Taylor & Francis
Taylor & Francis Group

Pathogens and Global Health

ISSN: (Print) (Online) Journal homepage: https://www.tandfonline.com/loi/ypgh20

Determination of genetic diversity of multidrug-resistant *Mycobacterium tuberculosis* strains in Turkey using 15 locus MIRU-VNTR and spoligotyping methods

Burcu Gürer Giray, Ahmet Aslantürk, Hülya Şimşek, Didem Özgür, Selçuk Kılıç & Gönül Aslan

To cite this article: Burcu Gürer Giray, Ahmet Aslantürk, Hülya Şimşek, Didem Özgür, Selçuk Kılıç & Gönül Aslan (2022): Determination of genetic diversity of multidrug-resistant *Mycobacterium tuberculosis* strains in Turkey using 15 locus MIRU-VNTR and spoligotyping methods, Pathogens and Global Health, DOI: 10.1080/20477724.2022.2084807

To link to this article: https://doi.org/10.1080/20477724.2022.2084807

Published online: 01 Jun 2022.

PATHOGENS AND GLOBAL HEALTH
https://doi.org/10.1080/20477724.2022.2084807

Taylor & Francis
Taylor & Francis Group

Determination of genetic diversity of multidrug-resistant *Mycobacterium tuberculosis* strains in Turkey using 15 locus MIRU-VNTR and spoligotyping methods

Burcu Gürer Giray[a], Ahmet Aslantürk[b], Hülya Şimşek[c], Didem Özgür[d], Selçuk Kılıç[e] and Gönül Aslan[f]

[a] Ankara Provincial Health Directorate Public Health Molecular Diagnosis Laboratory, Ankara, Turkey; [b]National Tuberculosis Reference Laboratory, Public Health Institution of Turkey (PHIT), Ankara, Turkey; [c]Faculty of Medicine, Department of Medical Microbiology, Yozgat Bozok University, Yozgat, Turkey; [d]Department of Medical Microbiology, Kafkas University, Kars, Turkey; [e]Department of Medical CBRN, University of Health Sciences, Institute of Dedenxe Health Sciences, Ankara, Turkey; [f]Mersin University Faculty of Medicine, Department of Medical Microbiology, Mersin, Turkey

ABSTRACT

Tuberculosis (TB) remains the leading cause of deaths from infectious disease worldwide. Nowadays, the tendency of *Mycobacterium tuberculosis* complex (MTBC) to spread between continents due to uncontrolled migration movements shows that TB is a global health problem. The number of studies for the detection of MTBC strains' epidemiological features in areas with TB spread risk using molecular-based methods such as spoligotyping and Mycobacterial Interspersed Repetitive Unit (MIRU) Variable Number Tandem Repeats (VNTR) at the clonal level is insufficient. In this study, it was aimed to determine the phylogenetic relationships of MTBC strains at the species level by spoligotyping and 15 locus MIRU-VNTR (MIRU-VNTR$_{15}$) molecular methods of 96 multidrug-resistant (MDR) MTBC strains isolated from sputum samples of patients with a preliminary diagnosis of pulmonary TB or suspected contact history those sent to National Tuberculosis Reference Laboratory from the centers that are members of the Tuberculosis Laboratory Surveillance Network. The phylogenetic relationship between 96 MDR-TB strains was investigated with the combination of bead-based spoligotyping and MIRU-VNTR$_{15}$ methods on the MAGPIX® Milliplex Map device. In this study, it was determined that the T1 family is more common in our country and LAM7-TUR family is less common than the Beijing family unlike other studies. It was determined that the strains in the same cluster had different locus profiles, and there was no transmission from the same clone in the clonal typing we performed with spoligotyping and MIRU-VNTR$_{15}$.

KEYWORDS

Mycobacterium tuberculosis complex; spoligotyping; MIRU-VNTR; MDR-TB; tuberculosis surveillance

1. Introduction

Tuberculosis (TB) which is caused by *Mycobacterium tuberculosis complex* (MTBC) ranks among the top three causes of death due to infectious diseases worldwide [1]. According to the World Health Organization (WHO) 2020 data, it is estimated that there are approximately 10 million TB cases per year and 1.2 million patients die of TB. Multidrug-resistant (MDR)-TB cases that were resistant at least to isoniazid (INH) and rifampicin (RIF) have emerged as the global TB incidence peaked around 2003. According to the WHO 2020 report, there are a total of 206,030 MDR-TB cases worldwide [2]. The number of MDR-TB cases was 191, and the number of extensively drug-resistant (XDR)-TB, a rare type of MDR-TB that were resistant to any fluoroquinolone and at least to one of the three injectable second-line drugs (i.e. amikacin, kanamycin, or capreomycin), cases was reported as 2 in the national report published in our country in 2019 [2,3]. It is accepted that the rapid spread of MTBC strains such as Chinese Beijing lineage, which is at risk for developing MDR/TB worldwide due to reasons such as travel and migration in addition to the increase of MDR and XDR-TB incidence, plays a role in TBs becoming a global health problem [1,2,4]. Conducting molecular epidemiological studies on the temporal and spatial changes of different lineages and genotypes in MTBC enables the determination of the dynamics and epidemics of transmission routes, the evaluation of the success of control programs and the development of strategies for disease prevention and control [5]. Methods based on DNA polymorphism are used for genotyping of MTBC strains [5]. For this purpose, spoligotyping methods based on polymerase chain reaction (PCR) with MIRU-VNTR targeting the polymorphism of MTBC strains with different numbers of repetitive and randomly distributed sequences in the bacterial genome have been developed [6–9]. Spoligotyping is a PCR-based reverse hybridization method. There are 36 base pair direct repeat (DR) loci in varying numbers preserved in MTBC genome. There are sequences called spacers with a length of 35–41 base pair between these DR loci. Genetic relationship between strains can be determined based on the number of DR

CONTACT Burcu Gürer Giray burcuggurer@gmail.com Molecular Diagnosis Laboratory, Public Health Institution of Ankara, Turkey

copies and the presence or absence of spacer sequences between these repeat units in the spoligotyping method [5,6]. In the MTBC genome, 10–100-bp-long minisatellite-like 41 loci scattered between genes and operons, named MIRUs, have been identified. MTBC strains are genotyped by analysis of VNTR copy number polymorphism at selected MIRU loci in the MIRU-VNTR method [8]. MIRU-VNTR typing method is the fastest method used to determine epidemiological features of MTBC clones due to its advantages such as high discrimination power and standardization [5–9]. The discriminatory power of the spoligotyping method is lower than that of the MIRU-VNTR method. Although spoligotyping yields good results in analyzing large populations, it can also be more useful to track strains in small populations than MIRU-VNTR as well [8]. An improved approach in which MIRU-VNTR method expanded with extra loci and enhanced by adding spoligotyping method [7] achieved better effectiveness in terms of surveillance, especially in the differentiation of Beijing lineage, which plays an important role in the spread of drug resistance to different geographical areas. There is not enough data for molecular typing of MTBC strains in our country. In this study, it was aimed to determine the genotypic characteristics of MDR-TB isolates in our country by spoligotyping and MIRU-VNTR methods while investigating the susceptibility to the resistance of the strains circulating in the country.

2. Method

2.1. Clinical isolates and identification

A total of 96 MDR MTBC isolates that were isolated from sputum samples of patients aged 18 years and older with confirmed pulmonary TB and prediagnosed pulmonary TB and sent from 26 different provinces between January 2018 and May 2019 to Ankara National Tuberculosis Reference Laboratory, T.C. Ministry of Health General Directorate of Public Health, were included in the study. Microscopic examination was performed with acid-fast staining and inoculated into Löwenstein–Jensen (LJ) medium and BD BACTEC™ MGIT™ 960 (BD Mycobacteria Culture System; Becton Dickinson, Cockeysville, MD) systems after decontamination and homogenization processes with NaOH-NALC were applied to clinical samples. Isolates from clinical specimens were identified as MTBC using routine biochemical and phenotypic tests [8].

2.2. Antibiotic susceptibility testing

Streptomycin (SM), INH, RIF and ethambutol (ETB) resistance of MTBC strains was evaluated using BD BACTEC™ MGIT™ 960 (BD Mycobacteria Culture System; Becton Dickinson, Cockeysville, MD) systems in accordance with the manufacturer's recommendations. Anti-TB drug resistance was defined as greater than 1% growth in the presence of 0.1 mg/ml INH, 2 mg/ml RIF, 2.5 mg/mL ETB and 2 mg/ml SM. Strains that were resistant to one or more of the INH, RIF or other drugs were considered MDR [9].

2.3. Spoligotyping

A total of 96 MDR-MTBC isolates were selected and studied using the spoligotyping method on the MAGPIX Milliplex Map device after DNA isolation (Mickle Tissue Disintegrator). The amplification of 36-bp-long DR regions was performed with Beamadex Tuberculosis Spoligotyping (Genoscreen, Lille, France) in the PCR step of this molecular method. Hybridization was performed using TB-SPOL-MagPlex beads after PCR. PCR products were loaded into the MAGPIX® Milliplex Map (Merck Millipore, USA) instrument in accordance with commercial kit recommendations after hybridization. Spoligotyping analysis was performed with Luminex X Potent. Blotting data provided by the device was loaded into the SITVIT2 (http://www.pasteur guadeloupe.fr:8081/SITVIT2) database by applying the analysis key, and clusters and families were determined with Octal code format during the interpretation of the results [10].

2.5. MIRU-VNTR

In this study, 15 locus MIRU-VNTR method was used. Primers used by Alonso-Rodríguez et al. were synthesized for the targeted MIRU loci [11]. The number of MIRU alleles of amplification products was determined by electrophoresis, and the number of alleles in each MIRU locus was evaluated using the MIRU-VNTR Plus database (https://www.miru-vntrplus.org/MIRU/searchdb.faces) as a result of PCR [12,13] .

3. Results

Fifty-three (55.2%) of the TB patients were male and 43 (44.8%) were female out of the 96 MTBC MDR isolates included in the study, and their mean age was 46.8. It was observed that the highest rate of MDR MTBC isolate (17.2%) was clustered in İstanbul when the geographical distribution of MDR-TB strains was examined. It was determined that 92 (95.83%) isolates genotyped by spoligotyping method were included in 14 major spoligofamily clusters. One (1.045%) isolate was evaluated as atypical strain in the SITVIT2 database. It was found out that two (2.08%) isolates (SIT 1736 and SIT 1196) were not included in any cluster. One isolate (1.045%) was excluded because of contamination. The largest spoligofamily cluster was the T1 family with 38 (40.86%) isolates followed by the Beijing family

with 16 (17.2%) isolates, LAM9 with 9 (9.67%) isolates and LAM7-TUR with 8 (8.6%) isolates according to the spoligotyping results, while it was observed that 22 (23.65%) isolates were separated into other subsets (Table 1). It was found that the T1 family displayed 14 different Spoligo International Types (SITs) including 17, 7, and 3 strains and the others 1 strain each, respectively, in the SITVIT2 database. On the other hand, the Beijing family showed 16 (17.2%) isolates and 4 different SIT patterns, consisting of 10 strains, 4 strains and 1 strain in the SITVIT2 database. Nine isolates (9.67%) and the LAM9 family were found to demonstrate six different SIT patterns consisting of four strains and one strain each. It was observed that 8 (8.6%) isolates and LAM7-TUR formed three different SIT patterns consisting of six strains and one strain each (Table 1). After data comparison in MIRU-VNTR Plus database for MIRU-VNTR$_{15}$ clonal typing of isolates included in the study, it was determined that the strains did not spread from the same clone because they did not have the same locus profile. The T1 family with the highest number of strains was divided into five subsets with member numbers ranging from 1 to 4 with the combination of MIRU-VNTR$_{15}$, and 22 isolates showed unique profiles (Table 2). Five of the 16 isolates belonging to the Beijing family, the second largest cluster, exhibited unique profiles in the MIRU-VNTR$_{15}$ combination, while the remaining 11 strains were gathered in three clusters with member numbers ranging from three to five (Table 2). Six of the eight isolates belonging to the LAM7-TUR family, which is the fourth largest cluster, were included in a cluster, while the remaining two isolates showed unique profiles. It was observed that the LAM7-TUR family, which is unique to our country, does not have a common MIRU-VNTR pattern (Table 2).

4. Discussion

TB incidence has increased in many developed countries as a result of the neglect of TB control programs due to the wars experienced in the last 30 years throughout the world and migration due to socioeconomic problems [1–3]. Molecular epidemiological studies contribute to the identification of circulating origins in the community, detection of transmission sources and the development of new programs in tuberculosis control and treatment. The spoligotyping method used for this purpose is considered a fast and consistent method, and it is reported that the multiplex microbead-based spoligotyping technique is an easy-to-apply, reliable and reproducible method that allows multiple samples to be studied simultaneously in particular [10,14]. A microbead-based method was used in the spoligotyping method during this study for the first time in our country. Two of the 96 MDR MTBC isolates were not classified under any known lineage when compared with the strains in the SITVIT2 database, and one isolate was identified as atypical in this study. It was determined that the largest cluster of 93 MDR MTBC isolates that could be classified in the study was T1 with 38 (40.86%) isolates and Beijing family with 16 (17.2%) isolates. It was determined that the T1 family formed 14 different SITs, and this distribution was SIT53 with 17 samples, SIT 1580 with 7 samples and SIT 535 with 3 samples. Another 11 different SITs in the T1 family are represented by one sample (Table 2). LAM7-TUR (21%) family is the most common family, and SIT41 constitutes the largest cluster in this family, while SIT53 is determined to be the largest cluster [15] in T1 (16.3%) family, which ranks second in the study performed with 245 MTBC isolates isolated from Malatya and Ankara regions, considering the studies carried out throughout Turkey. It was found that 112 isolates (50.9%) were in the T family in the spoligotyping of 220 MTBC isolates, 67 of which were drug resistant, in another study. It was reported that SIT53 (25%) was the most common family with 55 isolates, LAM7-TUR (11.8%) with 26 isolates and the Haarlem family (10.9%) with 24 isolates constituted the three largest clusters [16] within this family. Although there are differences in other families, our study is compatible with the studies in our country since the major family is T1 family and the largest cluster is SIT53, while the largest cluster is SIT41 in LAM7-TUR family in all MDR-TB strains isolated from 26 different provinces in our country [17]. A total of 200 drug-resistant isolates from four different regions of Turkey were examined by Durmaz et al. [17], and they reported that isolates were clustered as SIT41 (22.5%) and SIT1261 (4.5%) in the LAM7-TUR family, SIT53 (19.5%) in the T family, SIT50 (6.5%) in the H3 family and SIT 47 (3.5%) in the H1 family. In addition, the global distribution of MTBC groups has been reported as LAM (33.5%), T (29%), Haarlem (14%), and S (3%) families [17]. In our study, it was observed that the most common family was T and clustering was in the SIT 53 in contrary to the previous studies. LAM and T as families and SIT53, in terms of its type, in which isolates were clustered are considered partially compatible with our study based on the provided data. It was reported that the most common families were T1 (64.0%) and LAM7-TUR (18.0%), and no strain belonging to the Beijing family was detected in a study performed with PCR-RFLP and spoligotyping method in 55 MDR-TB samples in Gaziantep [18]. In a study conducted with 450 MTBC strains around Malatya, it was reported that the T family was the most common family in the region with 38.6% followed by LAM7-TUR (27.3%) and Haarlem (14.8%)

Table 1. Spoligo lineage, family and SIT clusters in which MDR-TB isolates cluster.

MTBC spoligo lineage	Sublineage	Occurrence rate (%)	Total strain count	SIT clusters
Lineage 1	EAI 5	2.2	2	SIT 2740
Lineage 2	Beijing	17.2	16	SIT 269
				SIT 1
				SIT 255
				SIT 2101
Lineage 3	Cas1 Delhi	1.1	1	SIT 22
Lineage 4	T1	40.86	38	SIT 1105
				SIT 1318
				SIT 1580
				SIT 1761
				SIT 1793
				SIT 53
				SIT 535
				SIT 798
				SIT 86
				SIT 373
				SIT 266
				SIT 2513
				SIT 2032
				SIT 2541
Lineage 4	T2	1.1	1	SIT 1622
Lineage 4	T5 Rus 1	6.5	6	SIT 254
Lineage 4	LAM 2	1.1	1	SIT 1588
Lineage 4	LAM 3	1.1	1	SIT 1280
Lineage 4	LAM 7- TUR	8.6	8	SIT 186
				SIT 41
				SIT 367
Lineage 4	LAM 9	9.67	9	SIT 161
				SIT 162
				SIT 1800
				SIT 2201
				SIT 2648
				SIT 42
Lineage 4	H1	2.2	2	SIT 1165
				SIT 47
Lineage 4	H3	5.4	5	SIT 2211
				SIT 335
				SIT 35
				SIT 36
				SIT 511
Lineage 4	X2	1.1	1	SIT 282
Lineage 4	manuan	1.1	1	SIT 523

strains [19]. It was reported that 23.9% of 145 MTBC isolates were clustered in the LAM7-TUR family followed by strains belonging to the T1 family with a rate of 22.5% in another study conducted in the same geographical area with one-year intervals [20].

In our study, clustering of modern TB strains in the T family, presence of modern and ancestral MTBC strains that were almost equally distributed in Iran and existence of the T family that includes modern strains in Afghanistan show that modern and ancestral MTBC strains can be seen equally distributed in this wide area after a while. The same distribution of ancestral and modern TB strains in a wide geography can be demonstrated as a result of intense migration waves [21]. It has been pointed out that the human circulation, which exists because of tourism, trade and migration between our country and neighboring geographies, will cause changes in the MTBC population [1,6,21,22]. In a study conducted in Georgia, 26% of the isolates were found in Beijing, 18% in LAM, 12% in Ural and 5% in Haarlem [22]. T1 family was the most common family with SIT53 clustering with 25.7%, while SIT41 clustered in LAM7-TUR family ranked second with 5.4% in a study conducted in Bulgaria with 133 MTBC strains genotyped by spoligotyping and MIRU-VNTR method [23]. As seen in this study, the SIT 53 clustering of strains in the T1 family in our close neighbor is consistent with our study. Researchers attributed the prevalence of LAM7-TUR being above the world average and the interaction of MTBC genotypes in nearby geographies was due to migrations [24]. LAM7-TUR family was determined as one of the most common families in our study similar to the results of the study in Georgia and Bulgaria. It was reported that the dominant lineage was Ural-2 (New-1) (25.53%), and it was an atypical (23.4%) lineage restricted to Iran in a study performed on 47 MTBC isolates in western Iran. Spoligotypes were defined as 4162 and 4163 under the typical lineage in this study [25]. In another study conducted with 291 MTBC isolates in Iran, it was reported that the isolates formed 35 clusters and that the dominant cluster was the Ural (34.3%) family, while the largest cluster of SIT127 (15.8%) was in this family. It has been reported that the isolates with SIT 53 (6.1%) clustering in the T1 (18.2%) family provided in the study were isolated from patients living in the region

Table 2. MIRU-VNTR$_{15}$ profile of isolates belonging to Spoligotype T1, Beijing and LAM7-TUR families according to SIT clusters.

Spoligotype family	SIT cluster	Total strain count	MIRU-VNTR	Octal code
T1	SIT 1105	1	232,534,333,322,235	777,773,777,760,771
T1	SIT 1318	1	221,314,344,522,234	577,767,777,760,771
T1	SIT 1580	4	231,535,242,522,233	777,777,747,760,771
		3	232,534,334,422,233	
T1	SIT 1761	1	231,433,242,522,221	677,767,777,760,771
T1	SIT 1793	1	232,532,334,422,231	777,777,777,760,000
T1	SIT 53	3	231,534,242,922,225	777,777,777,760,771
		1	243,527,242,623,312	
		1	231,421,254,824,312	
		3	233,531,344,422,225	
		1	283,643,332,732,647	
		1	343,433,444,934,123	
		1	241,531,245,324,313	
		2	233,531,344,422,225	
		1	231,534,242,522,225	
		1	241,132,342,731,343	
		1	233,531,344,422,225	
		1	231,534,242,522,225	
T1	SIT 535	2	231,534,232,522,235	777,777,707,760,771
		1	234,532,231,522,233	
T1	SIT 798	1	232,534,333,422,233	437,777,777,760,771
T1	SIT 86	1	232,534,334,422,224	777,777,737,760,771
T1	SIT 373	1	232,532,243,522,233	777,777,767,760,771
T1	SIT 266	1	263,643,332,732,626	775,740,003,760,771
T1	SIT 2513	1	223,533,242,822,336	777,767,777,760,471
T1	SIT 2032	1	231,534,344,522,236	037627777760771
T1	SIT 2541	1	231,434,344,522,232	777,740,000,760,771
Beijing	SIT 1	3	213,753,446,824,433	000000000003771
		5	233,563,426,824,533	
		1	321,643,446,833,443	
		1	232,743,444,504,543	
Beijing	SIT 269	3	321,613,445,813,542	000000000000771
		1	321,742,445,803,542	
Beijing	SIT 255	1	332,843,346,731,444	000000000003671
Beijing	SIT 2101	1	332,642,445,712,545	000000000003400
LAM7-TUR	SIT 186	1	151,133,342,343,443	777,767,404,760,771
LAM7-TUR	SIT 41	6	223,523,132,333,333	777,777,404,760,771
			243,722,132,333,333	
			243,522,132,333,333	
			223,444,242,433,332	
			225,445,123,432,424	
			243,484,224,233,233	
LAM7-TUR	SIT 367	1	151,132,342,734,345	773,777,404,760,771

close to the Iraq-–Turkey border [26]. The reason for the high number of atypical lineages in the Iranian geography can be attributed to the intense human circulation and the excess of interaction within modern MTBC strains. In our study, the presence of an atypical strain and the existence of two SIT types that cannot be included in any family may be the result of the change in strains in these areas due to human traffic from regions close to our country. It was seen that drug-resistant strains are dominated by families of strains with different profiles and distributions when the studies conducted around the world were considered. The global emergence of the Beijing lineage was often associated with hypervirulence and drug resistance and occurred in the form of a direct spread throughout the world [27]. However, the T family includes strains that cannot be classified within the established genotypic families unlike Haarlem, LAM, CAS and EAI families with well-known phylogeographic features. It is considered to represent an evolutionarily mixed community within this context [28]. In conclusion, the data of this study showed that the Turkish MTBC population has a heterogeneous distribution. Although LAM7-TUR family was common in Anatolia in some studies, T1 family was found to be the most common spoligo family in this study. Although Beijing family was expected to be higher, T1 family was more common than Beijing family Among MDR-TB strains. Resistant MTBC strains began to cluster not only in the Beijing family but also in the T1 family, which represents a phylogeographically mixed community. The prevalence of SIT 53 and SIT41 in our country shows the importance of migration and human movements in the propagation of MTBC genotypes. Historical migration waves and intense human circulation for various reasons are taken into account as the main reasons for high prevalence of the SIT41 complex, which is thought to originate from the Turkish philogeography, and the SIT53 complex, which is thought to represent a mixed community. There are similarities in terms of frequently detected SITs and major families when the findings reported from different centers in our country are compared with this study. The fact that the

samples included in the study did not have a common MIRU-VNTR pattern with different families when the case was followed shows that there are no patients with re-infection and that there is no spread from a clone in the studied samples. The high aggregation rate detected as a result of spoligotyping reveals that it is insufficient to disclose true cross-contamination and should be confirmed with MIRU-VNTR, which has a higher discriminative power. Phylogenetic studies should be started with regions with stable and low sample flows in order to take advantage of MIRU-VNTR discrimination power, and families with strain clusters with clonal association should be followed to identify new members.

Acknowledgments

The authors would like to thank all clinicians and laboratory technicians who contributed to this study.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This work was supported by the Mersin Üniversitesi [2019-1-TP3-3148].

ORCID

Burcu Gürer Giray http://orcid.org/0000-0003-3165-8924

Ethical conduct of research

This study was approved by the Ethical Committee of Clinical Researches of Mersin University (Approval date: 19/12/2018 and Number: 2018/491).

References

[1] Furin J, Cox H, Pai M. Tuberculosis. Lancet. 2019;393 (10181):1642–1656.
[2] World Health Organization. Global tuberculosis report 2020. WHO: Geneva, 2020 . https://www.who.int/teams/global-tuberculosisprogramme/data .
[3] Tuberculosis war in Turkey 2019 report, PHIT, Ankara, 2020.
[4] Singh R, Dwivedi SP, Gaharwar US, et al. Recent updates on drug resistance in *Mycobacterium tuberculosis*. J Appl Microbiol. 2020;128(6):1547–1567.
[5] De Viedma D G, Pérez-Lago L. The evolution of genotyping strategies to detect, analyze, and control transmission of tuberculosis. Microbiol Spectr. 2018;6(5). DOI:10.1128/microbiolspec.MTBP-0002-2016
[6] Coll P, García de Viedma D. Molecular epidemiology of tuberculosis. Enferm Infecc Microbiol Clin. 2018;36 (4):233–240.
[7] Christianson S, Wolfe J, Orr P, et al. Evaluation of 24 locus MIRU-VNTR genotyping of *Mycobacterium tuberculosis* isolates in Canada. Tuberculosis. 2010;90(1):31–38.
[8] Pfyffer GE, Brown BA, Swenson JM, et al. Mycobacterium: general characteristics, isolation and staining procedures. In: editors, Murray PR, Baron EJ, Jorgensen JH, et al. Manuel of clinical microbiology. 8th ed. Washington DC: ASM Press; 2003:543–572.
[9] Loddenkemper R, Sagebiel D, Brendel A. Strategies against multidrug resistant tuberculosis. Eur Respir J. 2002;36:66–77.
[10] Molina-Moya B, Agonafir M, Blanco S, et al. Microbead-based spoligotyping of *Mycobacterium tuberculosis* from Ziehl-Neelsen-stained microscopy preparations in Ethiopia. Sci Rep. 2018;58(1):3987.
[11] Alonso-Rodríguez N, Martínez-Lirola M, Herránz M, et al. Evaluation of the new advanced 15-loci MIRU-VNTR genotyping tool in *Mycobacterium tuberculosis* molecular epidemiology studies. BMC Microbiology. 2008;8(1):34.
[12] Supply P, Mazars E, Lesjean S, et al. Variable human minisatellite-like regions in the *Mycobacterium tuberculosis* genome. Mol Microbiol. 2000;36(3):762–771.
[13] Weniger T, Krawczyk J, Supply P, et al. MIRU-VNTRplus: a web tool for polyphasic genotyping of *Mycobacterium tuberculosis* complex bacteria. Nucleic Acids Res. 2010 Jul;38(Web Server issue):W326–31.
[14] Shi J, Zheng D, Zhu Y. Role of MIRU-VNTR and spoligotyping in assessing the genetic diversity of *Mycobacterium tuberculosis* in Henan Province, China. BMC Infect Dis. 2018 3;18(1):447.
[15] Zozio T, Allix-Beguec C, Gunal S, et al. Genotyping of *Mycobacterium tuberculosis* clinical isolates in two cities of Turkey: description of a new family of genotypes that is phylogeographically specific for Asia Minor. BMC Microbiol. 2005;5(1):44.
[16] Otlu B, Durmaz R, Gunal S, et al. Beijing/W and major spoligotype families of *Mycobacterium tuberculosis* strains isolated from tuberculosis patients in Eastern Turkey. New Microbiol. 2009;32(3):255–263.
[17] Durmaz R, Zozio T, Gunal S, et al. Genetic diversity and major spoligotype families of drug-resistant *Mycobacterium tuberculosis* clinical isolates from different regions of Turkey. Infect Genet Evol. 2007;7(4):513–519.
[18] Karsligil T, Bayram A, Ekşi F. Determination of genotypic varieties and genotyping of multiple drug-resistant tuberculosis by the RFLP and spoligotyping methods. Turk J Med Sci. 2016;46:1502–1511.
[19] Gunal S. Determination of IS6110 RFLP (restriction fragment length polymorphism) and spoligotyping profiles of *Mycobacterium tuberculosis* isolates collected from different geographical regions of Turkey. Doctoral Thesis, 2006. İnönü University Faculty of Medicine, Malatya
[20] Durmaz R, Zozio T, Gunal S, et al. Population-based molecular epidemiological study of tuberculosis in Malatya, Turkey. J Clin Microbiol. 2007;45(12):4027–4035.
[21] Farnia P, Mohammad M, Varahram M. The recent-transmission of *Mycobacterium tuberculosis* strains among Iranian and Afghan relapse cases: a DNA-fingerprinting using RFLP and spoligotyping. BMC Infect Dis. 2008;8(1):109.
[22] Niemann S, Diel R, Khechinashvili G, et al. *Mycobacterium tuberculosis* Beijing lineage favors the spread of multidrug-resistant tuberculosis in the Republic of Georgia. J Clin Microbiol. 2010;48 (10):3544–3550.

[23] Valcheva V, Mokrousov I, Narvskaya O, et al. Utility of new 24-locus variable-number tandem repeat typing for discriminating *Mycobacterium tuberculosis* clinical isolates collected in Bulgaria. J Clin Microbiol. 2008;46 (9):3005–3011.
[24] Maghradze N, Jugheli L, Borrell S, et al. Classifying recurrent *Mycobacterium tuberculosis* cases in Georgia using MIRU-VNTR typing. PLoS ONE. 2019;14(10):e0223610.
[25] Ramazanzadeh R, Shakib P, Rouhi S, et al. Molecular epidemiology of *Mycobacterium tuberculosis* isolates in Iran using spoligotyping. New Microbes New Infect. 2020;38:100767.
[26] Haeili M, Darban-Sarokhalil D, Fooladi AAI, et al. Spoligotyping and drug resistance patterns of *Mycobacterium tuberculosis* isolates from five provinces of Iran. Microbiologyopen. 2013;2 (6):988–996.
[27] Nieto Ramirez LM, Ferro BE, Diaz G, et al. Genetic profiling of *Mycobacterium tuberculosis* revealed "modern" Beijing strains linked to MDR TB from Southwestern Colombia. PLoS ONE. 2020;15(4): e0224908.
[28] Barnes PF, Cave MD. Molecular epidemiology of tuberculosis. N Engl J Med. 2003;349(12):1149–1156.