-	-	-	-	-	-	-	-
Lineage4	URAL	-	-	-	-	-	-	-	-
Lineage4	UgandaI	-	-	-	-	-	-	-	-
Lineage2	Beijing	-	-	-	-	-	-	-	-
M. bovis	*M. bovis*	-	-	-	-	-	-	-	-
		1	12	13	-	13	-	5	8
	Total	13		13		13		13	

Note. MTBC: *Mycobacterium tuberculosis* complex; MIRU-VNTR: Mycobacterial interspersed repetitive units–Variable number tandem repeats; OFX: Ofloxacin; KAN: Kanamycin; CAP: Capreomycin; AMK: Amikacin; R: Resistant; S: Susceptible; CAS: Central Asian Strain; LAM: Latin American–Mediterranean; H37Rv: Reference strain of *Mycobacterium tuberculosis*.

Table 4. 24-Locus MIRU-VNTR Genotyping of MTBC Isolates by City

Lineages	Sub-lineages	Isfahan	Arak	Tehran	Qom	Yazd	Kashan	Shahrekord	Total
Lineage3	Delhi/CAS	25	5	2	1	1	1	-	35
Lineage4	NEW-1	20	2	2	-	-	-	1	25
Lineage4	LAM	8	-	-	-	-	-	-	8
Lineage4	UgandaI	1	-	-	-	-	-	-	1
Lineage2	Beijing	3	-	-	-	-	-	-	3
M. bovis	*M. bovis*	3	-	-	-	-	-	-	3
Lineage4	URAL	2	-	-	-	-	-	-	2
Lineage4	H37Rv	3	1	-	-	-	-	-	4
	Total	65	8	4	1	1	1	1	81

Note. MTBC: *Mycobacterium tuberculosis* complex; MIRU-VNTR: *Mycobacterial interspersed repetitive units–variable number tandem repeats*; CAS: Central Asian strain; LAM: Latin American Mediterranean; URAL: Ural lineage; H37Rv: Reference strain of *Mycobacterium tuberculosis*.

were identified exclusively in CC1 (n = 4; 4.9%).

Using 24-locus MIRU-VNTR genotyping, we identified 72 genetic patterns, including six clustered and 66 unique ones. The isolates from 15 Iranian patients (18.5%), comprising ten males and five females, constituted the six identified clusters. Three of these clusters, including the H37Rv sub-lineage (Lineage 4), the LAM sub-lineage (Lineage 4), and the *M. bovis* lineage, each contained three members. Additionally, three clusters, comprising the Delhi/CAS sub-lineage (Lineage 3) and two NEW-1 sub-lineages (Lineage 4), each had two members. CC1 included two clusters (each with two members) corresponding to the NEW-1 sub-lineages (Lineage 4), while CC2 contained one cluster consisting of three members with the LAM sub-lineage (Lineage 4).

All clusters were isolated from patients residing in Isfahan and Arak between 2015 and 2017 (two patients from Arak and 13 patients from Isfahan). The clinical specimens included sputum, trachea, abscess, and gastric lavage (Table S3, Supplementary file 1). Three clusters (n = 7), comprising the *M. bovis* lineage and two NEW-1 sub-lineages, were associated with isolates susceptible to INH, RIF, and EMB. In contrast, three clusters (n = 8), including LAM, H37Rv, and Delhi/CAS sub-lineages, contained isolates resistant to RIF or INH, with each cluster containing one resistant isolate (Table S4, Supplementary file 1).

Discussion

The MIRU-VNTR genotyping method has been widely utilized in numerous studies worldwide to assess the genetic diversity and transmission dynamics of MTB strains (24). Iran is located in the Middle East, bordering several countries with high rates of tuberculosis, including Turkmenistan, Afghanistan, Pakistan, and Armenia, Azerbaijan (25-28), and Tajikistan (26). Isfahan province is located in central Iran, and its capital, Isfahan, one of the largest cities in the country, also serves as a place of residence for a considerable number of Afghan immigrants.

The present study describes the genetic diversity of 81 MTB isolates, both drug susceptible and first- and second-line drug-resistant strains, obtained from PTB and EPTB patients over four years based on 24-locus MIRU-VNTR analysis. Our results showed that the Delhi/CAS and NEW-1 genotypes (sub-lineages) were the predominant genotype among both susceptible and drug-resistant isolates. UgandaI was the least frequent genotype in the

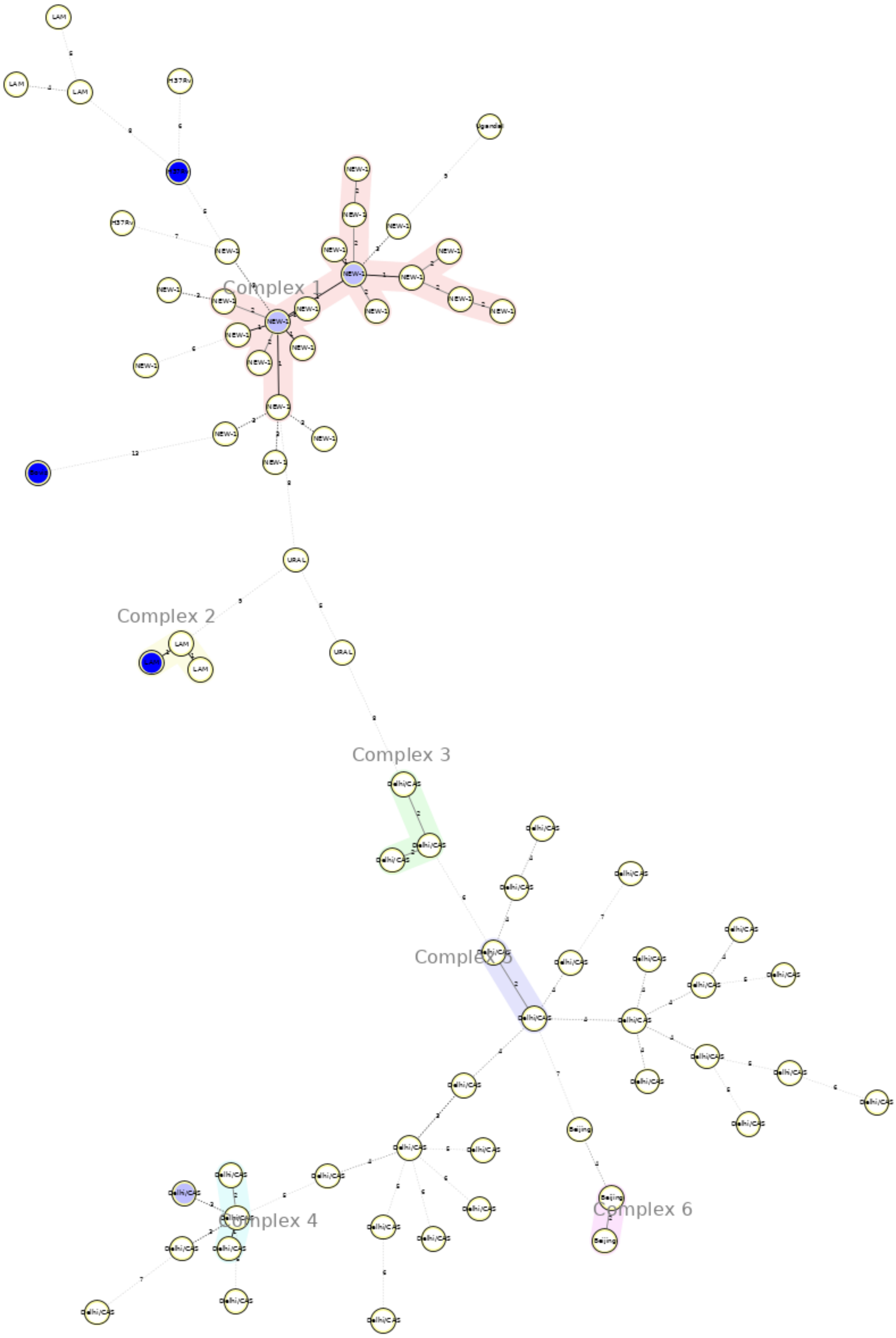


Figure 2. MST and Lineage Classification of 81 Mycobacterium Tuberculosis Complex 483 Isolates Genotyped Using 24-locus MIRU-VNTR Method. *Note*. MST: Minimum spanning tree; MIRU-VNTR: Mycobacterial interspersed repetitive units-Variable number tandem repeat

study, which was associated and was observed exclusively among susceptible isolates. Notably, of the eight identified genotypes, three—Beijing, *M. bovis*, and UgandaI—were found only in drug-susceptible isolates. In contrast, no genotype was found to be specific to drug-resistant isolates. Our results indicate that lineage 4 was the most prevalent, followed by lineages 3, 2, and the *M. bovis* lineage. Furthermore, the most prevalent sub-lineages were Delhi/CAS, NEW-1, LAM, H37Rv, Beijing, *M. bovis*, URAL, and UgandaI, respectively.

According to previous reports, countries in Central Asia and the Middle East, including India, Afghanistan, Saudi Arabia, and neighboring Pakistan, exhibit a high incidence of the Delhi/CAS genotype (23). Given Iran's shared borders with Pakistan and Afghanistan, along with the substantial Afghan immigrant population in Isfahan province, this may explain the predominance of the Delhi/CAS genotype in the central regions of the country.

Hadifar et al carried out a similar study in Tehran using 24-locus MIRU-VNTR genotyping (29) and reported Delhi/CAS and NEW-1 as the most prevalent genotypes, which aligns with the results of our study. Furthermore, Kargarpour Kamakoli et al recently reported similar results in Tehran (30).

A notable finding from a published systematic review by Hadifar et al is that the MTB genotypes NEW1, CAS, EAI, and T are the most predominant in Iran, while the West African, Cameroon, H37Rv, and TUR were reported at very low prevalence in Iran (< 2%) (31).

Skiba et al reported that the phylogeography of the NEW-1 genotype is specific to southern regions of Iran, suggesting a historical distribution of this genotype from Iran to Central and East Asia, likely via the Silk Road, where its historic traces were established (27). It may explain the high prevalence of the EW-1 genotype observed in Iran. Longitudinal studies from numerous countries have shown that the Beijing genotype constitutes an increasing proportion of global TB cases. Based on these reports, the Beijing genotype is the most dominant lineage in East Asia, accounting for over 50% of all studied isolates. In contrast to our results, the Beijing genotype has also been reported as predominant in Central, Northern, and Southeastern Asia, while representing only a minor proportion in Austral Africa, West, South, and Central Asia, Australasia, Northern Europe, and South and North America (32).

The Beijing genotype has also been associated with a higher risk of acquired antibiotic resistance, reduced protective efficacy of BCG vaccination, and increased disease severity (9,33,34). Among Taiwanese indigenous populations, a strong relationship has been reported between the Beijing lineage and MDR-TB (9,35). Furthermore, 39.0% of the Beijing isolates, representing 97.1% of the total isolates reported in South Korea, were obtained from patients with MDR-TB or XDR-TB (9,36). Contrary to the representation, we did not find a similar relationship in the present study, likely due to the limited number of Beijing lineage isolates identified.

Zenteno-Cuevas et al reported the H, T, and LAM genotypes as the most prevalent genotypes in Mexico (37), which does not support our findings. The difference can be attributed to the long geographical distance between the studied regions. Contrary to our findings, in which Delhi/CAS and NEW-1 were prevalent among MDR isolates, Tarashi et al, in a meta-Analysis using spoligotyping methods, reported a higher prevalence of the Haarlem and Beijing genotypes among MDR-TB patients in Iran (38). Additionally, the highest reported prevalence of multidrug resistance was observed in the CAS and Beijing genotypes (31). Notably, in the present study, only a small number of MDR isolates (n = 6) were identified, which limits the ability to determine dominant genotypes associated with drug resistance.

In countries with a low TB incidence, such as Australia, high genotypic diversity reflects the infections among foreign-born patients who were infected and migrated from around the globe to Australia, rather than local transmission (21,39,40). In contrast, reports from Russia and Asia have emphasized a higher degree of homoplasy within the Beijing strain family (20,36,41). Since the Beijing genotype is mainly prevalent in East Asia and the proportion of immigrants from these regions to Iran is relatively low, this genotype may have been underrepresented in the present study. Although previous studies have demonstrated a strong association between the Beijing genotype and drug-resistant strains in other countries, all three Beijing isolates identified in our study were not resistant, which can be attributed to the same reasons mentioned above.

Conclusion

The results of the present study indicate that the Delhi/CAS (Lineage 3) genotype was the predominant genotype circulating in the studied regions. In addition, the frequency of isoniazid-resistant isolates was significantly higher in the Delhi/CAS genotype compared with other genotypes. However, no genotype specifically associated with drug resistance was identified in this study.

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Supervision: Bahram Nasr Esfahani.
Writing-original draft: Marzieh Safari.
Writing-review & editing: Bahram Nasr Esfahani, Sharareh Moghim.

Competing Interests

The authors declare no conflict of interests.

Ethical Approval

The present study was ethically reviewed and approved by the Ethics Committee of Isfahan University of Medical Sciences under the ethical code IR.MUI.REC.1396.3.190.

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Supplementary Files

Supplementary file 1 contains Tables S1-S4.

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