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ORIGINAL ARTICLE

Genotypic diversity of *Mycobacterium tuberculosis* strains collected from immigrant patients in Mashhad, Iran using MIRU-VNTR method

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ABSTRACT

Background and Objectives: This research aimed to explore the genetic diversity and phylogenetic relationships of *Mycobacterium tuberculosis* (*Mtb*) strains, as well as to assess their drug susceptibility, specifically in strains isolated from immigrant patients attending the Referral Tuberculosis Laboratory in Mashhad.

Materials and Methods: A total of 52 sputum samples isolated from patients were examined utilizing the Mycobacterial Interspersed Repetitive-Unit Variable Number of Tandem Repeats (MIRU-VNTR). Drug-susceptibility testing against rifampin (RIF) and isoniazid (INH) was measured utilizing the proportional strategy. Thereafter, for more examination, Xpert MTB/RIF and multiplex allele-specific PCR (MAS-PCR) was performed to determine RIF and INH-resistance within the *Mtb* strains.

Results: Among 52 *Mtb* isolates, 2 (3.8%) were resistant to rifampin and one isolate was resistant to both INH and RIF and considered as multidrug-resistance (MDR) isolate. According to MIRU-VNTR, the most prominent genetic-variation patterns of these samples, were related to NEW-1 (n=18, 34.6%), followed by CAS/Delhi (n=17, 32.7%), Haarlem (n=12, 23%), Uganda I (n=2, 3.8%), S (n=1, 1.9%), Beijing (n=1, 1.9%), and unknown (n=1, 1.9%) genotypes. The statistical analysis showed that the estimated percentage of the recent TB-transmission in this study was 0.21%.

Conclusion: The result of this study indicated a great diversity of MTBC circulating among Afghan-immigrants which might be one of the reasons for the infection to become active. The relatively high percentage of resistant isolates in the studied population shows the importance of screening the immigrants especially at the entry borders and treatment and follow up of patients, to control TB-incidence in country.

Keywords: *Mycobacterium tuberculosis*; Mycobacterial interspersed repetitive-unit variable number of tandem repeats; Multidrug resistance

INTRODUCTION

According to the current World Health Organization (WHO) reports, tuberculosis (TB) is considered the leading deadly infectious cause of death, a position which was challenged from 2020 to 2021 by COVID-19. The causative agent of TB is an intracellular, acid-fast bacterium from the *Mycobacterium*

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tuberculosis strains family, which is mainly transmitted through breathing. Based on global reports, the number of newly diagnosed cases of tuberculosis in 2023, is reported to be 7.5 million people, which is the highest number since the start of global tuberculosis monitoring by the WHO in 1995 (1, 2). Although tuberculosis is under control in Iran, geographic conditions and migration trends from neighboring countries, especially Afghanistan and Pakistan have raised concerns in regards to managing the disease. These two countries are among the 30 nations with the highest rates of TB incidence. According to a 2023 WHO report; the prevalence of tuberculosis in these countries is 258 and 185 cases per 100,000 people, respectively; which is 18 and 12 times higher than in Iran. Based on the statistics published by the International Organization for Migration, more than one million migrants have settled in Iran during the first half of 2024, with more than %97 of them being Afghans (3, 4). The increasing migration rate has directly affected the status of TB in Iran. In the past two decades, 15% of identified tuberculosis cases in Iran were related to immigrants, which has recently increased to 25 percent (3). The presence of a floating population of different nationals, especially Afghan nationals, in Khorasan-Razavi province, particularly in Mashhad, has caused a TB incidence rate of 4.3 per 100,000 people, which is higher than the national average. One of the important factors in controlling the disease and preventing the spread of TB is identifying sources of infection and screening (4). Due to the long incubation period of pathogenic *Mycobacteria*, identifying their type and determining drug sensitivity takes about 2 to 4 months. Therefore, using innovative methods for rapid and accurate diagnosis of this disease and its various strains is very important (5). Different species of *Mycobacteria* can be identified through molecular and PCR-based typing methods. Using molecular typing techniques in epidemiological studies of this disease can play a significant role in identifying the source of infection, prevailing circulating strains, and tracking transmission routes between countries. Moreover, the growing concern over drug-resistant TB (DR-TB) makes genetic typing a critical tool in identifying resistant strains. Molecular tools are capable of detecting specific mutations in genes linked to drug resistance (5).

The most common PCR-based genotyping methods used in different studies, include MIRU-VNTR, Repetitive sequence-based PCR (rep-PCR), Spoligotyping, and Random Amplified Polymorphic DNA (RAPD) (6). Among these methods, the MIRU-VNTR technique attracts much attention among researchers in epidemiological and phylogenetic studies, due to its high discrimination power and reproducibility, the ability to create numerical codes for each sample and check it in global databases, as well as the ease and cost-effectiveness of doing it (6). This is used in the genotyping of strains by numbering copies of VNTR present in 12, 15, or 24 identified loci (7, 8). In this study, we used the 12-locus MIRU-VNTR method to determine phylogenic and genotyping relationship and transmission dynamics among *Mycobacterium tuberculosis* strains isolated from the Afghan immigrants referred to the Referral Tuberculosis Laboratory of Mashhad, Iran, to outline the epidemiological and phylogenetic profile of the studied strains and investigate the potential impact of the tuberculosis disease pattern in migrant patients on the genetic landscape of circulating *Mtb* in Mashhad. Additionally, the pattern of drug sensitivity in identified strains was assessed by using molecular and proportional methods.

MATERIALS AND METHODS

Samples. In the present cross-sectional investigation, a total of 52 sputum samples were gathered from smear-positive tuberculosis patients, specifically Afghan immigrants, who were referred to the Referral Tuberculosis Laboratory located in Mashhad, between 21 March and 21 November 2023. Furthermore, demographic data pertaining to the patients, such as gender, age, clinical symptoms, and residential addresses, were documented using designated forms.

Phenotypic procedures: preparation of smear and culture. All sputum samples were digested and decontaminated by the modified Petroff method and then all the slides were stained with Ziehl-Nelson. After culturing in Lowenstein–Jensen medium, incubation at 37°C for up to two months with weekly follow-up, was performed.

Conventional drug susceptibility testing (proportional method). As a first-line, the susceptibility of isolates against to RIF, and INH was evaluated using the proportional method suggested by WHO/IUTLD (9); RMP: 40 mg/L, and INH: 0.2 mg/L. The

LJ mediums were incubated for 43 days. For quality control of DST method, *Mycobacterium tuberculosis* H37Rv strain (ATCC 27294) was used.

DNA extraction. Genomic DNA from all positive cultures was extracted using the boiling method, following the guidelines established by Van Soolingen. A loopful of bacterial growth was placed into a microfuge tube containing 200 µL of TB lysis buffer. The sample was subjected to heat treatment at 96°C for 30 minutes, then centrifuged at 4600 g for 12 minutes. The supernatant was then transferred to a new tube and underwent an additional heat treatment at 80°C for 45 minutes. Subsequently, the quality of the extracted DNA was assessed using the Nanodrop method. The contents of the tube were stored at -20°C until needed for PCR applications.

PCR-16SrRNA and PCR-IS6110. To identify the genus of mycobacteria, the Huard method was utilized to amplify a 543 bp fragment of the 16S rRNA gene, employing the primer pair listed in Table 1 (10). For the identification of the *Mtb* complex, a 190 bp fragment of the IS6110 sequence using the INS1 and INS2 primers was amplified. The primers are further described in Table 1.

Xpert MTB/RIF assay. All isolates were evaluated by Xpert® MTB/RIF assay (Cepheid Sunnyvale, CA, United States) to detect *Mtb* and also determine the rifampin resistance, based on the kit instruction (9).

Multiplex Allele Specific PCR (MAS PCR) for detection of INH and RIF resistance. Based on our previous study, a MAS-PCR was conducted to focus on the mabA-inhA promoter region and codon 315 of the katG gene (-15). Additionally, a separate MAS-PCR was performed to target codons 516, 526, and 531 of the rpoB gene (9).

MIRU-VNTR. For this purpose, we used the MIRU-VNTR method, which is a PCR-based method that utilizes 12 specific pairs of primers to amplify *Mycobacterial* sporadic duplicated units. After amplifying of *Mycobacterium* sporadic duplicate unite, the number of copies per locus was calculated by electrophoresis of PCR product in agarose gel (11). *Mtb* H37Rv strain and distilled water were used as positive and negative control respectively.

Allelic diversity and Hunter-Gaston discriminatory index. Hunter-Gaston discriminatory index (HGDI) was utilized for determining the allelic diversity (h) for each locus of MIRU-VNTR that would be in a range of 0.00 to 1.00 (12). Based on this comparison h=0.00 demonstrates the lack of allelic variation, while h=1.00 indicates the highest amount of allelic variation (11).

Transmission and clustering rate estimate. The clustering rate was determined using the equation "R=nc/n," which indicates that a higher differentiation power of the method corresponds to a lower clustering rate. Furthermore, the recent transmission rate of TB was assessed using the formula "(nc - c)/n," where "n" represents the total number of cases examined, "NC" denotes the total number of clustered instances, and "c" signifies the number of clusters. This approach categorizes patients into two distinct groups: clustered and non-clustered. In cases where the isolates are clustered, it suggests that tuberculosis has been transmitted recently. Conversely, in instances where the isolates are singletons, the infection is attributed to the reactivation of a latent infection (13).

Phylogenetic analysis. For investigating, the genetic association of MTBC isolates, data were analyzed by MIRU-VNTR plus (http://www.miru-vntrplus.org) database. Then, in the next step, for the determination of Clonal strains and the phylogenic relationship between MIRU-VNTR profiles, the obtained data were used to draw the Minimum Spanning Trees (MST) (14). The locus changes were characterized by thick, thin, and spot-

Table 1. Primer sequence.

Gene	Primer sequence	Length (bp)
16SrRNA	5'-ACC TCC TTT CTA AGG AGC ACC-3'	543 bp
	5'-GAT GCT CGC AAC CAC TAT CCA-3'	
IS6110	5'-ATC CTG CGA GCG TAG GCG TCG G-3'	190 bp
	5'-CAG GAC CAC GAT CGC TGA TCC GG-3'	

ted lines that each represented one, two, or more of the two-locus variants, respectively. A dendrogram was drawn by the unweighted-pair group procedure with an arithmetic mean (UPGMA) algorithm and the genetic association was examined (14, 15).

Ethics approval. This project was approved by the ethical committee of Ferdowsi University of Mashhad, Mashhad, Iran (Code: IR.UM.REC.1403.284).

RESULTS

Phenotypic identification and drug susceptibility testing (DST). A total of 52, smear- and culture-positive MTB isolates from immigrant patients who live in Mashhad, were included that were characterized by genomic methods for gene targets of *16SrRNA* and *IS6110*. A total of 21 (40%) men and 31 (60%) women aged of 12-83 years old were included. Out of 52 *Mycobacterium tuberculosis* (*Mtb*) isolates, three cases (5.7%) exhibited resistance, which included one case (1.9%) demonstrating MDR to both INH and RIF, as well as two cases (3.8%) showing mono-resistance to rifampin.

Xpert MTB/RIF assay. The drug susceptibility testing approach revealed three cases of rifampin resistance, which were evaluated using the GeneXpert MTB/RIF assay. This assay confirmed that all three sputum samples exhibited resistance to rifampicin. Statistically, there was no significant difference in the detection of rifampin between the GeneXpert MTB/RIF assay and the traditional drug susceptibility testing method ($p > 0.05$).

MAS-PCR. The results from the MAS-PCR assay revealed a single phenotypic case of isoniazid (INH) resistance, which was classified as an INH-resistant strain. This particular isolate exhibited mutations in both codon 315 and the inhA promoter region. Additionally, among the three phenotypic cases of rifampin resistance, all were confirmed by MAS-PCR as rifampicin-resistant strains, with mutations occurring specifically in codon 531.

The MIRU-VNTR analysis. Our investigation revealed that among the 52 isolates examined, 51 exhibited similarities with the MIRU-VNTR plus database. This included the NEW-1 genotype (n=18, 34.6%), followed by CAS/Delhi (n=17, 32.7%), Haarlem (n=12, 23%), Uganda I (n=2, 3.8%), S (n=1, 1.9%), Beijing (n=1, 1.9%), and one isolate categorized as unknown (n=1, 1.9%). A phylogenetic tree was constructed using the UPGMA algorithm to explore the genetic relationships among the *M. tuberculosis* complex (MTBC) isolates (Fig. 1). To enhance the identification of clonal strains within the isolates, Minimum Spanning Trees (MST) were created based on Single Locus Variants (SLV) and Double Locus Variants (DLV). The analysis based on SLV identified 17 isolates grouped into 2 clonal strains, while the DLV analysis revealed 45 isolates classified into 4 clonal strains, as illustrated in Figs. 2 and 3. Specifically, the SLV analysis identified two clonal strains of *M. tuberculosis*: the first clonal strain (CC1), associated with the Delhi/CAS genotype, comprised 13 isolates, and the second clonal strain (CC2), linked to the Haarlem genotype, included 4 isolates. The DLV analysis further categorized the *M. tuberculosis* strains into four distinct strains.

Allelic diversity and discriminatory power. The findings from the HGDI indicated a value of 0.98184 for the MIRU-VNTR typing technique. Furthermore, the allelic diversity (h) for each locus was assessed, revealing that Miru-26 exhibited the greatest diversity (h=0.89%) among the 12 alleles analyzed. In contrast, Miru-10 and Miru-16 were identified as having high diversity ($h > 0.6$). Conversely, Miru-24 demonstrated low allelic variability ($h < 0.3$).

Estimation of transmission. The minimum estimate for TB caused by recent transfer was 0.21%.

Drug susceptibility results. Out of 52 examined samples, two rifampin-resistant samples and one MDR sample (resistant to rifampin and isoniazid) were detected.

DISCUSSION

Based on previous studies, migration can be considered as one of the most important factors affecting the spread of tuberculosis. Immigrants may have contracted tuberculosis during their residence in their home or indeed after immigration. Immigrants' poor living conditions due to economic, cultural, and health poverty in their areas of residence can be considered a predisposing factor for the transformation

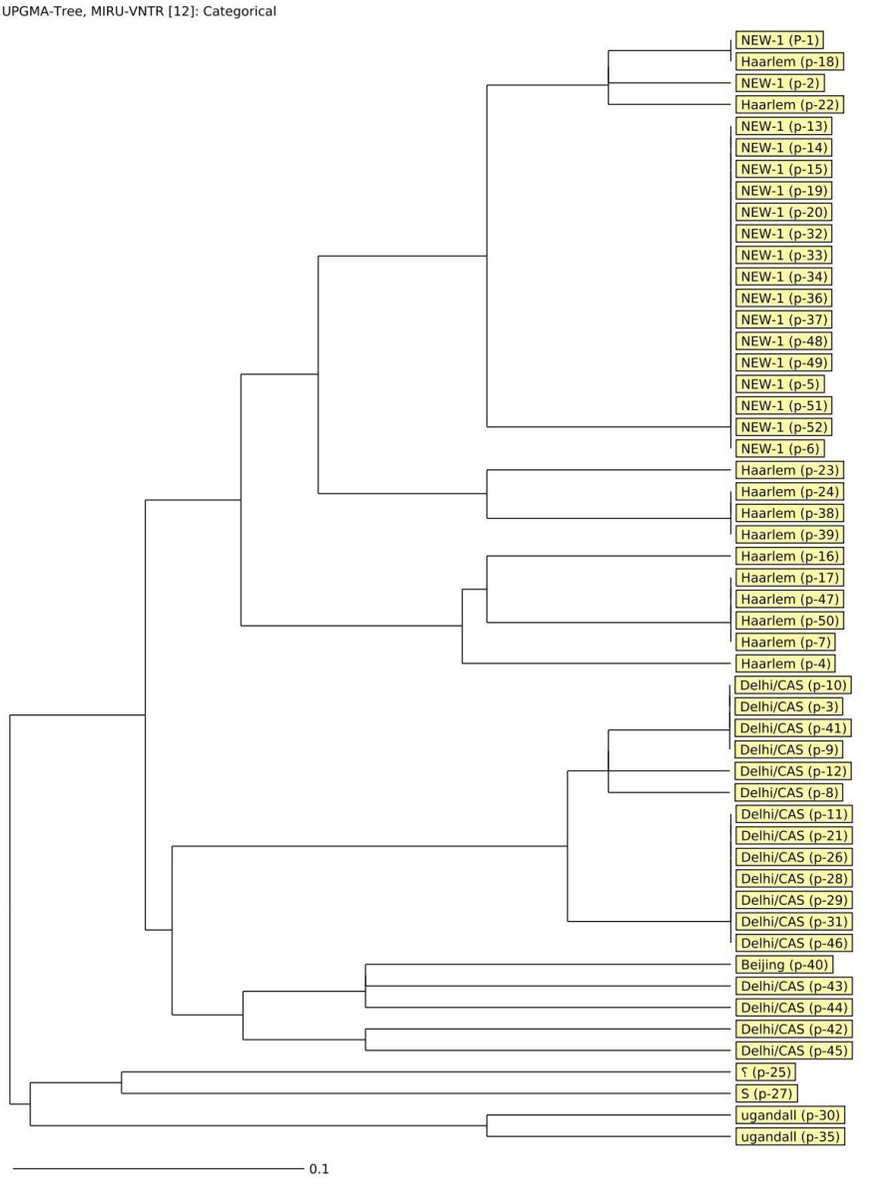

Fig. 1. The genetic relationship of 52 isolates of *Mycobacterium tuberculosis* strains isolated by UPGMA algorithm for MIRU-VNTR patterns.

of latent tuberculosis into active disease. Therefore, distinguishing between relapse (reactivation of the original infection) and re-infection (a new infection from a different strain) is one of the challenges in managing TB in migrant populations. Afghan immigrants, especially those who had poor living conditions, are at a heightened risk of tuberculosis due to various factors such as the potential for exposure to TB in their crowded living conditions and also their limited access to healthcare (16, 17). In this study, drug sensitivity tests and genotyping studies based on the MIRU-VNTR 12-locus method were performed on 52 samples of tubercular immigrants living in Mashhad. Among the 52 tested samples, two resistant patients to rifampin and one MDR patient (resistant to rifampin and isoniazid) were identified. Based on the patterns obtained from our genotyping results, NEW-1 (18 isolates - 34.6%), DELHI/CAS (17 isolates - 32.7%), HAARLEM (12 isolates - 23%), UGANDA (2 isolates - 3.8%), and S and BEIJING, 1.9%, were classified. The HGDI rate for the MIRU-VNTR method was 98/184% and the minimum

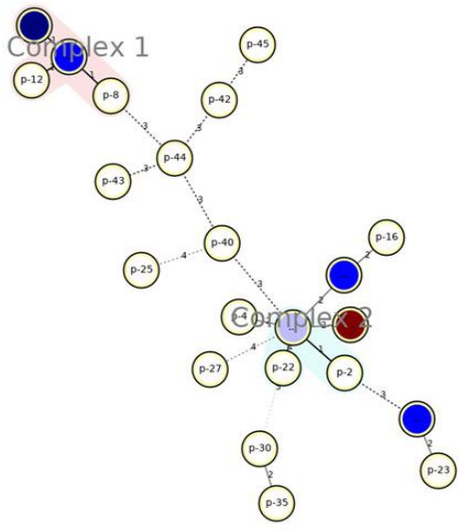

Fig. 2. Phylogenetic relationship of 52 *Mycobacterium tuberculosis* isolates based on MST drawn based on SLV.

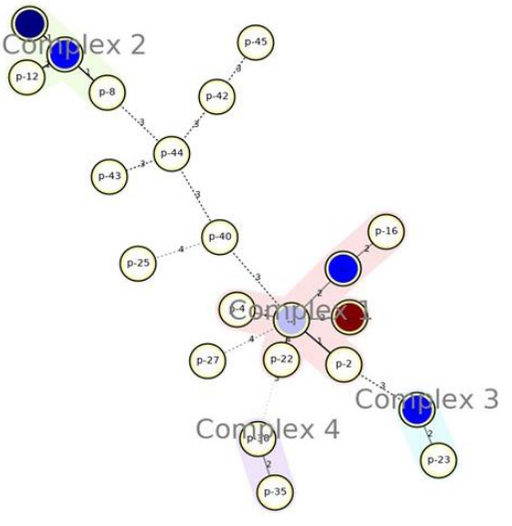

Fig. 3. Phylogenetic relationship of 52 *Mycobacterium tuberculosis* isolates based on MST drawn based on DLV.

estimate of TB due to recent transmission was calculated to be 21%. Of the 12 loci investigated, 3 loci had high diversity, two loci had moderate diversity, and the other loci had low allelic diversity. MIRU 26 and MIRU 24 had h=0.89 and h=0, respectively, showing the highest and lowest diversity.

The most common genotype known in the investigated samples was NEW-1, with a frequency of 34.6, which was consistent with the previous studies conducted on the samples of tuberculosis patients from Herat, Afghanistan, with a frequency rate of 45%, and the previous results of Khorasan Razavi province with a frequency rate of 53%. According to the widespread distribution of this genotype in previous studies, Iran has been considered a route for spreading NEW-1 to other regions throughout history (18). Our results also suggest the DELHI/CAS genotype in second place with a frequency of 32.7%, which was also in line with the previous studies on tuberculosis patients in Herat, Afghanistan, with a frequency of 21.8%, and Khorasan Razavi with a frequency rate of 24.8%. It should be noted that the DELHI/CAS genotype has a high frequency in Iran's neighboring countries, including, Pakistan, Afghanistan and Saudi Arabia (19, 20). Another study on TB patients in Qom, Iran, also suggested the DELHI/CAS and NEW-1 genotypes as predominant genotypes with a frequency of 43% and 27%, respectively (19). Haarlem genotype was identified as the third circulating genotype with 23%, which was also reported in the study of tuberculosis patients of Herat, Afghanistan, with a frequency of 18.8% and 5% in the study of tuberculosis patients of Khorasan Razavi province (21, 22). Our result suggested a frequency rate of 1.91% for the Beijing genotype, while it was estimated at 1.81% and 1.7% to 24.4% in Herat and Iran's previous reports, respectively (23). The only MDR sample identified in this study is related to the Beijing genotype. The Beijing genotype has a global distribution, and in most studies, the drug-resistant strains are related to this genotype (24). Probably, the "genotype circulating between MDR" are the strains related to this genotype that are circulating in neighboring countries of Iran. In the current study, the minimum rate of TB caused by recent transmission in the immigrant patients living in Mashhad was calculated to be 21%. In the studies conducted in Golestan and East Azarbaijan province using 12, 15, and 24 locus methods, the minimum rate of tuberculosis caused by recent transmission was between 5% and 27%, and in studies conducted in different places such as China, London, and the Netherlands, it has been reported to be between 13.5% and 35% (25, 26).

CONCLUSION

However, our results showed a high genomic diversity of circulating *M. tuberculosis* strains in the immigrant population of Khorasan Razavi. This finding could indicate that immigrants had latent tu-

berculosis infection before entering Iran, and due to economic and cultural poverty and inadequate sanitary conditions in their collective residences, which are often located in poor areas of Iran, the tuberculosis infection transforms into active disease. Therefor; considering the proper control of tuberculosis in Iran, it seems that to prevent the spread of the disease by immigrants, careful planning is needed. As a first step, controlling entry borders and screening migrants is one of the most important measures in decreasing the prevalence of the disease in our country. The next step is to continuously implement health programs to monitor the status of migrants and treat and follow up of infected cases.

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