Cellular and Molecular Biology

Asgharzadeh *et al. Cell. Mol. Biol.*2016, 62 (8): 15-20
ISSN: 1165-158X
doi: 10.14715/cmb/ 2016.62.8.3

Original Research

Strain differentiation of *Mycobacterium tuberculosis* for epidemiology in northwest of Iran

M. Asgharzadeh[1], H. Samadi Kafil[2*], M. Pourostadi[3], N. Asadi Faezi[4], J. Rashedi[5], B. Mahdavipour[6]

[1] Biotechnology Research Center, Faculty of Paramedicine, Tabriz University of Medical Sciences, Tabriz, Iran
[2] Drug Applied Research Center, Tabriz University of Medical Sciences, Tabriz, Iran.
[3] Hematology and Oncology Research Center, Tabriz University of Medical Sciences, Tabriz, Iran
[4] Immunology Research Center, Tabriz University of Medical Sciences, Tabriz, Iran
[5] Tuberculosis and Lung Diseases Research Center, Faculty of Paramedicine, Tabriz University of Medical science, Tabriz, Iran
[6] Department of Laboratory Science, Faculty of Paramedicine, Tabriz university of Medical Sciences, Tabriz, Iran

Abstract: Tuberculosis is a major health problem throughout the world and there are still a great number of people with the disease. Planning for controlling tuberculosis is required to identify the sources of infection and screening for the disease. The aim of this study is the differentiation of *Mycobacterium tuberculosis* strains for better understands the spread of the disease in North West Iran. In this study, 194 positive cultures of *M. tuberculosis* in North West Iran were evaluated by the MIRU-VNTR method. MIRU-12 differentiated the 194 isolates into 138 different patterns, comprising 30 clusters and 108 unique patterns (HGDI=0.9930). The largest cluster contained twelve isolates. The results showed that the majority of TB cases in North West Iran are due to reactivation and the 12-MIRU typing method can be used as a first-line method for strain differentiation of *M. tuberculosis*.

Key words: Tuberculosis, transmission, differentiation, MIRU genotyping.

Introduction

Nowadays, despite efforts to control tuberculosis and directly observed therapy short-course (DOTS) adopted by the world health organization (WHO), global TB, especially in developing countries is increasing (1). Nearly a third of the world's population is infected with *M. tuberculosis* but only ten percent of the patients develop active disease (2). According to the WHO in 2013, nine million new cases of TB occurred worldwide and as result, 1.5 million people lost their lives. Of these, 360,000 cases were positive for HIV (human immunodeficiency virus) (3) and most lived in poor socioeconomic conditions(4). The world TB control and containment program is currently associated with two global threats. One is the HIV epidemic (5) and the other is the prevalence of drug-resistant TB (6). Therefore, the use of methods that can identify the transmission of TB, especially multi-drug resistant TB (MDR-TB) should be effective in controlling this disease. To control TB the sources of the infection needs to be identified (7). With detection and treatment of the disease (8) using molecular epidemiology will helps Identifying the patterns of disease transmission and increasing our understand on the mode of transmission and spread of disease (9) This enables us to distinguish different mechanism of TB infection including recurrence of TB from recent infection transmission (10).

Several different typing methods are developed for the molecular epidemiology that make the study of strain diversity and dynamics of the disease possible, beside the evaluation of tuberculosis control programs (11). One of the molecular typing methods is MIRU-VNTR (Mycobacterial Interspersed Repetitive Units-Variable Number Tandem Repeats). The mycobacterium interspersed repetitive units are 40-100 base pair elements that are tandem repeats dispersed in the *M. tuberculosis* complex genome. There are a total of 41 MIRU loci, of which 12 are usually selected for genotyping: 2, 4, 10, 16, 20, 23, 24, 26, 27, 31, 39 and 40 (12). Different strains of *M. tuberculosis* have different numbers of repetitions in different positions. This repetition has been investigated by PCR (Polymerase Chain Reaction) and the number of repeats is calculated according to the size of the amplified products (13), Therefore it is an appropriate method to analyze the genetic diversity of strains. The aim of this study is the strain differentiation of *M. tuberculosis*, to help gain a better understanding of the spread of the disease in North West Iran and in this way helping to choose preventive measures to reduce TB in the country.

Materials and Methods

Mycobacterial isolates

All isolates of *M. tuberculosis* were collected from patients who were referred to central tuberculosis laboratories in North West of Iran. The study population comprised all patients from whom at least one sample was positive for *M. tuberculosis* by culture. One hundred and ninety-six of the *M. tuberculosis* complexes were collected from two central tuberculosis laboratories in North West Iran. The isolates were identified as

Received March 14, 2016; **Accepted** July 21, 2016; **Published** July 31, 2016

*** Corresponding author:** Dr. Hossein Samadi Kafil, Drug Applied Research Center, Faculty of Medical Sciences, Tabriz University of Medical Sciences, 5166614766Tabriz, Iran. Email: Kafilhs@tbzmed.ac.ir

Table 1. Discriminatory power of MIRU typing in the present study.

Distinct pattern	Unique pattern	No. of clusters	Isolated included in clusters	HGDI
138	108	30	86	0.9930*

*Highest degree for HGDI (Hunter-Gaston discriminatory index) is 1 that shows highest discriminatory power.

M. tuberculosis by Ziehl-Neelsen staining and standard microbiological tests, including production and growth rate on Lowenstein Jensen medium (14)

MIRU-VNTR typing

Extraction of DNA from *M. tuberculosis* isolates was performed by using lysozyme, SDS, Proteinase K and CTAB(15, 16). PCR was performed in 20µl volumes that contained 10-100ng DNA, 0.05µM of specific primers (17), 1.5Mm $MgCl_2$, 100µM dATP, dCTP, dGTP and dTTP, 0.5mM KCl, 20mM Tris-HCl, pH 8.4 and 1.25U DNA polymerase (SinaClon, Tehran, Iran). PCR was performed with an initial 7min denaturizing step at 94°C and final extension step at 72°C. The temperature cycles for different types of PCRs were as follows: 35 cycles of 45s at 94°C, annealing temperature for 45s and 72°C for 45s plus 72°C for 55s. The annealing temperature were as follows: 65, 63, 68, 65, 59, 67, 59, 65, 64, 63, 68 and 65°C for MIRU loci 2, 4, 10, 16, 20, 23, 24, 26, 27, 31, 39 and 40, respectively. In all PCRs negative controls consisted of the PCR components in reaction mixtures lacking *M. tuberculosis* DNA. PCR products were electrophoresed in 1.5% agarose gel and after staining with 0.5 micro g/ml ethidium bromide were visualized under UV light. The sizes of fragments were determined in comparison with a 100bp DNA ladder plus size marker (Fermentas, Lithuania).

Statistical analysis

All isolates included in this study were classified into two groups, clustered and non-clustered. A cluster was defined as two or more isolates with identical MIRU-VNTR types. Isolates with an unmatched genetic pattern were considered non-clustered. Clustered isolates were assumed to have arisen from recent transmission. The MIRU-VNTR allelic diversity (h) at a locus was calculated as $h=1-\sum\chi_i^2/n(n1)$, where χ_i is the frequency of the *i*th allele at the locus and n is the total number of strains in the typing scheme. The Hunter_Gaston discriminatory index (HGDI) was used as a numerical index for MIRU-VNTR discriminatory power (18). The minimum estimate of the proportion of tuberculosis cases related to recent transmission was calculated as (number of clustered isolates-number of clustered)/total number of isolates (19). Categorical data were compared by Chi-square test (or Fisher's exact test). P-values below 0.05 were considered significant. Online *MIRU-VNTRplus* tools were used for cluster analysis of the isolates (http://www.miru-vntrplus.org/).

Results

Sample of positive-culture tuberculosis (196) were prepared from sputum (n=137), bronchial fluid (n=32), gastric lavage (n=4), urine (n=8), cerebrospinal fluid (n=4), synovial fluid (n=3), peritoneal fluid (n=2), pleural fluid (n=2), biopsy (n=2) and exudates or aspirate abscess (n=2). Two of these samples were excluded due to cross-contamination. The remaining 194 isolates that were studied (128 isolates from East Azerbaijan and 66 isolates from West Azerbaijan) included 98 (50.51%) samples from males and 96 (49.49%) samples from females with a ratio male to female of 1.02. In East Azerbaijan, 57.81% of patients were female and in West Azerbaijan 33.33% of patients were female. The age range of the patients varied from newborn (40 days old) to 80 years old. By using the MIRU-VNTR method, 138 distinct patterns were detected that contained 30 clustered samples and 108 (55.67%) unique samples (Table 1). The level of clustering in this study was 44.33%: 46.88% in East Azerbaijan and 39.39% in West Azerbaijan. The largest cluster had 12 members, with seven members from West Azerbaijan and five members from East Azerbaijan. Eight were female and four were male. In several clusters there was no epidemiological connection, however, 1 five-member cluster, 2 four-member clusters, 9 three-member clusters and 17 three-member clusters observed (Table 2). Among these 30 clusters, 16 clusters were from Tabriz, most with two members. Two clusters from Urmia were unique and 12 clusters were from both Tabriz and Urmia, most of which had more than two members. Allelic diversity in 12 MIRU-VNTR are presented in Table 3. Loci 10, 26 and 40 had high discriminatory power (>0.6). Loci 4, 16, 20, 23, 24, 27

Table 2. MIRU clusters for the isolates in the present study.

MIRU Pattern [a]	NO. of isolates in cluster
2333-2325-2322	12
2342-2524-3323	5
2343-2525-3323	4
2332-1515-3324	4
2323-1525-3322	3
2333-1624-3322	3
2323-1525-3321	3
2332-1524-3324	3
2333-2516-3323	3
2233-2525-3322	3
2332-2525-2324	3
2332-1325-3322	3
2243-2325-3322	3
2331-1515-3423	2
2333-1515-3322	2
2341-2512-3322	2
2323-2513-3323	2
2223-2525-3323	2
2323-1512-3223	2
2333-1323-2322	2
2333-2323-3322	2
1324-1515-3324	2
2332-2524-3322	2
2363-2523-3323	2
2332-2615-3322	2
2333-2322-3322	2
2253-2525-2323	2
2331-1513-3324	2
2233-2523-3323	2
2323-2526-3313	2

[a] order of MRIU loci 2,4,10,16,20,23,24,26,27,31,39,40.

Table 3. MIRU–VNTR allelic distribution in isolates of the present study.

Locus	Number of isolates with the specified MIRU copy number											Allelic diversity
	0	1	2	3	4	5	6	7	8	9	10	
2	1	11	177	5								0.16
4		4	41	145	4							0.39
10		1	40	96	32	11	7	4	2	1		0.68
16		16	47	118	12	11						0.56
20		53	139	2		1						0.41
23	1	1	2	37	5	127	19	1	1			0.51
24		61	131	2								0.44
26		8	27	21	53	75	8	2				0.74
27		6	40	146	2							0.39
31		17	158	18	1							0.32
39		9	183	2								0.10
40		5	67	81	32	7	2					0.67

and 31 had intermediate discriminatory power ($0.3 \leq \chi \leq 0.6$) and loci 2 and 39 had low discriminatory power. Among the 12 loci in the MIRU-VNTR, locus 26 had the highest diversity (h=0.74). By using the convenient MIRU method, relatively high discriminatory power was obtained (HGDI=0.9930). In this study, almost one third of TB cases were due to recent transfer: 86 recurrent minus 30 recent = (86-30)/194 = 29%. To determine the risk factors associated with the recent transfer, 86 patients in the cluster were compared with 108 patients out of the cluster (Table 4). No significant association was observed in the clustering for sex, previous BCG vaccination, PPD positive skin test, diabetes, smoking and having a TB patient in the family ($p > 0.05$) While patients older than 60 years old were stayed within the cluster ($p<0.073$). There was also a significant relationship between the site of infection ($p<0.015$), hospitalization during the past year ($p<0.053$) and placement within the categories. Patients who had a history of anti-TB drugs had the most significant association with categories ($p<0.004$).

Discussion

M. tuberculosis infection can develop immediately after infection acquisition or after many years of acquisition. This may be due to reactivation of previous infection, or recurrent infection. Genotyping is essential to distinguish between re-infection and re-activation. The IS6110 method is often used as a standard technique but it is difficult and time-consuming, so in the last decade the MIRU-VNTR method has been used as a alternative method for strain differentiation due to its high speed and simplicity. It also has a high discriminatory power that enables study of the population genetics of *M. tuberculosis* (20) especially when a sufficient number of samples are examined.

In this study, the MIRU-VNTR method was used to determine the genetic diversity of strains and to study the dynamics of TB transmission in Northwest Iran. There was great variation in the MIRU-VNTR patterns, as of 194 isolates, 86 (44.33%) could be placed into thirty clusters. This is very similar to the 43.3% cluster

Table 4. Risk factor for clustering in the North West of Iran.

Risk factor	No. of patients (%)	No. of clustered patients (%)	No. of non-clustered patients (%)	P value
Age (years)				
< 40	55(28.35)	21(24.42)	34(31.48)	0.265
41 – 59	60(30.93)	24(27.91)	36(33.33)	0.405
> 60	79(40.72)	41(47.67)	38(35.19)	0.073
Sex				
Male	98(50.51)	43(50)	55(50.93)	0.895
Female	96(49.49)	43(50)	53(49.07)	0.895
Site of TB				
Pulmonary	171(88.14)	81(94.19)	90(83.33)	**0.015**
Extra pulmonary	23(11.86)	5(5.81)	18(16.67)	**0.015**
Previous BCG vaccination	72(37.11)	28(32.56)	44(40.74)	0.229
PPD positive skin test	98(50.52)	42(48.84)	56(51.85)	0.670
Diabetic patients	26(13.40)	14(16.28)	12(11.11)	0.287
Smoking	64(32.99)	28(32.56)	36(33.33)	0.907
Previous hospitalization (during last year)	78(40.21)	41(47.67)	37(34.26)	0.053
Previous TB treatment	16(8.25)	2(2.33)	14(12.96)	**0.004**
Underlying disorders	14(7.22)	7(8.14)	7(6.48)	0.652
History of family TB	34(17.53)	14(16.28)	20(18.52)	0.676
History of contact tracing	33(17.01)	14(16.28)	19(17.59)	0.804

in our previous study in this area (17). Only slight increases of clustering were observed in this latest study, therefore it seems progress in TB control in the region has not occurred. However fewer clusters occurred than in the total found in three provinces of Iran (21), where 61.98% clustering occurred, or the 57.446% clustering in Xi'an city, West China (22), and around 57.5% in Almeria, South East Spain (23). In North West Iran there is no migration from Afghanistan and Pakistan, so the control of TB is generally acceptable and the Beijing strain has not been observed. However, in the Sistan and Baluchistan provinces of Iran, because of common borders to countries with a high TB prevalence and Afghan immigrants, the TB level is high and HIV infection is greater than in Kermanshah (21). In Iran's capital, Tehran, the level of clustering is greater because of widespread marginalization and migrants from all over Iran and Afghanistan. In Xi'an (West China) the majority of the strains belong to the Beijing family (22). MIRU-VNTR typing in Beijing strains is limited and the power of differentiation is low (24). As the IS6110-RFLP method is not efficient for strains with less than six bands, it does not give accurate clustering (25).

In the present study, the clustering rate of tuberculosis strains was 44.33%, indicating that most TB cases in the region were caused by reactivation until recent population movements in this area. Although urbanization has increased, especially in the city of Tabriz, due to the lack of immigration from other countries, clustering is low in this area and the majority of the strains had a unique pattern. Therefore, in this study, 29% of TB cases were due to recent transmission. This is high compared to 14.4% in London (26), but is less when compared to 37% in Casablanca, Morocco (27). The level of infection control is a major factor. However, compared with a level of 26.8% in a recent study in our region, it shows a slight increase. This may be due to the increased level of HIV in the region and reduction of revenue is due to economic downturn. Overall, one strategy to prevent further spread of TB appears to be via the treatment of latent tuberculosis infection.

Great changes in MIRU-VNTR allele variation are seen in different geographical areas. In Paris, France (20) locus 39 (h=2) had the lowest diversity, locus 26 had the highest diversity (h=0.67), the next being locus 10 (h=0.69), then locus 40 (h=0.74). In Kerala, India 40 had the highest diversity among the 12 locus MIRU (28), and in a loci similar study in Singapore, locus 26 and locus 10 had the highest diversities (29), so extensive genetic diversity of different strains occurs in different regions of the world.

In this study, we examined gender as a risk factor but sex was not identified as a factor in the spread of bacteria ($p> 0.05$). However, a study in Tehran (30), found male gender was a risk factor for TB clustering and 73.2% were males inside clusters (30) Fewer women than men had TB, possibly due to the low literacy level among women, their low level of employment, more poverty among females and also the low prevalence of HIV in the region compared with men. Generally, a young age is known as a risk factor for recent transmission (26). In a study in Tehran, 85.7% of patients in a cluster were under 35 years old (31). Despite recent transmission in young people, in our study, most of the patients in a cluster were more than 60 years old [Table 4]. This was probably due to unemployment, low living standards of the elderly, low prevalence of HIV in the region, which is more common in young people and the sacrifice of the elderly, with reduced family incomes causing elderly family members more food poverty, thereby increasing TB transmission. In contrast to Farnia's study in Tehran(30), a relationship between TB and clustering was observed ($p< 0.05$). As bacteria are transmitted through coughing and sneezing (11), pulmonary cases are the main sources of infection and as a result, there are more cases of pulmonary tuberculosis than of extra pulmonary tuberculosis within clusters. In addition, people who were hospitalized during the previous year are more likely to be in a cluster because there is not an acceptable level of control and surveillance in the regional hospital.

The discriminatory power of 12-MIRU in this study was 0.9930 for all samples, with 138 having a distinct pattern [Table 1]. In a similar study in the USA, the HGDI on 259 isolates was 0.995 (32), while in Taiwan, the HGDI on the 502 samples collected was 0.951 (33) and in Xi'an, China on 195 isolates was determined as 0.948 (22). In the studies from Taiwan and China, a significant number of strains belonged to the Beijing family, leading to a low HGDI. So far, Beijing strains have not been identified in North West Iran, so the relative genetic diversity of strains is high. An increasing aging population and the activation of latent infection is effective in giving a higher discriminatory power in countries like Iran, so the MIRU method can be a good way to differentiate strains in this population base. In contrast, the spoligotyping method used alone has low discriminatory power (25)·(32), and although IS6110-RFLP gives good results, it is time-consuming. It is better suited for retrospective analysis.

In this study 30 MIRU patterns were obtained, 12 of which were common in both Western and Eastern Azerbaijan. The largest cluster with 12 samples was common among the two provinces (7 samples from the West Azerbaijan province and 5 samples from the East Azerbaijan province). This shows that TB transmission occurs between these two provinces, probably due to being adjacent provinces and the high mobility between them. For example, many of the residents in small towns in the West Azerbaijan province had been referred to the Tabriz in the East Azerbaijan province for medical treatment and work, so it seems probable that this has led to infection with the strains of *M. tuberculosis* circulating in the area. If TB case detection is improved, the health care of West Azerbaijan province increased and the mobility between the two provinces thereby reduced, this should limit TB transmission between the two provinces. (17), that mostly contained intra-cluster samples. This suggests that detection, treatment in the region was not sufficient, and untreated patients were thus able to transmit the disease to new people. This indicates the way forward to control the spread of infection.

It can be concluded that the 12-MIRU typing method is a simple, reliable and repeatable method that has enough power to assess the genetic diversity and identify sources of TB infection, when a sufficient number of samples have been examined. This technique can be

used as a first-line method to differentiate *M. tuberculosis* strains in population-based studies.

Acknowledgments
We thank Iranian National Science Foundation and Drug Applied Research Center for cooperation's and financial support and Tuberculosis Research Center for collaborations.

References

1. Merza MA, Farnia P, Salih AM, Masjedi MR, Velayati AA. First insight into the drug resistance pattern of *Mycobacterium tuberculosis* in Dohuk, Iraq: Using spoligotyping and MIRU-VNTR to characterize multidrug resistant strains. J infect public health. 2011;4(1):41-7.
2. Leylabadlo HE, Kafil HS, Yousefi M, Aghazadeh M, Asgharzadeh M. Pulmonary Tuberculosis Diagnosis:Where We Are?.Tuberc Respir Dis 2016;79:134-142
3. World Health Organization Global Tuberculosis Report 2013. WHO Press, Geneva, Switzerland. 2013.
4. Sissolak D, Bamford C, Mehtar S. The potential to transmit *Mycobacterium tuberculosis* at a South African tertiary teaching hospital. Intern J Infect Dis. 2010;14(5):e423-e8.
5. Sharma S, Mohan A, Kadhiravan T. HIV-TB co-infection: epidemiology, diagnosis & management. Indian J Med Res. 2005;121(4):550-67.
6. Krüüner A, Hoffner SE, Sillastu H, Danilovits M, Levina K, Svenson SB, et al. Spread of drug-resistant pulmonary tuberculosis in Estonia. J Clin Microbiol. 2001;39(9):3339-45.
7. Asgharzadeh M, Shahbabian K, Kafil HS, Rafi A. Use of DNA fingerprinting in identifying the source case of tuberculosis in East Azarbaijan province of Iran. J Med Sci. 2007;7(3):418-21.
8. Borgdorff MW, Nagelkerke NJ, De Haas PE, van Soolingen D. Transmission of *Mycobacterium tuberculosis* depending on the age and sex of source cases. Am J Epidemiol. 2001;154(10):934-43.
9. Borgdorff M, Nagelkerke N, Van Soolingen D, Broekmans J. Transmission of tuberculosis between people of different ages in The Netherlands: an analysis using DNA fingerprinting. Interna J Tubercul Lung Dis. 1999;3(3):202-6.
10. Bandera A, Gori A, Catozzi L, Degli Esposti A, Marchetti G, Molteni C, et al. Molecular epidemiology study of exogenous reinfection in an area with a low incidence of tuberculosis. J Clin Microbiol. 2001;39(6):2213-8.
11. Asgharzadeh M, Kafil HS, Pourostadi M. Source Case Identification and Control of Tuberculosis by Molecular Epidemiology.J Mazandaran Univ Med Sci. 2014;24(115):180-91.
12. Supply P, Mazars E, Lesjean S, Vincent V, Gicquel B, Locht C. Variable human minisatellite-like regions in the *Mycobacterium tuberculosis* genome. Molecul Microbiol. 2000;36(3):762-71.
13. Blackwood K, Wolfe J, Kabani A. Application of mycobacterial interspersed repetitive unit typing to Manitoba tuberculosis cases: can restriction fragment length polymorphism be forgotten? J Clin Microbiol. 2004;42(11):5001-6.
14. Runyon E. Identification of mycobacterial pathogens utilizing colony characteristics. Am J Clin Pathol. 1970;54(4):578-86.
15. Asgharzadeh M, Kafil HS, Khakpour M. Comparison of mycobacterial interspersed repetitive unit-variable number tandem repeat and IS6110-RFLP methods in identifying epidemiological links in patients with tuberculosis in Northwest of Iran. Annal Microbiol. 2008;58(2):333-9.
16. van Soolingen D, Hermans PW, de Haas PE, Soll DR, JD. vE. Occurrence and stability of insertion sequences in *Mycobacterium tuberculosis* complex strains: evaluation of an insertion sequence-dependent DNA polymorphism as a tool in the epidemiology of tuberculosis. J Clin Microbiol. 1991;29(11):2578-86.
17. Asgharzadeh M, Khakpour M, Salehi TZ, Kafil HS. Use of mycobacterial interspersed repetitive unit-variable-number tandem repeat typing to study *Mycobacterium tuberculosis* isolates from East Azarbaijan province of Iran. Pakistan journal of biological sciences: Pakistan J Biol Sci. 2007;10(21):3769-77.
18. Hunter PR, Gaston MA. Numerical index of the discriminatory ability of typing systems: an application of Simpson's index of diversity. J Clin Microbiol. 1988;26(11):2465-6.
19. Asgharzadeh M, Kafil HS, Roudsary AA, Hanifi GR. Tuberculosis transmission in Northwest of Iran: using MIRU-VNTR, ETR-VNTR and IS6110-RFLP methods. Infect, Genet Evol. 2011;11(1):124-31.
20. Mazars E, Lesjean S, Banuls A-L, Gilbert M, Vincent V, Gicquel B, et al. High-resolution minisatellite-based typing as a portable approach to global analysis of *Mycobacterium tuberculosis* molecular epidemiology. Pnas. 2001;98(4):1901-6.
21. Zamani S, Aflaki M, Fooladi AAI, Darban-Sarokhalil D, Bameri Z, Khazaee 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.
22. Zhou A, Nawaz M, Xue X, Karakousis P, Yao Y, Xu J. Molecular genotyping of *Mycobacterium tuberculosis* in Xi'an, China, using MIRU-VNTR typing. Intern J Tubercul Lung Dis. 2011;15(4):517-22.
23. de Viedma DG, Rodríguez NA, Andres S, Lirola MM, Ruiz-Serrano M, Bouza E, et al. Evaluation of alternatives to RFLP for the analysis of clustered cases of tuberculosis. Intern J Tubercul Lung Dis. 2006;10(4):454-9.
24. Kam KM, Yip CW, Tse LW, Wong KL, Lam TK, Kremer K, et al. Utility of mycobacterial interspersed repetitive unit typing for differentiating multidrug-resistant *Mycobacterium tuberculosis* isolates of the Beijing family. J Clin Microbiol. 2005;43(1):306-13.
25. Jonsson J, Hoffner S, Berggren I, Bruchfeld J, Ghebremichael S, Pennhag A, et al. Comparison between RFLP and MIRU-VNTR genotyping of mycobacterium tuberculosis strains isolated in stockholm 2009 to 2011. PloS one. 2014;9(4).
26. Maguire H, Dale J, McHugh T, Butcher P, Gillespie S, Costetsos A, et al. Molecular epidemiology of tuberculosis in London 1995–7 showing low rate of active transmission. Thorax. 2002;57(7):617-22.
27. Tazi L, Reintjes R, Banuls A-L. Tuberculosis transmission in a high incidence area: a retrospective molecular epidemiological study of *Mycobacterium tuberculosis* in Casablanca, Morocco. Infect, Genet Evol. 2007;7(5):636-44.
28. Joseph BV, Soman S, Radhakrishnan I, Hill V, Dhanasooraj D, Kumar RA, et al. Molecular epidemiology of *Mycobacterium tuberculosis* isolates from Kerala, India using IS6110-RFLP, spoligotyping and MIRU-VNTRs. Infect, Genet Evol. 2013;16:157-64.
29. Sun Y-J, Bellamy R, Lee AS, Ng ST, Ravindran S, Wong S-Y, et al. Use of mycobacterial interspersed repetitive unit-variable-number tandem repeat typing to examine genetic diversity of *Mycobacterium tuberculosis* in Singapore. J Clin Microbiol. 2004;42(5):1986-93.
30. Farnia P, Mohammadi F, Masjedi MR, Varnerot A, Zarifi AZ, Tabatabee J. Evaluation of tuberculosis transmission in Tehran: using RFLP and spoloigotyping methods. J Infect. 2004;49(2):94-101.
31. Farnia P, Mohammadi F, Masjedi MR, Varnerot A, Zarifi AZ, Tabatabee J, et al. Evaluation of tuberculosis transmission in Tehran: using RFLP and spoloigotyping methods. J Infect. 2004;49(2):94-101.
32. Cowan LS, Diem L, Monson T, Wand P, Temporado D, Oemig TV, et al. Evaluation of a two-step approach for large-scale, pros-

pective genotyping of *Mycobacterium tuberculosis* isolates in the United States. J Clin Microbiol. 2005;43(2):688-95.
33. Chin PJ, Jou R. A modified automated high-throughput mycobacterial interspersed repetitive unit method for genotyping *Mycobacterium tuberculosis*. Diagn Microbiol Infect Dis. 2005;53(4):325-7.