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*Original Research*

# MIRU-VNTR 15 loci capability in diagnosis of Beijing M. Tuberculosis strains in comparison with Real Time PCR

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**Abstract:** Beijing strain has great importance among *Mycobacterium tuberculosis* (MTB) genotypes due to their drug resistance and pathogenicity. Determination of MTB genotypes and comparison of MIRU-VNTR 15 loci and real-time PCR methods for Beijing family identification was the main objective in this study. This study was conducted on 92 confirmed MTB isolates, 31 and 61 of which previously were determined by real- time PCR as Beijing and non-Beijing, respectively. Allelic diversity, clustering rate, phylogenetic tree, clonal complexes and molecular genotypes of isolates were determined by MIRU-VNTR 15 loci on its online software. In addition MIRU-VNTR 15 loci were performed for 16 non-tuberculosis isolates. Concordance between MIRU-VNTR 15 loci and real Time PCR in determination of Beijing genotype was 95.6(89.2-98.8). 74 different pattern of MIRU-VNTR were detected in 92 MTB isolates. 69 patterns were unique and 5 clusters were determined. Largest clusters contain 11 and 6 members. The clustering rate was 19.56%. Among 15 loci, Mtub04 and MIRU10 have the highest and MIRU04 had the lowest discriminatory power. In non-Beijing isolates, New1 and Delhi/CAS with 25% and 16.3% were the most prevalent MTB genotypes. None of the non-Tuberculosis isolates had the complete MIRU-VNTR 15 loci pattern. The results of this study showed that MIRU-VNTR 15 loci, in addition to being able to differentiate MTB genotypes, can distinguish non-tuberculosis species from MTB strains, but for the exact differentiation of Beijing MTB genotypes, it may be necessary to increase the patterns known in MIRU-VNTR data base.

***Key words:*** *Mycobacterium tuberculosis*; Beijing genotype; MIRU-VNTR; Real time PCR.

## Introduction

Tuberculosis (TB) has affected human beings throughout history and despite the availability of efficacious treatment for decades; TB remains a major global health problem (1). Usage different molecular methods for typing of *Mycobacterium tuberculosis* (MTB) determined that different genotype or lineages have various capacity to stability on macrophage, escape from BCG vaccination, spreading rate, pathogenicity and even antibiotic susceptibility (2-4), so that Beijing family among them found more attention. The relation between Beijing isolates and multidrug resistance or treatment failure in TB were confirmed in many studies (4-6), made it as the most important MTB genotypes, although its relation to drug resistance was questioned in a new study which carried out in china (7). The scientist believed that this genotype faster distributed in the community and so that higher bacilli were founded in patient's sputum smear (8).

Beijing genotype is belong to lineage 2 of MTB which also referred to as 'East Asian lineage' (9) and first described in 1995 by van Soolingen and founded it is responsible for about 85% of tuberculosis in china (10), but in a short period of time, this genotype reported in different part of the world, so that it is the causative agent of 50% and 13% tuberculosis in east Asia and world, respectively (11).

The prevalence of this genotype in different part of Iran were assessed and was between 3.2% (12) to 10 %(6) and 15.6% (13) in referral hospital, Mycobacterial Research center (MRC) in Tehran, Iran. Golestan province in north of Iran and southeast Caspian sea is the second tuberculosis area in country with11.6% - 13.9% Beijing genotype (14, 15) which is predominant in extra-pulmonary tuberculosis and young patients.

Different molecular method including IS6110 RFLP, Spoligotyping, Single neucleotide polymorphism (SNP), Large Sequence Polymorphisms (LSP) and MIRU-VNTR were designed to diagnosis of Beijing and other genotypes of MTB. In 2006 Hillemann and colleagues used Real Time PCR with two different primers and probes for identification and differentiation Beijing and non- Beijing strains (16). The accuracy of Hillemann Real Time PCR in comparison to other molecular method especially MIRU-VNTR, which is ongoing and widespread, was not determined. MIRU-VNTR is a simple, reproducible, non-expensive method for genotyping which introduced in typing of MTB and its application is increasing (17). Establishment of MIRU-VNTRplus data base as a strong, applicable and user friendly online data base, made it as favorite method. In this web application, comparisons can be based on MIRU-, spoligo-, RD-, SNP-, susceptibility-typing data, or by a combination of different data types (https://www.miru-vntrplus.org), but its correlation

with Real Time PCR is not clarified. Determination of concurrence of MIRU-VNTR 15 loci and Real-Time PCR methods in Beijing family identification was the main objective in this study.

## Materials and Methods

### Patient's information

This study was conducted on 31 Beijing and 62 non-Beijing MTB isolates which previously identified by Hillemann Real-time PCR method (15). MTB isolates collected from 67 and 25 men and women TB patient's respectively in Golestan province, north of Iran and southeast of Caspian Sea in 2011. Tuberculosis patients had age range from 1 to 84 years (mean 42.4±20.7years). In 80 cases, MTB was isolated from sputum and in 12 cases the samples were obtained from other biological specimens.

### MIRU VNTR typing

DNA extraction was carried out by boiling method (18), briefly three to five fresh colonies of each MTB isolates were grown on Lowenstein Jensen media Merck Co. Germany inoculated in TE buffer, the suspension was placed in a boiling water bath for 15 min and its supernatant after centrifugation were used. Purity and concentration of DNA extract were determined and adjusted by spectrophotometer (Eppendorf Co. Germany). The extracted DNA was used for MIRU-VNTR 15 loci according Supply method (17).

PCR fragments for all VNTR loci were generated individually in monoplex PCRs using sets of primers. The list of 15 primers, master mix and PCR program was performed according supply method (17). The size of PCR product for each locus were detected on 1.5% gel agarose electrophoresis and their tandem repeat were determined and finally, a 15-digit number was obtained for each locus, each numerical value reflects the number of repeats in tandem at each locus. Distilled water and H37RV was used as negative and positive control, respectively. In addition DNA extract of 16 non-tuberculosis mycobacteria (NTM) including; *M.simiae*, *M.fortuitum*, *M.capra and M.abscessus* were applied for assess of MIRU-VNTR15 loci method.

15-digit number for each MTB isolates were entered in MIRU-VNTRplus database and different analysis such as identification by Similarity distance cut-off of less than 0.3, Clustering rate (the isolates with identical MIRU-VNTR patterns), Allelic diversity of a loci, radial tree for determination of phylogenetic relation among MTB isolates by UPGMA method and determination of clonal complexes by plot Minimum Spanning Tree were performed (https://www.miru-vntrplus.org). HGDI was calculated as the Hunter and Gaston suggested (19).

### Comparison between two method

The concurrency of Real Time PCR and MIRU-VNTR15 loci for identification of Beijing sub-lineage was carried out by medcal online software (https://www.medcalc.org/calc/diagnostic_test.php).

## Results

74 different patterns of MIRU-VNTR were detected in 92 MTB isolates. 69 patterns were unique and 5 clusters were determined. The largest cluster contained 11 members followed by a cluster include 6 members and the three other clusters contained only 2 members. The clustering rate was 19.56%. None of the patients within a cluster lived in the same cities of the province and family. Among 15 loci only MIRU04 was low discriminative (h <0.3) and 7 loci showed high discriminatory power with h≥0.6 (Table 1). HGDI of MIRU-VNTR method was 0.982.

New-1 and Delhi/CAS were the most prevalent MTB genotypes in non- Beijing isolates of this region. The MIRU-VNTR patterns in 24 isolates were not match with any standard profile in MIRU-VNTRplus database

**Table1.** Allelic diversity and HGDI of 15 MIRU-VNTR loci in 92 MTB isolates in Golestan province, southeast of Caspian Sea.

| Locus* | 424 | 577 | 580 | 802 | 960 | 1644 | 1955 | 2163b | 2165 | 2401 | 2996 | 3192 | 3690 | 4052 | 4156 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Allele** | MTUB04 | ETR C | MIRU 04 | MIRU40 | MIRU10 | MIRU16 | MTUB21 | QUB11b | ETR A | MTUB30 | MIRU26 | MIRU31 | MTUB39 | QUB26 | QUB4156 |
| 1 | 10 | - | 2 | 10 | 1 | 4 | 1 | - | - | - | 4 | - | - | - | 1 |
| 2 | 24 | 31 | 89 | 8 | 30 | 10 | 4 | 60 | 6 | 60 | 4 | - | 11 | - | 61 |
| 3 | 16 | - | 1 | 63 | 34 | 49 | 14 | 4 | 33 | 13 | - | 30 | 73 | 2 | 5 |
| 4 | 37 | 56 | - | 10 | 9 | 29 | 20 | 1 | 52 | 19 | 9 | 27 | 7 | 5 | 23 |
| 5 | 4 | 3 | - | 1 | 17 | - | 47 | 1 | 1 | - | 54 | 34 | 1 | 11 | 1 |
| 6 | 1 | 1 | - | - | 1 | - | 4 | 26 | - | - | 11 | - | - | 4 | 1 |
| 7 | - | - | - | - | - | - | 2 | - | - | - | 10 | 1 | - | 24 | - |
| 8 | - | - | - | - | - | - | - | - | - | - | - | - | - | 46 | - |
| 12 | - | 1 | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Total | 6 | 5 | 3 | 5 | 6 | 4 | 7 | 5 | 4 | 3 | 6 | 4 | 4 | 6 | 6 |
| H | 0.72 | 0.51 | 0.05 | 0.49 | 0.71 | 0.6 | 0.66 | 0.49 | 0.54 | 0.51 | 0.61 | 0.67 | 0.34 | 0.66 | 0.49 |

*Position of the locus in H37RV genome **Name of the locus.

**Table2.** Distribution of sub-lineages of MTB isolates in Golestan province, southeast of Caspian Sea.

| MTB genotypes | | Number | Mean Age (year) | City** | Type of sample | |
|---|---|---|---|---|---|---|
| | | | | | Sputum | others |
| Beijing | | 27 | 34.1±21.5 | 51.8% | 21*** | 6 |
| Non-Beijing | NEW 1* | 26 | 56.2±20.8 | 61.5% | 24 | 2 |
| | Delhi/CAS | 15 | 41.3±16.2 | 60% | 15 | 0 |
| | Unknown | 24 | 44.4±15.3 | 62.5% | 20 | 4 |
| Total | | 92 | 42.4±20.7 | 58.6% | 80 | 12 |

* In three cases more than one sub-lineage were diagnosed (mismatched) which accounted in new1 family, ** the patients who livening in towns of Golestan province, southeast of Caspian Sea and *** the number of samples which MTB isolated from them, N; number of patients in each group.

and named as Unknown genotype. Mean age in patients with Beijing MTB was lower than other genotypes (Table 2).

UPGMA Radial Tree showed the distribution 92 MTB isolates among known genotypes (Figure 1), it showed the unrelated isolates that named unknown. Most of the unknown MTB isolates related to Delhi/CAS genotype but is different from them, which need to further studies for recognition the real genotypes especially for isolates 29,63,66,68.

The plot of minimum spanning tree for 92 MTB isolates in this study showed 12 clonal complexes with at least 2 to 25 members (Figure 2). The largest clonal complex (CC2) with 25 members (3 clusters with 11, 6 and 3 members) was belonging to Beijing family. Clonal complexes 1(13 members), 5 (4members) and CC10-12(each comprised 2 member) belong to NEW-1 genotype. Most Delhi/CAS isolates; comprised in CC4, CC 7 and CC 9.

Comparison between Real Time PCR and MIRU-VNTR 15 loci efficacy in determination of Beijing genotype was major objective of this study. We found that agreement between two methods for identification of 31 Beijing and 61 non-Beijing isolates was >95% ( table 3), but there were four disagreement in Beijing isolates, as one of them was known as NEW-1 and another one diagnosed as Delhi/CAS and 2 other cases were unknown based on MIRU-VNTR method.

None of 16 NTM species have complete pattern of MIRU-VNTR 15 loci method. QUB11b and Mtub39 were more prevalent loci in these mycobacteria but MIRU16 and ETRA loci weren't amplified in none of tested NTM.

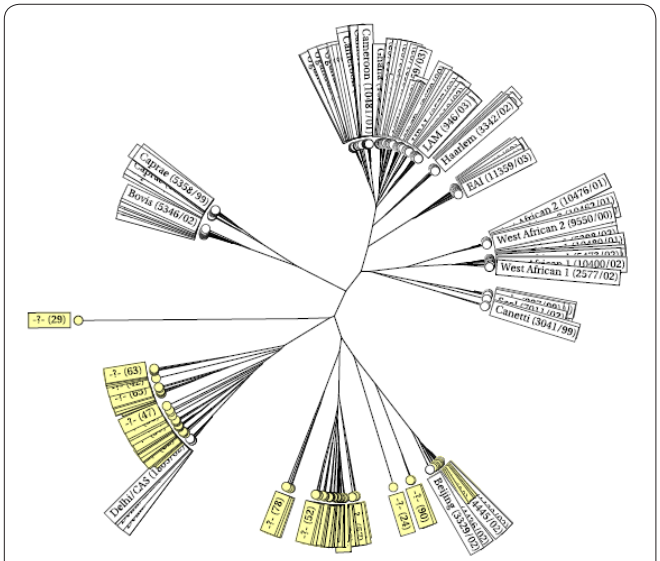


**Figure 1.** UPGMA Radial Tree using MIRU–VNTR 15 loci of 92 MTB isolates in Golestan province, southeast of Caspian Sea. Yellow color shows the isolates of this study.

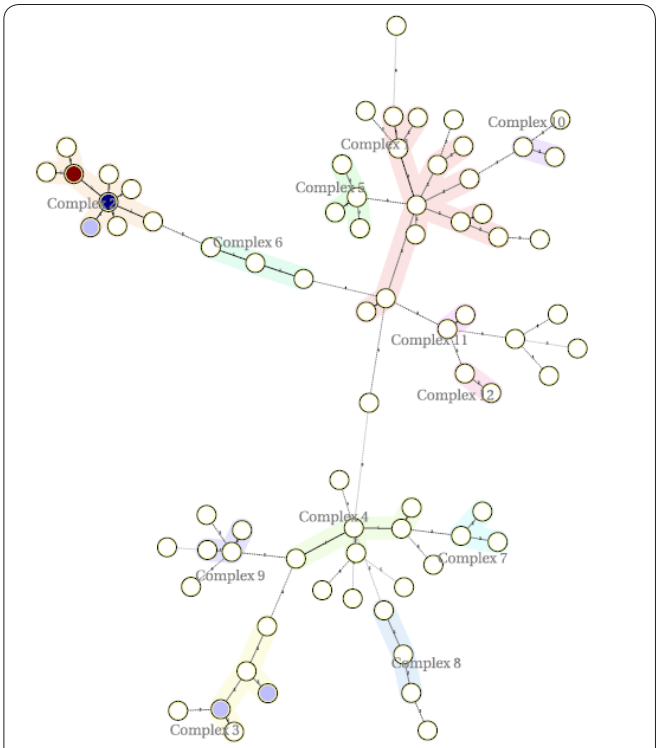


**Figure 2.** Minimum spanning tree of the 92 M. tuberculosis clinical isolates based on double-locus variants with 12 clonal complexes.

## Discussion

Although the agreement of real Time PCR and MIRU-VNTR methods in the diagnosis of the Beijing genotype is more than 95%, but the existence of 4 disagreements is very important. Regarding the specificity of the primer and probe of Beijing in the real Time method and confirmation of this result by genome non-amplification in the presence of a non-Beijing primer, it seems that it is necessary to rely on the results of the Hellman method. On this basis, it should be assumed that in MIRU-VNTR 15 loci method, some cases of Beijing were not properly identified and there is the need to add some new profile to 186 standards MIRU- VNTR profiles. Although proper determination of this 4 isolates by other molecular methods such as SNP, LSPs, IS6110 RFLP and spoligotyping for final decision about addition them to 186 standard profiles is necessary.

Like most studies conducted in Iran, genetic variation among MTB strains is very high and in fingerprinting

by MIRU-VNTR method most isolates have unique pattern (14, 20, 21, 22 and 23). Clustering rate in this study was about 20% which it means that the most TB patients in this area acquired their pathogenic organism previously and recent transmission of tuberculosis is low. We don't know, Why the clustering rate of MTB isolates in some area is less than expected? Torkaman et al. study showed that in Iran reactivation of latent TB is the major reason of tuberculosis and recent transmission have minor role (13). Among different genotypes, Beijing family showed the highest clustering rate (59.2%) and most of them included in Clonal Complex 2, with three clusters (11, 6 and 2 members), which means the high relationship between the members of this family as described by Zhou 2017 in china (9) but New-1 and Delhi/CAS members were mostly singleton or made small Clonal Complex. The existence of a large clone complex among the Beijing genotype confirms that this strain has recently been expanding in the province.

In this study 7 loci were defined as highly discriminative and among them MTUB04 and MIRU10 were the most prevalent MIRU VNTR loci in our area such as other reports from Iranian studies (22-26). on the other hand MIRU04 had the lowest discriminatory power as Azimi et al study. These similarities indicate the probability of circulation of the related MTB isolates in Iran (23).

Regardless of Beijing, NEW-1 and Delhi/CAS were the most prevalent MTB genotypes in this region. New-1 genotype showed high distribution in the southeast of Caspian Sea which confirm Mokrousov et al. findings. They found that the NEW-1 genotype of MTB (or its subtype) firstly introduced from south and east and has increasing circulation in Iran and its neighbors and the capacity to rapidly acquire drug resistance (27). Mansoori et al in a recently published study found new 1 as the second frequent genotype of MTB in Golestan province which is confirmed our finding but in opposite they mentioned Delhi/CAS genotype as the most prevalent MTB genotype (14). In the studies were performed in Khorasan province, in Sotuheast of Iran and border with Afghanistan, Harllem and Delhi/CAS genotypes of MTB had a high prevalence (14, 26) which is similar to our finding. CAS genotype is distribute in India, Bangladesh and Pakistan (28) and most part of Iran (23, 29)

Our data showed that none of tested NTM such as *M.simiae, M. fortuitum, M.capra and M.abscessus,* had all 15 loci which used in MIRU-VNTR method, it also confirms the ability of this technique in diagnosis of MTB from NTM.

Although the concordance MIRU-VNTR 15 loci and real- time PCR methods was high, failure to recognition four Beijing isolates by MIRU-VNTR 15 loci method with cutoff less than 0.3, suggests that it is the time to consider in standard strain in MIRU-VNTR plus profiles.

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**Competing interests**

The authors declare no competing interests

**Contributorship Statement**

All authors contributed to the research concept, discussed the results and contributed to the final manuscript. Maya BabaiiKochaksareii; carried out the experiment, contributed to sample preparation. Hami Kaboosi; contributed to the interpretation of the results and involved in planning and Ezzat A. Ghaemi; designed and directed the project and Critical revision.

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