



Genetic Variation of the *Mycobacterium tuberculosis* in North of Iran; the Golestan Province

Maya Babai Kochkaksaraei¹, Hami Kaboosi¹ and Ezzat Allah Ghaemi^{2,*}

¹Department of Microbiology, Ayatollah Amoli Branch, Islamic Azad University, Amol, Iran

²Infectious Diseases Research Center, Golestan University of Medical Sciences, Gorgan, Iran

*Corresponding author: Professor, Infectious Diseases Research Center, Golestan University of Medical Sciences, Gorgan, Iran. Email: eghaemi@yahoo.com

Received 2019 March 13; Revised 2019 June 10; Accepted 2019 July 28.

Abstract

Background: Genotyping of *Mycobacterium tuberculosis* (M.tub) is an essential step for several purposes, including the epidemiological studies and the tuberculosis (TB) control programs. Golestan province in the Southeast of the Caspian Sea is the second Iranian high burden TB province.

Objectives: This study aimed to determine the genetic diversity of M.tub isolates in Golestan province located in the north of Iran.

Methods: In this cross-sectional study, all non-repetitive confirmed M.tub isolates, which were collected from patients with TB in Golestan province in 2016, were used for genotyping. After DNA extraction, PCR was done for 15 loci of mycobacterial interspersed repetitive unit-variable-number of tandem repeats (MIRU-VNTRs) for the 162 M.tub isolates. Then genetic diversity and genetic relationships between them were assessed by best match analysis using tools on MIRU-VNTRplus database. Antibiotic susceptibility patterns of M.tub isolates was determined by the proportional method. Statistical analysis was done by SPSS and R software.

Results: Out of 162 M.tub isolates, 156 genotype patterns were obtained, which 150 of which were unique. Nine of 15 loci were highly discriminative. The clustering rates were 3.7%. The prevalence of polyclonal infection was 2.46%. Also, HGDI and AHGDI were 0.999 and 0.979, respectively. The New-1 (22.2%) and Beijing (13.6%) sub-lineages had the highest prevalence in this region. Although there was no significant correlation between demographic criteria and M.tub genotypes, still Beijing isolates showed more treatment failure (18.2% vs. 0.7%) and resistance to streptomycin (40.9% vs. 7.8%) compared to others ($P < 0.05$). The assessment of the minimum spanning tree showed that the members of the clonal complex were limited except for members of Beijing. The PCA analysis showed that the combination of Qub1b and Mtub30 loci was diagnostic for Beijing sub-lineage.

Conclusions: Owing to the high genotypic diversity of M.tub isolates in this region, relying on tuberculosis control program just based on close contact treatment is not sufficient, and we require to apply another approach.

Keywords: Genetic Diversity, Golestan Province, Iran, Minisatellite Repeats, MIRU VNTR Typing, *Mycobacterium tuberculosis*, Polymerase Chain Reaction, Variation

1. Background

Achieving the goals of the World Health Organization (WHO) to eradicate tuberculosis (TB) until 2035 requires the accurate identification of different types of *Mycobacterium tuberculosis* (M.tub) in different parts of the world (1). Molecular genotyping of M.tub is one of the valid methods for understanding TB transmission to trace outbreaks and investigate epidemiological relationships among TB cases in different locations and to discriminate the re-infection from reactivation (2).

Different genotypes of M.tub have different potential on pathogenicity, spreading rate, drug resistance, and different clinical characteristics. Genetic diversity determination of M.tub is important for understanding the trans-

mission dynamics of M.tub and effective controlling the emergence of drug-resistant strains. For each successful planning to control TB, it is necessary to identify the genotype pattern of the region, their drug resistance, and understand the way of TB transmission (3).

Among the various molecular genotyping techniques, the mycobacterial interspersed repetitive unit-variable-number of tandem repeats (MIRU-VNTRs) method is very popular due to the lack of complexity, small amounts of DNA requirement, digital format enabling the portability of results and creation of international databases for routine and research purposes in addition to low cost and repeatability (4). Application of 15 loci MIRU-VNTR method in developing countries is more attractive compared to 24 MIRU VNTR loci due to the simplicity of the procedure and

lowering the cost and time. The HGDI and clustering rates in both 15 and 24 loci MIRU-VNTR methods are very similar without a significant difference between them (5).

Health authorities of Iran had considerable successes in TB control over the last 50 years and accordingly the incidence of TB dropped from 142 cases in 1964 to 12.6 in 100,000 population in 2016 (6, 7). Golestan province is the second most prevalent TB area in Iran following the Sistan-Baluchestan province with an incidence rate of 38 to 45.5 cases per 100,000 population in 1999 to 2017 (8, 9). Most *M.tub* isolates in this area are sensitive to the first-line anti TB drug, and only about 2.3% of them are Multi Drug-resistant (MDR) (10).

It seems that the residents of Golestan province exposed to various types of *M.tub* strains due to the proximity to the high TB burden countries like Turkmenistan and Afghanistan. Also, the massive migration of job seekers from Sistan-Baluchestan province due to the requirement of manpower in agriculture, the migration and trafficking of Turkmens of Iran and Turkmenistan, high population density in some places, populous households (11), and ethnic and geographical diversity (8) increase the possibility of exposure to *M.Tub* strains. In a recent study, we found 13.9% Beijing strain in this area, and in another investigation, Mansoori et al. showed CAS/Delhi, NEW-1 and Beijing genotypes as the most prevalent genotypes in this region (12, 13).

2. Objectives

Determination of *M.tub* genotypes among Golestanian patients with TB in 2016 was the main objective of this study.

3. Methods

3.1. Sample Collection

In this cross-sectional study, 215 *M.tub* suspected samples were collected from TB suspected patients in Golestan province in the north of Iran in 2016. Fifty-three samples were excluded from the study due to repeated samples and Non-Tuberculosis Mycobacteria (NTM). The remaining 162 culture-positive Non-repetitive *M.tub* samples subjected to the evaluation by census strategy. Golestan Province with an area of 20,380 km², is one of the 31 provinces of Iran located in the southeast of the Caspian sea with almost 1.8 Million population residing in 14 cities. Grogan is the capital city of the province and Turkman, Fars, and Sistani-Baluch are the three prevalent ethnic group in this region (https://en.wikipedia.org/wiki/Golestan_Province).

According to the strategy of the Iranian ministry of public health, the sample of all suspected patients with TB was referred to the city's health center. Smear-positive samples were sent for culture process to Gonbad (in the east of province) or Gorgan health centers. The bacterial culture was performed on Lowenstein Jensen media after treating the sample by Petroff's method. Culture positive isolates subjected to biochemical tests for final confirmation (12) and antibiogram were carried out in the same Lab. Two months after treatment with 4 anti TB drugs, a new sample obtained from each patient and subjected to Ziehl-Neelsen method for the evaluation of treatment achievements. All these centers are funded and controlled by governmental health authorities. The mycobacterial colonies and demographic data of patients with TB in this study were gathered from the Gorgan health center. The study proposal was approved by the ethics committee of Golestan University of Medical Sciences (IR.GOUMS.REC.1394.288) and written informed consent was completed for each participant through their city health center. Based on the above arrangement and strategy, we included 162 *M.tub* isolates which collected from 162 patients with TB.

3.2. Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing on 162 isolates confirmed *M.tub* against four first-line anti-tuberculosis drugs was carried out by the proportional method (14).

3.3. Fifteen Loci MIRU-VNTR Analysis

The genomic DNA was extracted as described by the manufacturer and directly used for PCR-based analysis of 15 loci MIRU-VNTR typing. The PCR amplification for each locus was carried out with the specific primers and methods, according to the manufacturer's protocol (15) in a thermocycler (PeqLab, peqSTAR thermocycler, USA). The presence and size of PCR products for each locus were detected on 1.5% gel agarose electrophoresis. The number of repetitions of each locus was determined by the size of the amplicon and comparison with the H37RV PCR product of that locus, and finally, a 15-digit number was obtained for each locus. Each test was repeated at least two times. The H37RV strain and distilled water were used as a positive and negative control of the MIRU-VNTR method, respectively. For quality control, some of the PCR products were sent for sequencing by the MacroGen company and results were compared to standard defined results, according to NCBI BLAST. For further analysis, the 15-digit number of an allelic profile of each *M.tub* isolate was surveyed in the MIRU-VNTRplus database, available at <http://www.miru-vntrplus.org>. Lineage identification was made by the best match analysis and tree-based identification with tools

on the MIRU-VNTRplus database. The genetic distance between each genotype was compared with the data from reference strains with a distance of < 0.3 (16).

In order to investigate the phylogenetic relationships within the sample and identify clonal complexes, a plot of the minimum spanning tree (MST) was constructed using the MIRU-VNTRplus database with 15 loci based on (SLV) and double locus variation (DLV).

3.4. Statistical Analysis

The Chi-square, Fisher’s exact test, and ANOVA were used for statistical analysis of data by the SPSS Statistics Software for Windows, version 18.0 (SPSS Corp, Chicago, Ill., USA). The Hunter-Gaston discriminatory index (HGDI) was calculated as described by Sola et al. (17) and Adjusted HGDI by Mokrousov 2017 suggestion (18). Genetic relationships among isolates were assessed applying the categorical coefficient and UPGMA. Based on a distance cut-off of 0 and the same patterns in 15 loci, a cluster was defined, and clustering rates were calculated as already described (19). The PCA was performed in package FactoMineR of R software (https://www.r-project.org) to determine which group of MIRU-VNTR loci could identify a specific M.tub sub-lineage (20).

4. Results

4.1. Clinical Isolates

Among 215 M.tub suspected isolates that were evaluated in 2016, 199 isolated strains from 162 patients were confirmed as M.tub, according to biochemical test results and 16 isolates were NTM. In cases, which isolated more than one M.tub, only one isolate was selected for this study. Finally, 162 M.tub, which isolated from 162 patients with TB, were studied. The demographic data of these patients are presented in Tables 1 and 2. In brief, the mean age of the patients was 50.3 ± 19.3 years, 50% were female, and 92.6% had pulmonary TB. Most patients (87 cases, 53.7%) belonged to Fars race. Only two, four, and one M.tub isolates were resistant to isoniazid (INH), rifampicin (RIF), and Ethambutol (ETB), respectively but 20 (12.3%) cases were resistant to streptomycin (STP).

4.2. Allele Frequencies of the Isolates

In this study, 156 different patterns of MIRU-VNTR were detected in 162 M.tub isolates. Moreover, 150 unique patterns and 6 clusters (each of clusters with two members) were determined. The clustering rate was 3.7%, and none of the patients within a cluster belonged to a family; they lived in different parts of the province. The phylogenetic relationship of these isolates is presented in Figure 1.

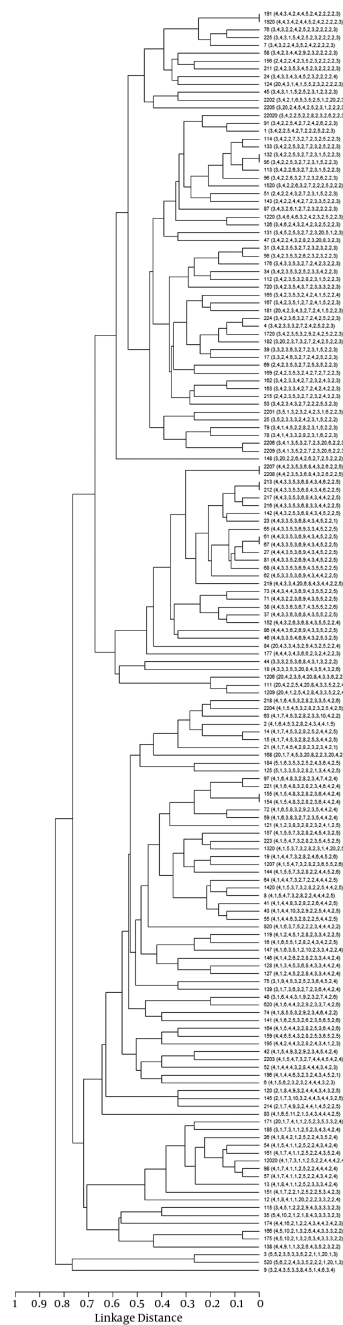


Figure 1. Phylogenetic tree and genetic relationships among of 162 M. tuberculosis isolates from Golestan province, north of Iran.

The maximum copy number of tandem repeat in a locus was 16 repeats for MIRU 10, which was isolated from a 14-year-old girl in Gorgan city. The MIRU 10 and MIRU 26 loci with 11 variable alleles and MIRU 04 and MTUB 30 with only three variable alleles were shown the maximum

Table 1. The Prevalence of Different Sub-Lineage of MTB in Golestan Province Based on Demographic Data

| Sub-Lineage | No. (%) | Age, y ^a | Single ^b | Sex, Male (%) ^c | Urban ^d | Race, % | | | |
|------------------|------------------|---------------------|---------------------|----------------------------|--------------------|-------------|-------------|-------------|------------|
| | | | | | | Fars | Sistani | Turkman | other |
| Beijing | 22 (13.58) | 41.57 | 4 (19) | 13 (61.9) | 11 (52.4) | 52.4 | 38.1 | 9.5 | 0.0 |
| Cameron | 4 (2.47) | 41.00 | 1 (25) | 1 (25) | 3 (75) | 100.0 | 0.0 | 0.0 | 0.0 |
| Delhi/CAS | 15 (9.26) | 55.54 | 1 (7.1) | 7 (50) | 9 (64.3) | 78.6 | 0.0 | 21.4 | 0.0 |
| New-1 | 36 (22.22) | 54.67 | 2 (5.4) | 21 (56.8) | 21 (56.8) | 51.4 | 32.4 | 13.5 | 2.7 |
| Tur | 1 (0.62) | 57.00 | 0 | 1 (100) | 0 | 100.0 | 0.0 | 0.0 | 0.0 |
| Multiple matches | 2 (1.23) | 42.67 | 0 | 0 | 1 (100) | 100.0 | 0.0 | 0.0 | 0.0 |
| Ural | 3 (1.85) | 34.00 | 0 | 3 (100) | 1 (33.3) | 66.7 | 0.0 | 33.3 | 0.0 |
| Unknown | 79 (48.77) | 50.70 | 7 (8.5) | 35 (42.7) | 41 (50) | 46.3 | 36.6 | 14.6 | 2.4 |
| Total | 162 (100) | 50.33 | 15 (9.2) | 81 (49.7) | 87 (53.4) | 53.4 | 30.7 | 14.1 | 1.8 |
| P value | | 0.2 | 0.9 | 0.2 | | 0.7 | | | |

^aThe ages average (years) in patients with TB based on sub-linages of *M. tuberculosis*.

^bNo. (%) of no married patients with TB.

^cNo. (%) of males.

^dPeople who live in city for each sub-lineage group.

Table 2. The Frequency of *M. tuberculosis* Sub-Linages in Golestan Province Based on Laboratory Findings and Anti-TB Drug Resistance

| Sub-Lineage | Smear Negative ^a | 2, 3+ Before ^b | 2, 3+ After ^c | % Resistance to | | | |
|--------------------|-----------------------------|---------------------------|--------------------------|-----------------|-----------------|-----------------|--------------|
| | | | | INH | RIF | ETB | STP |
| Beijing (22) | 9.1 | 63.6 | 18.2 | 0 | 0 | 0 | 40.9 |
| Cameron (4) | 0 | 75 | 0 | 0 | 0 | 0 | 0 |
| Delhi/CAS (15) | 20 | 20 | 0 | 0 | 6.7 | 0 | 0 |
| New-1 (36) | 11.1 | 33.3 | 0 | 0 | 0 | 0 | 5.6 |
| Tur (1) | 0 | 100 | 0 | 0 | 0 | 0 | 0 |
| Multiple match (2) | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Ural (3) | 0 | 100 | 0 | 0 | 0 | 0 | 0 |
| Unknown (79) | 13.9 | 37.9 | 1.3 | 2.5 | 3.8 | 1.3 | 11.4 |
| P value | > 0.5 | 0.09 | 0.009 | > 0.5 | > 0.5 | > 0.5 | 0.01* |
| Total (162) | 12.3 | 40.7 | 3.1 | 1.2 | 2.5 | 0.6 | 12.3 |

Abbreviations: ETB, Ethambutol; INH, Isoniazid; RIF, rifampicin; STP, Streptomycin.

^aThe percent of smear negative TB patients in the time of diagnosis

^bThe percent of two or three plus smear positive TB patients in the time of diagnosis

^cThe percent of two or three plus smear positive TB patients after two months antiTB treatment

and minimum allelic variation in this study, respectively. Also, MTUB04, MIRU10, MIRU26, MTUB21, QUB 26, MIRU31, QUB4156, MIRU16, and ETRA were highly discriminative ($h \geq 0.6$) and only MIRU04 ($h < 0.3$) showed low discriminatory power. The HGDI and AHGDI for 15 loci MIRU-VNTR typing in this study were 0.999 and 0.979, respectively (Table 3).

4.3. Clonal and Polyclonal Infections

In four *M.tub* isolates, 2 bands were found at least in one locus. In three isolates, copy number variation has

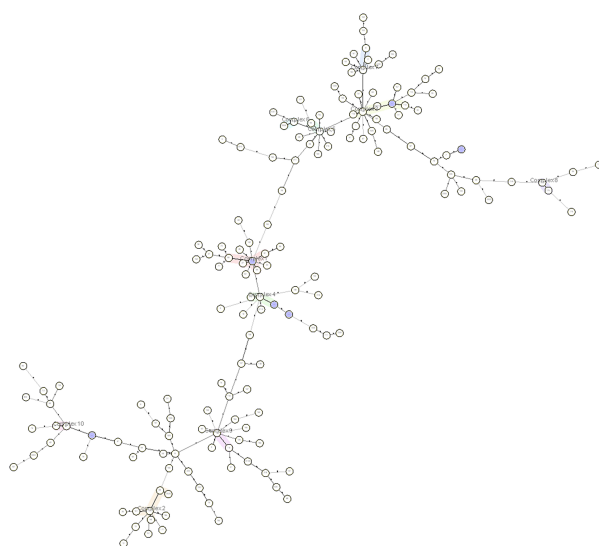
been seen on MIRU26, Qub26, and ETRC and in one isolate; both *Mtub*21 and *Mtub*39 have two bands; thus the frequency of clonal and polyclonal infections was 2.46% in this population.

4.4. The Minimum Spanning Tree

In the single locus variation (SLV) minimum spanning tree, ten clonal complexes (CCs), including 32 isolates, were determined. The largest CC contained six isolates (Figure 2). In DLV, 14 CCs were determined with 72 isolates, which the largest CC contained 21 isolates (Data not shown).

Table 3. Global HGDI and HGDI of Each 15 MIRU VNTR Loci in Sub-Lineages of MTB

| | Global HGDI | Beijing | Cameron | Delhi | New1 | Unknown | Ural |
|---------|-------------|---------|---------|-------|------|---------|------|
| MTUB04 | 0.8 | 0.57 | 0 | 0.69 | 0.61 | 0.8 | 0 |
| ETRC | 0.57 | 0.09 | 0 | 0 | 0.15 | 0.57 | 0.67 |
| MIRU04 | 0.04 | 0 | 0 | 0 | 0 | 0.09 | 0 |
| MIRU40 | 0.51 | 0.27 | 0.67 | 0 | 0.41 | 0.63 | 0.67 |
| MIRU10 | 0.82 | 0.27 | 0.67 | 0.61 | 0.3 | 0.88 | 0 |
| MIRU16 | 0.71 | 0.25 | 0.67 | 0.38 | 0.56 | 0.72 | 0 |
| MTUB21 | 0.78 | 0.57 | 0.5 | 0.4 | 0.57 | 0.8 | 0 |
| QUB11B | 0.46 | 0 | 0.5 | 0 | 0.05 | 0.42 | 0.67 |
| ETRA | 0.6 | 0 | 0.67 | 0 | 0.16 | 0.62 | 0.67 |
| MTUB30 | 0.4 | 0.18 | 0 | 0 | 0 | 0.35 | 0.67 |
| MIRU26 | 0.78 | 0.42 | 0.67 | 0.73 | 0.7 | 0.81 | 0 |
| MIRU31 | 0.73 | 0.27 | 0 | 0.58 | 0.11 | 0.78 | 0.67 |
| MTUB39 | 0.55 | 0 | 0.83 | 0.6 | 0.56 | 0.58 | 0.67 |
| QUB26 | 0.79 | 0.57 | 0.5 | 0.38 | 0.46 | 0.78 | 0.67 |
| QUB4156 | 0.64 | 0.09 | 0 | 0.38 | 0.16 | 0.73 | 0 |

**Figure 2.** Minimum spanning tree (MST) of 162 *M. tuberculosis* clinical isolates in Golestan province, north of Iran. Clonal complexes are highlighted on the tree (CCI-10).

4.5. Distribution of Identified Sub-Lineages

The prevalence of sub-lineages of 162 *M. tub* isolates based on MIRU-VNTR_{plus} online software and their distribution according to demographic criteria are reported in Table 2. *M. tub* isolates in this region were classified into six known sub-lineages, and more than 48% of isolates were unknown. New-1 and Beijing sub-lineages, including 36 (22.2%) and 22 (13.6%) isolates, were the most prevalent known lineages, respectively. Genotypes from two isolates

(1.2%) had multiple matches, clarifying that their MIRU-VNTR patterns belonged to two or more families by MIRU-VNTR.

HGDI of each locus for sub-lineages is presented in Table 3. None of the loci had high HGDI for Beijing, but in Delhi/CAS and new-1 sub-lineages, MIRU26 and Cameroon MTUB39 showed the highest discriminatory power. In evaluating MST among isolates that belong to Beijing sub-lineage, our data showed that almost all isolates indicated

one CC, but for other sub-lineages more variation on CC occurred. In the evaluation by PCA assay, the individual plot distinguished the Beijing and the Delhi/CAS sub-lineage that formed two distinct groups, while other families were classified into a cloud. The combination of loci Qub11b and Mtub30 was diagnostic for Beijing sub-lineage, and MIRU26, MIRU40, MIRU16, Qub4156 were diagnostic for Delhi/CAS sub-lineage in this area (Figure 3).

4.6. Sputum Smear Results Among MTB Genotypes

Totally, 12.3% of patients had smear-negative results at the primary diagnostic step. This feature in patients infected by Delhi/CAS strain was 21.4%. Sixty-one percent of Beijing and 75% of Cameron were 2+ or 3+ in primary diagnosis. The evaluation of sputum smear in two months after the treatment showed that in 14.3% of Beijing strains smear remained positive, which is significantly more than other M.tub sub-lineages (Table 4).

4.7. Drug Resistance Among M.tub Genotypes

MDR strain was not diagnosed among 162 M.tub isolates. Only two, four, and one M.tub isolates were resistant to INH, RIF, and ETB, respectively. Twenty isolates (12.3%) were resistant to streptomycin. Resistance to streptomycin in Beijing lineage was statistically more than other groups ($P = 0.02$), but there was no significant difference between resistance to other drugs and M.tub sub-lineages.

5. Discussion

The presence of 150 unique MIRU-VNTR patterns among 162 isolates (92.6%), confirmed that 15 loci MIRU-VNTR method had enough power to differentiate M.tub isolates and on the other hand, showed that M.tub isolates in northern Iran had great diversity and dispersion. This finding is similar to previous studies conducted by this method in Iran (21-23), except for the Zamani et al. study which found about 70% unique patterns in isolates from three different provinces in Iran (24).

If we consider each cluster as a recent transmission of M.tub (25), this finding showed that the recent transmission is limited in Golestan province. The high frequency of unique patterns indicates that TB reactivation may play an important role in the spread of TB in the region (5). The high diversity of M.tub genotypes in our region may be due to high migration from other provinces such as Sistan-Baluchestan to this area, significant traffic of the inhabitants of this region to Turkmenistan country or the ethnic diversity in this area. A previous study in Sistan-Baluchestan province shows that the diversity of unique

patterns in this area is more than other provinces, which is consistent with our findings (24).

Mokrousov et al. concluded that HGDI overestimates the discriminatory power of a typing method and suggests increasing the HGDI's discriminatory power to more than 0.95 and the use of the Adjusted Hunter Gaston Index (18). In the present study, HGDI and AHGDI for 15 loci MIRU-VNTR typing method was 0.999 and 0.979, respectively, which is consistent with the results of other studies and confirms the ability of this method to effectively distinguish M.tub genotypes for subsequent studies (26-28).

The prevalence of mixed infection, which is especially important in the cases of MDR strain (29) in this study, was 2.46% (4 out of 162 cases) which is similar to a large population study in the Netherland with 2.2% mixed infection (30); however, in the study of Nathavitharana this prevalence was about 9% (31), which is much higher than our region. In this study, MIRU10 and some other loci had $h > 0.78$, which is similar to a recent study in Tehran (21) and is in contrast to Alonso-Rodriguez et al. which they found MIRU10, locus had the lowest levels of HGDI (27). On the other hand, only MIRU04 locus showed very low allele variation in this study that is similar to others (28, 32, 33).

One of the important statistical methods used to identify the importance of different locus at the level of the lineage and sub-lineage is the PCA method. The Beijing sub-lineage could be differentiated from the other lineage with Qub11b and Mtub30 loci. Also, the Delhi/CAS lineage distinguishing markers are MIRU26, MIRU40, MIRU16, and Qub4156 loci. In the case of another lineage, the particular locus did not show this discrimination power. The loci that helped to distinguish the Beijing strains in this region were different from those reported by Rasoahanitralisoa et al. and Mokrousov et al. (18, 34). Whether this difference is due to differences in genotypes of this lineage or other factors affecting it should be investigated in future studies.

In the present study, the New-1 sub-lineage with 22.2% was reported as the most prevalent sub-lineage. Similar data described in Azimi et al. study in Tehran (22.5%) and Mansoori study in this region (13, 21). The Delhi/CAS sub-lineage is prevalent in the countries of the Middle East and in the countries of the Silk Road, which is why it is also abundant in Iran. The prevalence of Delhi/CAS sub-lineage in studies conducted by Mansoori et al. and Feyisa et al. were more than our findings (13, 35). The relatively high frequency of Beijing family in Golestan province also has been reported in previous studies (12, 13) and its distribution remained constant over the last five years. Similar to the Mokrousov et al. study, our finding showed that almost all of the Beijing isolates were located in one clonal complex (18), indicating that Beijing family has recently expanded and there is still no chance for differentiation and

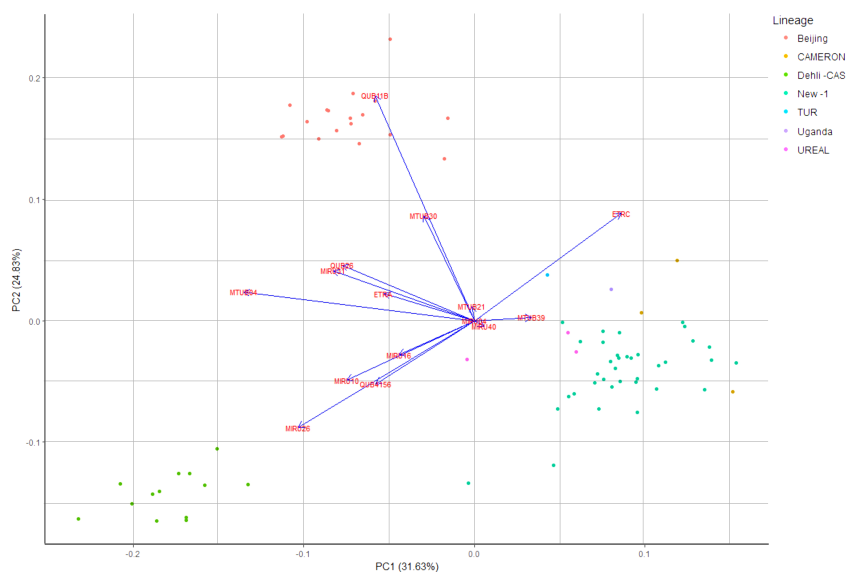


Figure 3. A two dimensional scatter plot-based PCA, based on 15 MIRU-VNTR individual alleles.

Table 4. Allelic Diversity Among 162 *M. tuberculosis* Isolates in South East Caspian Sea

| Tandem Repeat | Locus | | | | | | | | | | | | | | |
|---------------|-------|------|---------|---------|---------|---------|---------|--------|---------|---------|---------|---------|---------|---------|---------|
| | ETRA | ETRC | MIRU 10 | MIRU 16 | MIRU 26 | MIRU 40 | QUB 11b | QUB 26 | MTUB 30 | MTUB 39 | MTUB 04 | MTUB 21 | QUB4156 | MIRU 04 | MIRU 31 |
| 0 | 8 | 3 | - | - | - | 1 | 5 | - | - | - | 4 | 1 | 4 | - | - |
| 1 | - | 63 | 6 | 6 | 15 | 17 | 3 | - | - | 2 | 20 | 2 | 3 | 3 | 3 |
| 2 | 9 | 1 | 50 | 34 | 10 | 12 | 116 | 2 | 118 | 21 | 45 | 15 | 84 | 158 | 10 |
| 3 | 55 | 4 | 34 | 64 | 5 | 110 | 7 | 3 | 4 | 102 | 33 | 21 | 17 | 1 | 63 |
| 4 | 85 | 84 | 12 | 50 | 21 | 19 | 4 | 10 | 40 | 28 | 35 | 48 | 43 | - | 32 |
| 5 | 5 | 6 | 17 | 7 | 66 | 3 | 1 | 27 | - | 7 | 18 | 51 | 10 | - | 43 |
| 6 | - | 1 | 17 | 1 | 18 | - | 26 | 7 | - | - | 7 | 18 | 1 | - | 10 |
| 7 | - | - | 15 | - | 13 | - | - | 35 | - | 2 | - | 4 | - | - | 1 |
| 8 | - | - | 5 | - | 8 | - | - | 56 | - | - | - | 1 | - | - | - |
| 9 | - | - | 2 | - | 3 | - | - | 21 | - | - | - | - | - | - | - |
| 10 | - | - | 3 | - | 2 | - | - | 1 | - | - | - | 1 | - | - | - |
| 11 | - | - | - | - | 1 | - | - | - | - | - | - | - | - | - | - |
| 16 | - | - | 1 | - | - | - | - | - | - | - | - | - | - | - | - |
| Total | 5 | 7 | 11 | 6 | 11 | 6 | 7 | 9 | 3 | 6 | 7 | 10 | 7 | 3 | 7 |
| h | 0.6 | 0.57 | 0.82 | 0.71 | 0.78 | 0.51 | 0.46 | 0.79 | 0.4 | 0.55 | 0.8 | 0.78 | 0.64 | 0.04 | 0.73 |

the emergence of new genotypes in our region.

On the contrary to the expectation, the Beijing strain in this region did not show resistance to isoniazid and rifampicin, but 14% of those infected with Beijing isolates had a positive smear at the end of the second month of anti TB therapy. This indicated that treatment failure in Beijing *M.tub* is certainly more than other lineages. The findings of this study also indicated that in the most infected people with Beijing sub-lineage, TB was diagnosed later than usual, and patients mostly identified with 2+ or 3+ positive smears. Research to find out the cause of this phe-

nomenon is one of the most interesting topics for future studies. Among the known sub-lineages, more attention should be given to Beijing due to the relatively high prevalence, late detection and treatment failure and Delhi/CAS due to the high levels of smear-negative TB and resistance to rifampin.

More than 48% of the isolates did not match with the known pattern on the MIRU VNTRplus site (with distance < 0.3). The abundance of unknown genotypes suggests that new types of MTB may develop in the country, which needs to be identified. These isolates are distributed in the differ-

ent parts of the Golestan province and are not clustered. These isolates did not have a specific resistance pattern. For future studies, the identification of these isolates at the lineage or sub-lineage level is proposed by whole genome sequencing.

5.1. Conclusions

The analysis of data showed a high proportion of unique patterns and limited recent transmission among *M.tub* isolates in this region. These data reflect the high degree of *M. tuberculosis* diversity circulating within the autochthonous populations of Golestan province. Taken together, the low frequency of clusters, the low population in each clonal complex, and the high frequency of MIRU-VNTR patterns indicated that in the Golestan province, the transmission of person-to-person has a weak role in the spread of tuberculosis. Therefore, it seems that depending on the patient, close contact diagnosis, and the treatment as the sole TB control program is not enough to achieve the goals of eradicating tuberculosis in 2035 by the WHO, and it is necessary to take steps toward preventing the activation of the latent TB in the society.

Acknowledgments

This research is part of a Ph.D. thesis in Microbiology that was partly financed by the Infectious Diseases Research Center, Golestan University of Medical Sciences, Iran. We are sincerely grateful to Hesamaddin Shirzad-Aski, Maryam Shafipour, Masoumeh Taziki, Nasser Behnampour, and Samin Zamani for their kind help and cooperation.

Footnotes

Authors' Contribution: All authors participated in the writing and revision of this manuscript.

Conflict of Interests: There is no conflict of interest in this paper.

Ethical Approval: The study proposal was approved by the Ethics Committee of Golestan University of Medical Sciences (IR.GOUMS.REC.1394.288).

Funding/Support: This research is part of a Ph.D. thesis in Microbiology that was partly financed by the Infectious Diseases Research Center, Golestan University of Medical Sciences, Iran.

Patient Consent: Written informed consent has been obtained from the participants by their related city health center.

References

1. World Health Organization. *Global strategy and targets for tuberculosis prevention, care and control after 2015*. 2015. Available from: https://www.who.int/tb/post2015_strategy/en/.
2. Niemann S, Supply P. Diversity and evolution of Mycobacterium tuberculosis: Moving to whole-genome-based approaches. *Cold Spring Harb Perspect Med*. 2014;**4**(12). a021188. doi: 10.1101/cshperspect.a021188. [PubMed: 25190252]. [PubMed Central: PMC4292095].
3. Lopez-Avalos G, Gonzalez-Palomar G, Lopez-Rodriguez M, Vazquez-Chacon CA, Mora-Aguilera G, Gonzalez-Barrios JA, et al. Genetic diversity of Mycobacterium tuberculosis and transmission associated with first-line drug resistance: A first analysis in Jalisco, Mexico. *J Glob Antimicrob Resist*. 2017;**11**:90-7. doi: 10.1016/j.jgar.2017.07.004. [PubMed: 28760681].
4. Nikolayevskyy V, Trovato A, Broda A, Borroni E, Cirillo D, Drobniowski F. MIRU-VNTR genotyping of Mycobacterium tuberculosis strains using QIAxcel technology: A multicentre evaluation study. *PLoS One*. 2016;**11**(3). e0149435. doi: 10.1371/journal.pone.0149435. [PubMed: 26939051]. [PubMed Central: PMC4777483].
5. Perdigo J, Clemente S, Ramos J, Masakidi P, Machado D, Silva C, et al. Genetic diversity, transmission dynamics and drug resistance of Mycobacterium tuberculosis in Angola. *Sci Rep*. 2017;**7**:42814. doi: 10.1038/srep42814. [PubMed: 28230095]. [PubMed Central: PMC5322374].
6. Moosazadeh M, Nasehi M, Bahrapour A, Khanjani N, Sharafi S, Ahmadi S. Forecasting tuberculosis incidence in Iran using box-jenkins models. *Iran Red Crescent Med J*. 2014;**16**(5). e11779. doi: 10.5812/ircmj.11779. [PubMed: 25031852]. [PubMed Central: PMC4082512].
7. Marvi A, Asadi-Aliabadi M, Darabi M, Rostami-Maskopae F, Siamian H, Abedi G. Silent changes of tuberculosis in Iran (2005-2015): A joint-point regression analysis. *J Family Med Prim Care*. 2017;**6**(4):760-5. doi: 10.4103/jfmpc.jfmpc_190_17. [PubMed: 29564259]. [PubMed Central: PMC5848394].
8. Salek S, Salek S, Emami H, Masjedi MR, Velayati AA. Epidemiologic status of tuberculosis in Golestan province. *Tanaffos*. 2008;**7**(3):63-8.
9. Zahedi Bialvaei A, Asgharzadeh M, Aghazadeh M, Nourazarian M, Samadi Kafil H. Challenges of tuberculosis in Iran. *Jundishapur J Microbiol*. 2017;**10**(3). e37866. doi: 10.5812/jjm.37866.
10. Javid SN, Ghaemi EA, Amirmozaffari N, Rafiee S, Moradi A, Dadgar T. [Detection of isoniazid and rifampin resistant strain of Mycobacterium tuberculosis isolated from patients in Golestan province (North of Iran)]. *Med Lab J*. 2009;**3**(1). Persian.
11. Rafiee S, Besharat S, Jabbari A, Golalipour F, Nasermoaadeli A. Epidemiology of tuberculosis in northeast of Iran: A population-based study. *Iran J Med Sci*. 2009;**34**(3):193-7.
12. Erie H, Kaboosi H, Javid N, Shirzad-Aski H, Taziki M, Kochkarsaraei MB, et al. The high prevalence of Mycobacterium tuberculosis Beijing strain at an early age and extra-pulmonary tuberculosis cases. *Iran J Microbiol*. 2017;**9**(6):312-7. [PubMed: 29487728]. [PubMed Central: PMC5825930].
13. Mansoori N, Yaseri M, Vaziri F, Douraghi M. Genetic diversity of Mycobacterium tuberculosis complex isolates circulating in an area with high tuberculosis incidence: Using 24-locus MIRU-VNTR method. *Tuberculosis (Edinb)*. 2018;**112**:89-97. doi: 10.1016/j.tube.2018.08.003. [PubMed: 30205974].
14. Canetti G, Fox W, Khomenko A, Mahler HT, Menon NK, Mitchison DA, et al. Advances in techniques of testing mycobacterial drug sensitivity, and the use of sensitivity tests in tuberculosis control programmes. *Bull World Health Organ*. 1969;**41**(1):21-43. [PubMed: 5309084]. [PubMed Central: PMC2427409].
15. Supply P, Allix C, Lesjean S, Cardoso-Oelemann M, Rusch-Gerdes S, Willery E, et al. Proposal for standardization of optimized mycobacterial interspersed repetitive unit-variable-number tandem repeat typing of Mycobacterium tuberculosis. *J Clin Microbiol*. 2006;**44**(12):4498-510. doi: 10.1128/JCM.01392-06. [PubMed: 17005759]. [PubMed Central: PMC1698431].

16. Aze J, Sola C, Zhang J, Lafosse-Marin F, Yasmin M, Siddiqui R, et al. Genomics and machine learning for taxonomy consensus: The Mycobacterium tuberculosis complex paradigm. *PLoS One*. 2015;**10**(7):e0130912. doi: [10.1371/journal.pone.0130912](https://doi.org/10.1371/journal.pone.0130912). [PubMed: [26154264](https://pubmed.ncbi.nlm.nih.gov/26154264/)]. [PubMed Central: [PMC4496040](https://pubmed.ncbi.nlm.nih.gov/PMC4496040/)].
17. Sola C, Filliol I, Legrand E, Lesjean S, Loch C, Supply P, et al. Genotyping of the Mycobacterium tuberculosis complex using MIRUs: Association with VNTR and spoligotyping for molecular epidemiology and evolutionary genetics. *Infect Genet Evol*. 2003;**3**(2):125–33. [PubMed: [12809807](https://pubmed.ncbi.nlm.nih.gov/12809807/)].
18. Mokrousov I. Revisiting the Hunter Gaston discriminatory index: Note of caution and courses of change. *Tuberculosis (Edinb)*. 2017;**104**:20–3. doi: [10.1016/j.tube.2017.02.002](https://doi.org/10.1016/j.tube.2017.02.002). [PubMed: [28454645](https://pubmed.ncbi.nlm.nih.gov/28454645/)].
19. Wang J, Liu Y, Zhang CL, Ji BY, Zhang LZ, Shao YZ, et al. Genotypes and characteristics of clustering and drug susceptibility of Mycobacterium tuberculosis isolates collected in Heilongjiang Province, China. *J Clin Microbiol*. 2011;**49**(4):1354–62. doi: [10.1128/JCM.02274-10](https://doi.org/10.1128/JCM.02274-10). [PubMed: [21325562](https://pubmed.ncbi.nlm.nih.gov/21325562/)]. [PubMed Central: [PMC3122865](https://pubmed.ncbi.nlm.nih.gov/PMC3122865/)].
20. [No Author Listed]. *Team RC A language and environment for statistical computing. The R project for statistical computing*. Vienna, Austria; 2015. Available from: <https://www.r-project.org/>.
21. Azimi T, Nasiri MJ, Zamani S, Hashemi A, Goudarzi H, Imani Fooladi AA, et al. High genetic diversity among Mycobacterium tuberculosis strains in Tehran, Iran. *J Clin Tuberculosis Other Mycobacterial Dis*. 2018;**11**:1–6. doi: [10.1016/j.jctube.2018.01.001](https://doi.org/10.1016/j.jctube.2018.01.001).
22. Zaniani FR, Moghim S, Esfahani BN. Genetic diversity of drug-resistant Mycobacterium tuberculosis isolates in Isfahan province of Iran. *Adv Biomed Res*. 2018;**7**:23. doi: [10.4103/2277-9175.225594](https://doi.org/10.4103/2277-9175.225594). [PubMed: [29531921](https://pubmed.ncbi.nlm.nih.gov/29531921/)]. [PubMed Central: [PMC5840967](https://pubmed.ncbi.nlm.nih.gov/PMC5840967/)].
23. Baghbanian M, Zandi H, Zamani S, Javadpour S, Hamzehloo GR, Feizabadi MM. MIRU-VNTR analysis of Mycobacterium tuberculosis from Tehran, Sistan-Baluchestan, Kermanshah and Hormozgan during 2014 and 2015. *Cell Mol Biol (Noisy-le-grand)*. 2017;**63**(12):14–21. doi: [10.14715/cmb/2017.63.12.5](https://doi.org/10.14715/cmb/2017.63.12.5). [PubMed: [29307335](https://pubmed.ncbi.nlm.nih.gov/29307335/)].
24. Zamani S, Aflaki M, Fooladi AA, Darban-Sarokhalil D, Bameri Z, Khazaei S, et al. MIRU-VNTR analysis of the Mycobacterium tuberculosis isolates from three provinces of Iran. *Scand J Infect Dis*. 2013;**45**(2):124–30. doi: [10.3109/00365548.2012.717233](https://doi.org/10.3109/00365548.2012.717233). [PubMed: [22954102](https://pubmed.ncbi.nlm.nih.gov/22954102/)].
25. Khosravi AD, Shahraki AH, Dezfuli SK, Hashemzadeh M, Goodarzi H, Mohajeri P. Genetic diversity of multidrug-resistant Mycobacterium tuberculosis strains isolated from tuberculosis patients in Iran using MIRU-VNTR technique. *Kaohsiung J Med Sci*. 2017;**33**(11):550–7. doi: [10.1016/j.kjms.2017.06.011](https://doi.org/10.1016/j.kjms.2017.06.011). [PubMed: [29050672](https://pubmed.ncbi.nlm.nih.gov/29050672/)].
26. Zhou A, Nawaz M, Xue X, Karakousis PC, Yao Y, Xu J. Molecular genotyping of Mycobacterium tuberculosis in Xi'an, China, using MIRU-VNTR typing. *Int J Tuberc Lung Dis*. 2011;**15**(4):517–22. doi: [10.5588/ijtld.10.0495](https://doi.org/10.5588/ijtld.10.0495). [PubMed: [21396212](https://pubmed.ncbi.nlm.nih.gov/21396212/)].
27. Alonso-Rodriguez N, Martinez-Lirola M, Herranz M, Sanchez-Benitez M, Barroso P, Indal-Tb group, et al. Evaluation of the new advanced 15-loci MIRU-VNTR genotyping tool in Mycobacterium tuberculosis molecular epidemiology studies. *BMC Microbiol*. 2008;**8**:34. doi: [10.1186/1471-2180-8-34](https://doi.org/10.1186/1471-2180-8-34). [PubMed: [18339198](https://pubmed.ncbi.nlm.nih.gov/18339198/)]. [PubMed Central: [PMC2291470](https://pubmed.ncbi.nlm.nih.gov/PMC2291470/)].
28. Afaghi-Gharamaleki A, Moaddab S, Darbouy M, Ansarin K, Hanifian S. Determining the risk of intra-community transmission of tuberculosis in the northwest of Iran through 15 Loci Miru-Vntr typing. *Eur J Microbiol Immunol (Bp)*. 2017;**7**(1):46–54. doi: [10.1556/1886.2016.00033](https://doi.org/10.1556/1886.2016.00033). [PubMed: [28386470](https://pubmed.ncbi.nlm.nih.gov/28386470/)]. [PubMed Central: [PMC5372480](https://pubmed.ncbi.nlm.nih.gov/PMC5372480/)].
29. Cohen T, van Helden PD, Wilson D, Colijn C, McLaughlin MM, Abubakar I, et al. Mixed-strain mycobacterium tuberculosis infections and the implications for tuberculosis treatment and control. *Clin Microbiol Rev*. 2012;**25**(4):708–19. doi: [10.1128/CMR.00021-12](https://doi.org/10.1128/CMR.00021-12). [PubMed: [23034327](https://pubmed.ncbi.nlm.nih.gov/23034327/)]. [PubMed Central: [PMC3485752](https://pubmed.ncbi.nlm.nih.gov/PMC3485752/)].
30. Jajou R, Kamst M, van Hunen R, de Zwaan CC, Mulder A, Supply P, et al. Occurrence and nature of double alleles in variable-number tandem-repeat patterns of more than 8,000 Mycobacterium tuberculosis complex isolates in The Netherlands. *J Clin Microbiol*. 2018;**56**(2). doi: [10.1128/JCM.00761-17](https://doi.org/10.1128/JCM.00761-17). [PubMed: [29142049](https://pubmed.ncbi.nlm.nih.gov/29142049/)]. [PubMed Central: [PMC5786718](https://pubmed.ncbi.nlm.nih.gov/PMC5786718/)].
31. Nathavitharana RR, Shi CX, Chindelevitch L, Calderon R, Zhang Z, Galea JT, et al. Polyclonal pulmonary tuberculosis infections and risk for multidrug resistance, Lima, Peru. *Emerg Infect Dis*. 2017;**23**(11):1887–90. doi: [10.3201/eid2311.170077](https://doi.org/10.3201/eid2311.170077). [PubMed: [29048297](https://pubmed.ncbi.nlm.nih.gov/29048297/)]. [PubMed Central: [PMC5652442](https://pubmed.ncbi.nlm.nih.gov/PMC5652442/)].
32. Noguti EN, Leite CQ, Malaspina AC, Santos AC, Hirata RD, Hirata MH, et al. Genotyping of Mycobacterium tuberculosis isolates from a low-endemic setting in northwestern state of Parana in Southern Brazil. *Mem Inst Oswaldo Cruz*. 2010;**105**(6):779–85. doi: [10.1590/s0074-02762010000600008](https://doi.org/10.1590/s0074-02762010000600008). [PubMed: [20944992](https://pubmed.ncbi.nlm.nih.gov/20944992/)].
33. Guo JH, Xiang WL, Zhang G, Luo T, Xie N, Yang ZR, et al. Mycobacterial interspersed repetitive unit typing in Mycobacterium tuberculosis isolates from Sichuan province in China. *Indian J Med Res*. 2011;**134**:362–8. [PubMed: [21985820](https://pubmed.ncbi.nlm.nih.gov/21985820/)]. [PubMed Central: [PMC3193718](https://pubmed.ncbi.nlm.nih.gov/PMC3193718/)].
34. Rasoahanimitalisoa R, Rakotosamimanana N, Stucki D, Sola C, Gagneux S, Rasolof Razanamparany V. Evaluation of spoligotyping, SNPs and customised MIRU-VNTR combination for genotyping Mycobacterium tuberculosis clinical isolates in Madagascar. *PLoS One*. 2017;**12**(10):e0186088. doi: [10.1371/journal.pone.0186088](https://doi.org/10.1371/journal.pone.0186088). [PubMed: [29053711](https://pubmed.ncbi.nlm.nih.gov/29053711/)]. [PubMed Central: [PMC5650158](https://pubmed.ncbi.nlm.nih.gov/PMC5650158/)].
35. Feyisa SG, Haeili M, Zahednamazi F, Mosavari N, Taheri MM, Hamzehloo G, et al. Molecular characterization of Mycobacterium tuberculosis isolates from Tehran, Iran by restriction fragment length polymorphism analysis and spoligotyping. *Rev Soc Bras Med Trop*. 2016;**49**(2):204–10. doi: [10.1590/0037-8682-0405-2015](https://doi.org/10.1590/0037-8682-0405-2015). [PubMed: [27192590](https://pubmed.ncbi.nlm.nih.gov/27192590/)].