DETERMINING THE RISK OF INTRA-COMMUNITY TRANSMISSION OF TUBERCULOSIS IN THE NORTHWEST OF IRAN THROUGH 15 LOCI MIRU-VNTR TYPING

Ali Afaghi-Gharamaleki[1], Seyyedreza Moaddab[2,*], Mojtaba Darbouy[1], Khalil Ansarin[3], Shahram Hanifian[4]

[1]Department of Microbiology Science and Research Branch, Islamic Azad University, Fars, Iran
[2]Department of Laboratory Sciences, Paramedical Faculty, Tabriz University of Medical Sciences, Tabriz, Iran
[3]Tuberculosis and Lung Disease Research Center, Tabriz University of Medical Sciences, Tabriz, Iran
[4]Department of Food Science and Technology, Tabriz Branch, Islamic Azad University, Tabriz, Iran

This study was carried out in order to investigating the effect of travelling on the transmission of tuberculosis from high- to low-burden TB countries. Mycobacteria samples isolated from patients of distinct and relatively co-related countries (Azerbaijan Republic and Tabriz [located in the northwest of Iran]) were analyzed through 15 loci MIRU-VNTR typing method. PCR was done using special primers for each of the loci; then the number of allele repeats for all loci were determined by the size of their fragments. Finally, the created numeric patterns for each isolate were analyzed and clustered, using MIRU-VNTRplus.org website. All 119 isolates dispersing at 106 distinct patterns were composed of 10 clusters with 23 members and 96 unique patterns. Nine and five loci had high and moderate discriminatory power, respectively, but only one of them was poor in clustering. The study showed that 89.08% of TB cases involved resulted from the reactivation pattern and 10.92% were related to ongoing transmission. Although Azerbaijan Republic is a higher-burden TB region than Tabriz and Azerbaijan people make frequent tours to Tabriz to receive low or free medical services, the findings showed no TB transmission from the regions at least during the year of the study.

Keywords: tuberculosis, molecular epidemiology, 15 loci MIRU-VNTR, intra-community transmission, recent transmission

Introduction

Tuberculosis (TB) is a major health problem that affects millions of people around the globe, predominantly in low- and average-income countries. Worldwide, 9.6 million people are estimated to have fallen ill with TB in 2014: 5.4 million men, 3.2 million women, and 1.0 million children [1]. TB results by either recent transmission from particularly positive smear patients or reactivation of remote infections acquired in the past. The identification of transmission routes or sources of TB is very important, as there are their related appropriate prevention programs to control the disease. Since *Mycobacterium tuberculosis* is transmitted through airborne particles, eliminating the risk of transmission may completely be impossible, but using appropriate control programs could, to a large extent, reduce it. This entails fundamental methods that could initially prohibit the generation of infectious particles and then prevent their spread and reduce or eliminate them. The promptitude of diagnosis, isolation, and treatment of people with active tuberculosis is an effective control program [2, 3].

The epidemiology of TB in most low-incidence countries is characterized by a low rate of transmission among the population, and it is the cross-border migrations that bring about challenges [4]. Immigration into countries with different rates of TB could globally affect the features of the disease. There is evidence of increased risk of TB due to immigration from a high-prevalence area in the east of Iran. The increase of Afghans' migration from a high-burden TB country (Afghanistan) had resulted to increase of frequency as well as resistance in TB [5, 6].

* Corresponding author: Seyyedreza Moaddab; Department of Laboratory Sciences, Paramedical Faculty, Tabriz University of Medical Sciences, Tabriz, Iran

Although there are frequent trips between the Republic of Azerbaijan, a high-burden TB country, and Tabriz (located in the northwest of Iran), a low-burden city, it seems that the region does not suffer from those serious problems.

The "transmission index" has been introduced epidemiologically as a measure of transmission of TB. The transmission index is defined as the average total number of cases of TB attributable, directly or indirectly, to recent

Table 1. Designated loci with their characteristics and PCR primer sequences used in this study for the 15-loci set

Locus	Alias	MgCl₂ for PCR (mM) [*]	*M. tuberculosis* H₃₇Rv		PCR primer pairs Sequence (5′ to 3′)
			Rep. number and length (bp)	PCR fragment size[†]	
580	MIRU4	3	2 (77)	329	F: GCGCGAGAGCCCGAACTGC
					R: GCGCAGCAGAAACGCCAGC
2996	MIRU 26	3	3 (51)	387	F: TAGGTCTACCGTCGAAATCTGTGAC
					R: CATAGGCGACCAGGCGAATAG
802	MIRU 40	3	1 (54)	408	F: GGGTTGCTGGATGACAACGTGT
					R: GGGTGATCTCGGCGAAATCAGATA
960	MIRU 10	2	3 (53)	643	F: GTTCTTGACCAACTGCAGTCGTCC
					R: GCCACCTTGGTGATCAGCTACCT
1644	MIRU 16	2	2 (53)	671	F: TCGGTGATCGGGTCCAGTCCAAGTA
					R: CCCGTCGTGCAGCCCTGGTAC
3192	MIRU31	2	3 (53)	651	F: GTGCCGACGTGGTCTTGAT
					R: ACTGATTGGCTTCATACGGCTTTA
424	Mtub04	1.5	2 (51)	639	F: CTTGGCCGGCATCAAGCGCATTATT
					R: GGCAGCAGAGCCCGGGATTCTTC
577	ETR C	1.5	4 (58)	382	F: CGAGAGTGGCAGTGGCGGTTATCT
					R: AATGACTTGAACGCGCAAATTGTGA
2165	ETR A	1.5	3 (75)	420	F: AAATCGGTCCCATCACCTTCTTAT
					R: CGAAGCCTGGGGGTGCCCGCGATTT
2401	Mtub30	3	2 (58)	363	F: CTTGAAGCCCCGGTCTCATCTGT
					R: ACTTGAACCCCCACGCCCATTAGTA
3690	Mtub39	3	5 (58)	562	F: CGGTGGAGGCGATGAACGTCTTC
					R: TAGAGCGGCACGGGGGAAAGCTTAG
4156	QUB-4156	5	2 (59)	681	F: TGACCACGGATTGCTCTAGT
					R: GCCGGCGTCCATGTT
2163b	QUB-11b	1.5	5 (69)	412	F: CGTAAGGGGGATGCGGGAAATAGG
					R: CGAAGTGAATGGTGGCAT
1955	Mtub21	1.5	2 (57)	206	F: AGATCCCAGTTGTCGTCGTC
					R: CAACATCGCCTGGTTCTGTA
4052	QUB-26	1.5	5 (111)	708	F: AACGCTCAGCTGTCGGAT
					R: CGGCCGTGCCGGCCAGGTCCTTCCCGAT

[*]PCR Mixmaster contained 1.5 mM MgCl₂, and excess concentrations were created using 25 mM MgCl₂ solution.
[†]Data of the fragment sizes conformed to Ref. [10] except the fragment size of MIRU26, which conformed to Ref. [2] as well as the authors' observations.

transmission from a single source case [7]. DNA finger-printing of *M. tuberculosis* isolates is helpful for molecular epidemiologic investigations [8]. The number of DNA fingerprinting methods for *M. tuberculosis* has increased in recent years, but all have significant drawbacks, and only a few of them have proved fruitful. Although variable number tandem repeat (VNTR) loci were identified and named by Nakamura et al. [9], it was Supply et al. [10] who pioneered the study and classification of these microsatellites as mycobacterial interspersed repetitive units (MIRU-VNTR). These structures are composed of 40–100 bp repetitive sequences scattering over 41 locations throughout the chromosome of *M. tuberculosis*. MIRU-VNTR is a polymerase chain reaction (PCR)-based method for typing *M. tuberculosis* isolates that requires amplification followed by the analysis of the loci size. The number of repeated units can be determined by the size of the fragments produced by the amplification of the entire locus. Each isolate is typed by the number of copies of repeated unit sets in respective methods. In an effort to attain maximum discrimination, three sets of MIRU-VNTR were formulated: 12 loci, 15 loci, and 24 loci [10]. MIRU-VNTR analysis has the potential to become more discriminative, especially by utilizing additional loci. The addition of more loci increases the number of patterns observed in the sets [11].

This study is a molecular epidemiologic investigation of TB in Tabriz by using 15 loci MIRU-VNTR to determine the rate of TB transmission and intracommunity transmission in the Northwest of Iran.

Materials and methods

Patient population and bacterial isolates

The population included all 119 culture positive patients with TB who had referred to the central tuberculosis laboratory in Tabriz. Ninety-one isolates (76.5%) were obtained from individual cases from Tabriz, and the rest (28 isolates) from the Republic of Azerbaijan during the period from April 2013 to March 2014. The isolates were confirmed by culture and conventional diagnostic methods for *M. tuberculosis* such as niacin and catalase production and nitrate reduction [12].

DNA extraction and PCR

The used DNA for the PCR analysis was extracted by the cetyl-trimethyl ammonium bromide (CTAB) method described by Somerville et al. [13]. Fifteen loci MIRU-VNTR comprising Mtub04, 21, 30, 39; Qub11b, 26, 4156; ETRA; ETRC; and MIRU4, 10, 16, 26, 31, and 40 were individually amplified and analyzed as described by Supply et al. [10]. Each MIRU locus was amplified individually with specific primers for sequences flanking the MIRU units *(Table 1)*. PCRs were carried out using 2 ng of DNA

per reaction in 25 µl volume, 0.4 mM specific primers, and 12.5 µl Taq master kit (Ampliqon) containing Tris–HCl pH 8.5, $(NH_4)_2SO_4$, $MgCl_2$ (1.5–5 mM final concentration according to *Table 1*), 0.2% Tween 20, 0.4 mM of each dNTP, 0.2 Units/µl Amplicon Taq DNA Polymerase, inert red dye, and stabilizer. The PCR was performed under conditions of 7 min at 94 °C, 35 cycles as 45 s at 94 °C, 45 s at annealing temperature (locus related 59.5 to 67 °C), and 50 s at 72 °C and was then completed by a 7-minute extension step at 72 °C with a thermal cycler (Peqlab Primus 96). The electrophoresis was performed on 2% gel agarose to visualize the PCR products by a 100-bp DNA ladder (Thermo). All the experiments were conducted twice using standard positive ($H_{37}Rv$) and negative (Water) controls.

MIRU-VNTR typing

Using the results of standard $H_{37}Rv$ strain, the characteristics of the MIRU-VNTR were identified *(Table 1)*. The number of the repeated units for each isolate was calculated according to its fragment size and unit length and compared with the results of standard $H_{37}Rv$ strain. Thus, the results of all loci created the set 15 numeric patterns for each isolate. Algorithm data were entered and analyzed using the site www.MIRU-VNTRplus.org [14]. Finally, neighbor-joining dendrogram was calculated and drawn for typing method *(Fig. 1)* [15].

Clustering

The strain-clustering rates referred to as "DNA fingerprint clusters" are assumed to represent recent or ongoing transmission. A cluster is defined as two or more patterns with identical DNA fingerprints for each genotyping method. It assumes that a typical cluster of *n* people comprises one index patient with reactivated disease and *n* − 1 patients with recently acquired disease. The strain-clustering rate was calculated using the following equation:

$$\text{Strain-clustering rate} = (n_c - c)/n,$$

where n_c is the total number of strain clustered cases, *c* is the number of strain clusters, and *n* is the total number of cases in the sample [16].

The statistical analysis

To determine the discriminatory power of the used method, the study employed the Hunter–Gaston discriminatory index (HGDI) described by Hunter and Gaston as a numerical index [17]. The HGDI was calculated as follows:

$$\text{HGDI} = 1 - \frac{1}{N(N-1)} \sum_{j=1}^{S} n_j(n_j - 1),$$

where *N* is the total number of strains that were typed, *S* is the total number of MIRU-VNTR patterns, and n_j is the number of strains belonging to the *j*th pattern.
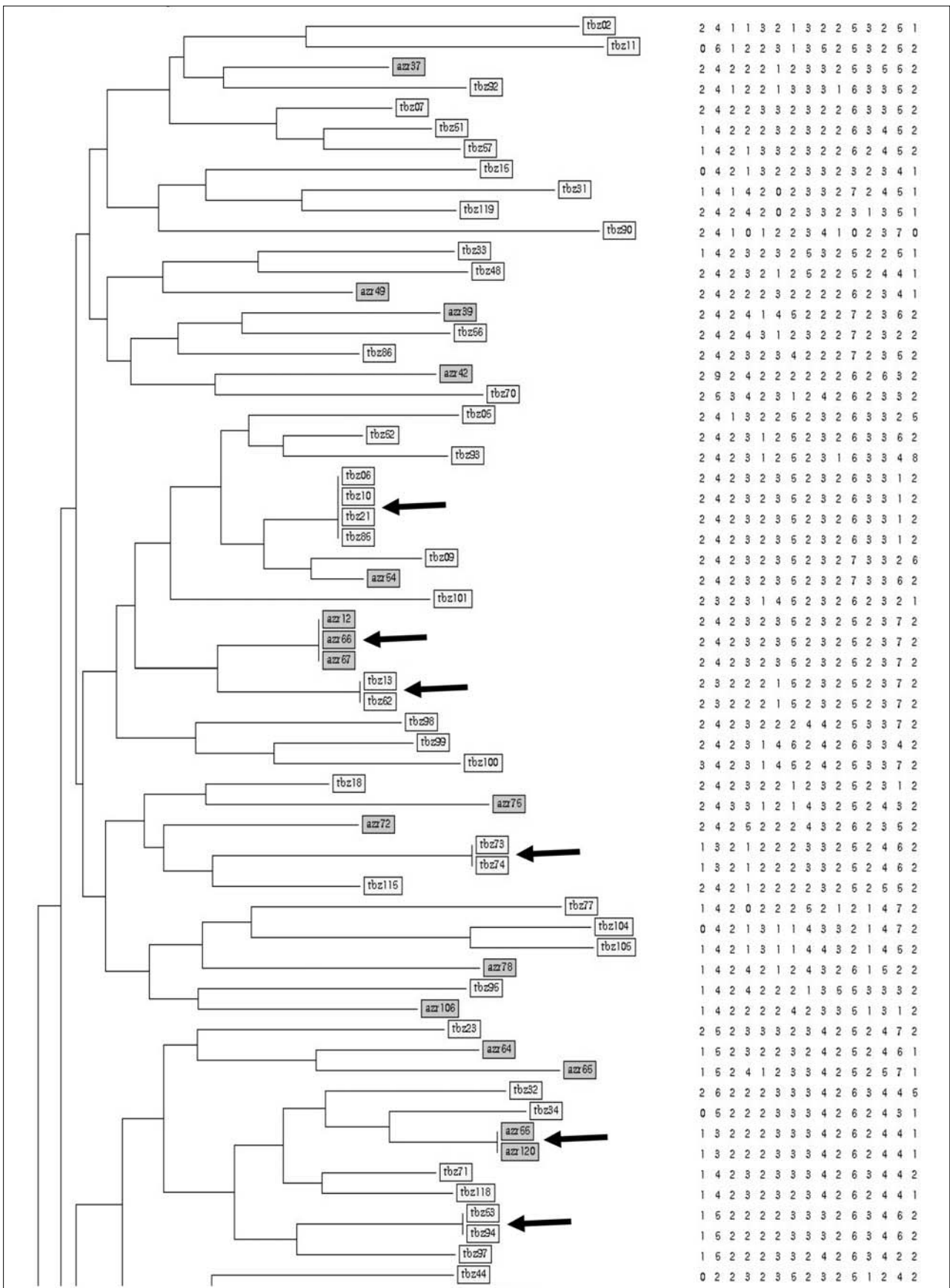
Fig. 1. NJ dendrogram of the genetic distances for typing of 119 used isolates in this study revealed ten clusters (iIndicated by arrows) and 106 distinct patterns. VNTR patterns for each of all isolates had been shown on the right. The set of each isolate left to right belongs to Mtub04, ETRC, Miru4, Miru40, Miru10, Miru16, Mtub21, Qub11b, ETRA, Mtub30, Miru26, Miru31, Mtub39, Qub26, and Qub4156, respectively. tbz, Tabrizian patients; azr, Azerbaijani patients
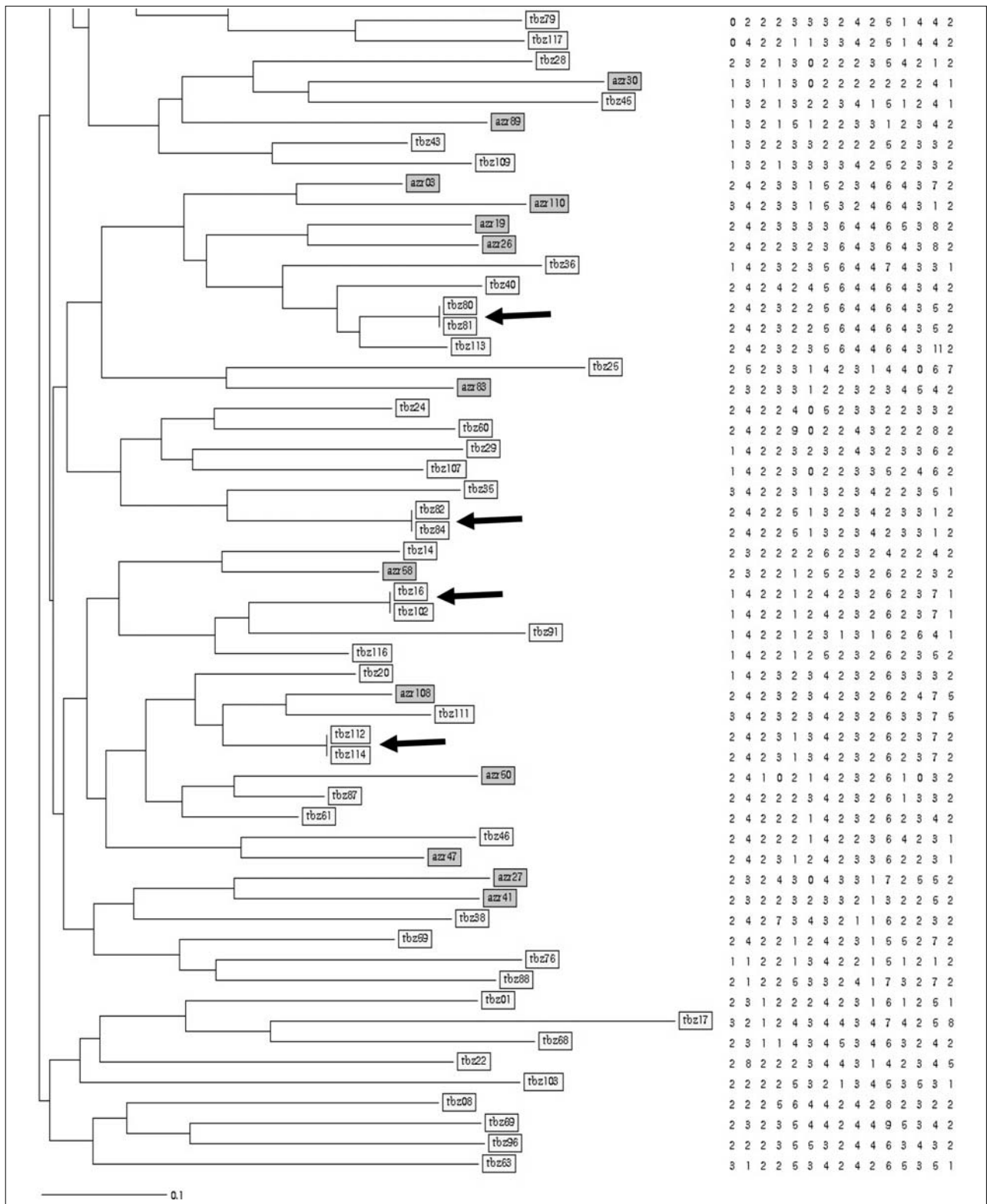
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Fig. 1. *(cont'd)*

Genetic diversity for a locus is calculated as $h = 1 - \sum x_i^2 [n/(n-1)]$, where x_i is the frequency of the ith allele at the locus, and n is the number of isolates [18].

Ethics statement

All samples of *M. tuberculosis* routinely isolate at the central tuberculosis laboratory in Tabriz for tuberculosis diag-

nosis. Since the study was carried out in parallel with the laboratory tests only to identify the microbial molecular characteristics without reference to the patients' medical data, it did not require any ethical approval or consent. Also, this research project has been approved by Tuberculosis and Lung Disease Research Committee numbered "5/76/594."

Results

The population

A total of 119 isolated mycobacteria were cultured and confirmed for *M. tuberculosis*. About 5% of whole population was excluded for diverse reasons (poor growth, insufficient sample, contamination etc.). The sample was drawn from Azerbaijan Republic and Tabriz to determine the probable intracommunity TB transmission. Ninety-one cases (76%) were from Tabriz, and the rest (24%) were from the Republic of Azerbaijan. There were 58 (59%) male and 61 (51%) female patients whose age range was between 15 and 86 years, with an average of 59 years.

Discriminatory power

The results of allelic diversity for 15 loci are summarized in *Table 2*. The discriminatory index of nine loci (MIRU10, 16, 26, 31, 40; Qub11b, 26; Mtub21, 39) showed high power ($h > 0.6$); five loci (Qub4156; Mtub04, 30; ETRA; ETRC) were dispersed to discriminate the isolates moderately ($0.3 \leq h \leq 0.6$), and only MIRU4 had a poorly discriminated index ($h < 0.3$). The discriminatory power of 15 loci MIRU-VNTR typing method was 0.9976 for the isolates involved in the study. *Table 3* comparatively summarizes the results of this study and other reports.

Table 2. The comparison of the discriminatory power of different MIRU loci in this study

Locus	Allele no.											Allelic diversity[*]	Discriminatory power
	0	1	2	3	4	5	6	7	8	9	11		
Miru4		11	106	2								0.19	Poor
Miru10		20	60	26	3	8	1			1		0.66	High
Miru16	8	21	36	45	8	1						0.72	High
Miru26	1	1	10	4	3	33	55	10	1	1		0.69	High
Miru31		14	55	33	13	4						0.68	High
Miru40	3	14	45	43	11	2		1				0.70	High
Qub11b		3	67	30	8	4	7					0.61	High
Qub26		11	7	18	24	23	11	21	3		1	0.84	High
Qub4156	1	26	83			5	1	1	2			0.46	Moderate
ETRA		1	18	65	34	1						0.59	Moderate
ETRC		3	6	21	76	9	2		1	1		0.55	Moderate
Mtub04	7	34	72	6								0.54	Moderate
Mtub21		7	32	25	25	28	2					0.78	High
Mtub30		15	75	12	16	1						0.55	Moderate
Mtub39	2		20	62	26	7	2					0.65	High

[*]Allelic diversity is defined as $h = 1 - \sum x_i^2$, where xi is the frequency of the *i*th allele at the locus

Table 3. The comparison of the discriminatory power combination of different MIRU loci in different countries [19]

Typing methods	All strains				
	HGDI[*]	No. of types	No. of unique types	No. of clusters	Percentage of clustering
15-locus (Supply)	0.990	291	269	22	17.2
24-locus (Supply)	0.999	303	287	16	11.7
12-locus (JATA)	0.999	302	284	18	12.6
16-locus (Gao)	0.9983	183	–	27	14.9
12-locus (Liu)	0.999	869	796	73	25.84
15-locus (this paper)	0.9976	106	96	10	19.33

[*]Hunter–Gaston discriminatory index

Clustering

The results of the clustering are shown in *Fig. 1*. All 119 isolates dispersing at 106 distinct patterns made up 10 clusters and 96 unique patterns. Twenty-three isolates (19.33%) were included in one of ten clusters. The largest cluster consisted of four patients of the same sex. The next cluster comprised of three patients with two females and one male. The rest contained only two patients per cluster with seven females and nine males. Accordingly, eighteen Tabrizi (18/91) and five Azeri (5/28) patients were clustered. Globally, according to 15 loci MIRU-VNTR method, at least 11% (23-10/119) of TB could be attributed to the recent infection developing to an active disease during the study. The sub-analysis of the clusters showed that all the strains existing in each cluster are restricted to the same geographical regions. The analysis of demographic factors such as sex, age, and nationality on clustering was not significant.

Discussion

The epidemiological investigation of TB is of great importance so that appropriate strategies for the prevention and elimination of the disease can be adopted [20]. Geographically, the incidence of the infection and, consequently, the disease burden vary greatly. Increased immigration contributes to an increased risk of TB among the resident population, but the results of the present study demonstrated that travels between Azerbaijan Republic and Tabriz did not have any significant effect on the features of TB in the region. Although there is a high immigration rate into Iran from some countries with a high prevalence of TB (especially Afghanistan), the researcher believes that the difficulty of learning Turkish language and subsequently difficulty in establishing relationship can be a reason for the small amount of immigration from other regions into Turkish-speaking area, so, epidemiologically, Azerbaijanis constitute a substantial proportion of tourists to Tabriz due to free or low medical services; however, Azerbaijan is counted as a higher-burden TB region than Tabriz. According to the World Health Organization (WHO) records, the estimated incidence of TB in Iran was 22, and the figure is lower in the studied regions according to the local reports, but the incidence rate of TB in the Republic of Azerbaijan was 76 per 100,000 [1].

This cross-sectional study was carried out in the northwest of Iran not only to determine the loci efficiency on the participants but also to describe the contemporary pattern of TB transmission in two distinct but co-related areas with different rates of TB.

In the cluster frequency analysis for all 119 TB cases (from both countries), 106 distinct patterns were identified. Twenty-three of the obtained isolates shared MIRU-VNTR patterns at one of 10 clusters, and 96 patients (81%) had unique patterns. The results showed that 18% and 20% of Azeri and Tabrizi patients were clustered respectively,

with the vast majority of patients (77%) being from Tabriz. The results revealed that, in the target region, 89% of TB cases were related to the reactivation pattern and 11% were due to recent transmission. The similar results have been reported for recent transmission across the world. Anderson *et al.* [21] used 24 loci MIRU-VNTR to investigate multidrug-resistant (MDR) isolates and estimated recent transmission index being 15% globally and, after adjustment for epidemiological links, 8.5%. Moreover, a report from China estimated 8.5% ongoing tuberculosis [22]. On the other hand, Asgharzadeh et al. [23] by using 12 loci MIRU-VNTR calculated the sharing of 26.6% TB transmission in almost the same as our studied region. Eimani et al., using *restriction fragment length polymorphism* (RFLP) method, came up with 66% for the ongoing transmission [24].

The subcluster analysis of the patients showed that all the existing clusters contained samples from a single area and the patients of the target nationalities had nothing in common. The results demonstrated no TB transmission between people of Tabriz and Azerbaijan, at least during the year the studied was being conducted. These findings do not agree with the results obtained by Asgharzadeh et al. [23]. Most likely, this disagreement lies in that the present study used additional number of loci with higher efficiency. Almost the same results have been reported by other researchers across the world. Varghese et al. concluded that the vast majority of clusters were not exclusively nationality specific and cross-national transmission of *M. tuberculosis* occurs among the immigrants and autochthonous in Eastern province of Saudi Arabia [25]. However, there is a report on the two regions with different TB burden similar results to those of the present study. The results of a study conducted in Denmark over 7 years showed almost no transmission between Somalis immigrants and Danes, while two-thirds of TB patients in Denmark are immigrants, half of which are from Somalia [26]. These results imply the possibility of TB controlling in the mixed populations from especially high- to low-burden TB countries.

The results of the loci performance showed that allelic discriminatory power of 15 loci was effective on the used isolates. The ability of 9 loci in the differentiation of isolates was high; only one locus (MIRU4) had a poor index, and the other five loci showed moderate power. The global discriminatory power of 15 loci MIRU-VNTR typing method (HGDI) was 0.9976 for the isolates. The value of HGDI was acceptable compared to other molecular methods such as RFLP and spoligotyping and even their composition.

Examining the risk factors based on demographic data such as sex, age, and nationality in clustering, it was observed that none of them was statistically significant ($p > 0.05$).

Conclusion

While TB occurs as a result of remote infection or ongoing transmission, it was estimated in this study that the bulk

of TB in the North-West of Iran is associated with the re-activation of *M. tuberculosis* in the body of previously infected patients, and the contribution of recent transmission is low. Nine of 15 loci used in this study (MIRU10, 16, 26, 31, 40; Qub11b, 26; Mtub21, 390) had high efficiency in genotyping. Furthermore, in spite of a high amount of travel from Azerbaijan, a high-burden TB country, to Tabriz, intracommunity transmission of the disease did not occur at least during the time when the study was being done.

Conflict of interest

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