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Genotyping of *Mycobacterium tuberculosis* isolates from northwest Iran for determination on the mechanism of transmission

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Abstract. Planning to control tuberculosis requires identification of dominant strains in the region, transmission patterns and risk factors that are possible by using molecular genotyping techniques. The aim of this study is to determine the transmission of tuberculosis in the northwest of Iran in order to better understand the spread of disease in northwest of Iran. In this study, 194 positive mycobacterium cultivars in northwest of Iran were investigated using exact tandem repeat-variable number tandem repeats (ETR-VNTR) method. The ETR-VNTR method was identified 55 different patterns in 194 isolates, which contained 25 clusters and 30 unique patterns, and the largest cluster had 33 isolates, and discriminatory power of ETR-VNTR method was determined 0.9322 in the examined samples. There are strains of *Mycobacterium tuberculosis* located in the northwest of Iran that infect people, and ETR-VNTR method can be used as a first-line method to examine the dynamics of tuberculosis transmission.

INTRODUCTION

Tuberculosis is one of the most common infectious diseases in the world, which, despite significant medical advances, is a health problem in most countries around the world, as nearly one-third of the world's population is infected with *Mycobacterium tuberculosis* (Murray 2004). According to the World Health Organization (WHO) in 2013, 9 million tuberculosis cases have occurred in the world, and 1.5 million people have died due to tuberculosis (WHO 2014), and drug resistant strains have increased in different countries (Krüüner *et al.*, 2001; Arora *et al.*, 2013). There is a potential danger to the world, so there is a strong need to develop and combat the control of all the diseases (Godfrey-Faussett *et al.*, 2000). The design of new control strategies, on the one hand, requires the identification of mechanisms and patterns of disease transmission, on the other hand, it is necessary to recognize the quick identification of the emergence and spread of new epidemics to prevent the spread of disease by treating infected people. Identifying strains of this bacterium is possible by increasing the knowledge of the

researchers about the genome of this bacterium (Cole *et al.*, 1998). So by genotyping of *M. tuberculosis* isolated from patients, identification of dominant strains in a region (Nabyonga *et al.*, 2011), transmission pattern (Mohammadi *et al.*, 2001; Maguire *et al.*, 2002), and risk factors (Farnia *et al.*, 2004; Singh *et al.*, 2005) is possible, according to the results, it can be used to control tuberculosis. Several molecular typing methods are used for genotyping and molecular epidemiological studies (Asgharzadeh and Samadi Kafil 2014), one of these methods is ETR-VNTR (exact tandem repeat-variable number tandem repeats), which is used for true continuous repeats for study, which are part of sequences that are repeated as a continuous variable, and 5 Locus A, B, C, D and E are used for typing the strains. Each ETR locus contains a variable number of repetitive sequences. They range from 53 to 79 bp, which are repeated. ETR is repeated continues in variable numbers in different strains of *M. tuberculosis*. These replications can be reviewed by PCR (polymerase chain reaction) method are based on the PPR method, and the number of repetitions is calculated according to the size of the duplicated products (Frothingham and Meeker-O'Connell 1998). As a result, it is a simple way to study the genetic diversity of strains in a region. The purpose of this research is to investigate the transmission of tuberculosis in the northwest of Iran by ETR-VNTR genotyping method, which can be helpful to better understanding the spread of disease, to take preventive decisions to reduce tuberculosis in Iran.

MATERIAL AND METHODS

Mycobacterium spp. isolates

All isolates of the mycobacterial complex were collected from patients who visited the Central Laboratories of Tuberculosis in the northwest of Iran. The population of study included all patients who had at least one positive sample for cultivating mycobacterium complex. 196 isolates were collected from two Central Laboratory laboratories in the northwest of Iran. Identification of mycobacterium complex was performed by using Ziehl-Neelsen stain staining and standard microbiological tests including niacin production capability, catalase activity, nitrate reconstruction, pigment production and growth rate in the lowenstein jensen medium (Runyon 1970; Asgharzadeh *et al.*, 2007b).

ETR-VNTR typing

DNA extraction from isolated bacteria was performed by using Lysozyme, SDS (sodium dodecyl sulfate), Proteinase K and CTAB (Cetyl trimethylammonium bromide) (Merck, Darmstadt, Germany) (Asgharzadeh *et al.*, 2008). PCR was performed in a volume of 20 µl which contains 10-100 ng of DNA, 0.5 µM of proprietary primers, 1.5 mM of $MgCl_2$, 100 µM of dNTP (Deoxynucleotide), 50 mM of KCl, 20mM of Tris-Cl (pH=8.4) and 1.25 unit recombinant taq polymerase DNA (Sinaclon Co, Tehran, Iran) (Asgharzadeh *et al.*, 2007a). The reaction was carried out in Thermal cycler in 35 cycles, which every cycle included denaturation step which was done at 94°C for 45 seconds, the annealing step was done at 94°C for 50 second and the extension step was done at 72°C for 65 second. The annealing temperatures of 66.68.69.63 and 63 were used for ETR-A, ETR-B, ETR-C, ETR-D and ETR-E respectively. Initial denaturation was performed at 94°C for 7 minutes and the final extension was carried out at 72°C for 7 minutes. In all PCR negative controls; there were all components of PCR except DNA of mycobacterium in the reaction mixture. PCR products were electrophoresed in 1.5% agarose gel in 0.7× TBE (Tris/Borate/EDTA) buffer. Finally, they were examined by staining with 0.5 µg/ml ethidium bromide against ultraviolet light from the transluminant. The size of the parts was determined by the size of the 100-bp DNA plus marker (Fermentas, Lithuania).

Statistical analysis

All isolates of this study were categorized into two groups consisting inclusive and non-inclusive categories. Isolates with a non-identical genetic pattern were considered

as non-inclusive in the categories, and a cluster containing two or more isolates which had the same ETR-VNTR pattern, and it was assumed that each cluster had an infectious source in which the disease is activated, and the rest have recently become infected. Allelic variation of ETR-VNTR (h) for each locus was calculated from the $h=1-\Sigma X_i^2/n(n-1)$ formula, and the X_i was the frequency of each allele in the locus, and n was the total number of strains. Hunter-Gaston discriminatory index (HGDI) was used to calculate the discriminatory power of ETR-VNTR (Hunter and Gaston 1988). Categorical information was compared with chi-square test (Fisher's exact test). P-value <0.05 was considered significant statistically.

RESULTS

Using ETR-VNTR method, 194 isolates (128 isolates from East Azerbaijan and 66 isolates from West Azerbaijan) were investigated, which the age of the patients were from 40 days old baby to 88 years old person. 55 different patterns were detected, including 25 clusters and 30 exclusive samples (16%) (Table 2). The level of clustering in this study was 84%, which was 88.28% in East Azerbaijan and 77.27% in Western Azerbaijan, the largest cluster includes 33 members, with 24 members from East Azerbaijan and 9 from West Azerbaijan, in which a significant number of them were not observed any epidemiological links. also 1 cluster of 26 members (15 members from East Azerbaijan and 11 members from West Azerbaijan), 1 cluster of 18 members (13 members from East Azerbaijan and 5 from West Azerbaijan), 2 clusters of 14 members, 1 cluster of 8 members, 2 clusters of 5 members, 3 clusters of 4 members, 1 cluster of 3 members and 13 clusters of 2 members were observed (Table 3). Among these 25 clusters, 8 clusters were exclusive to East Azerbaijan (5 clusters of 2 members and 3 clusters of 4 members) and 5 clusters were exclusive to Western Azerbaijan, which all of the clusters that were exclusive to West Azerbaijan were two-membered and in 13 clusters, East Azerbaijan and West Azerbaijan were common, which most of them were 5 members or more.

Table 1. Primers used in ETR-VNTR typing and size of repeat units

Locus name	primer sequences (5' 3')	length of repeat units (bp)
ETR-A	AAA TCG GTC CCA TCA CCT TCT TAT CGA AGC CTG GGG TGC CCG CGA TTT	75
ETR-B	GCG AAC ACC AGG ACA GCA TCA TG GGC ATG CCG GTG ATC GAG TGG	57
ETR-C	GTG AGT CGC TGC AGA ACC TGC AG GGC GTC TTG ACC TCC ACG AGT G	58
ETR-D	GCG CGA GAG CCC GAA CTG C GCG CAG CAG AAA CGC CAG C	77
ETR-E	ACT GAT TGG CTT CAT ACG GCT TTA GTG CCG ACG TGG TCT TGA T	53

Table 2. Discriminatory power of ETR typing in genotying of tuberculosis isolates in Iran

Distinct pattern	Unique pattern	No. of clusters	Isolates included in clusters	HGDI
55	30	25	164	0.9322

Highest degree for HGDI (Hunter-Gaston Discriminatory Index) is one that shows highest discriminatory power.

Table 3. ETR clusters pattern for the tuberculosis isolates from Northwest of Iran

ETR Pattern[a]	No. of isolates in cluster
31433	33
32433	26
42433	18
22433	14
42533	14
32323	8
21433	5
31434	5
42234	4
22432	4
55623	4
22232	3
32233	2
21633	2
22533	2
41433	2
31233	2
42733	2
23433	2
42443	2
42532	2
42234	2
32334	2
22334	2
22212	2

[a]order of ETR loci A, B, C, D, E.

The allelic diversity of ETR-VNTR is presented in Table 4, only the Locus A had a high level of difference (>0.6). The rest of the loci, including the A, B, C, D and E, had modest differences ($0.6 \geq X \geq 0.3$). Among the 5 loci, ETR-A had the highest ally variety (h=0.65). By using the ETR-VNTR method, the discriminatory power was obtained relatively low, so only 28% of tuberculosis cases were due to reactivation.

In order to determine the risk factors associated with the recent transmission, 164 patients within the cluster, were compared 30 patients out of cluster (Table 5). There is no significant relationship between clustering for age, sex, hospitalization during the last year, having a TB in the family, the history of TB treatment, smoking, diabetes, asthma, prison, and agricultural jobs and carpets, was observed ($p>0.05$). But people who had extra-pulmonary tuberculosis were placed less inside the cluster ($p<0.05$).

DISCUSSION

Nowadays, molecular typing methods are used to evaluate and improve TB control programs and to better understand the dynamics of TB transmission. Although the standard method is IS6110-RFLP, but due to inadequate power to distinguish the isolates less than 6 bands and the timing of obtaining results, other methods are used (Jonsson *et al.*, 2014), since spoligotyping alone has low selectivity, the genotyping methods based on PCR are used as a suitable method for diagnosis of tuberculosis due to the recurrence of infection from recent transmissions (Asgharzadeh *et al.*, 2011). In recent study, ETR-VNTR method was used to genotyping the *M. tuberculosis* strains in north-western Iran, so among 194 isolates, 164 isolates were located in 25 clusters, which the clustering rate was 84%, and the amount of clustering with this method is less than the rate of clustering in Hong Kong (Kam *et al.*, 2006), but from the clustering of 72%, Sola and colleagues were more likely to have

Table 4. ETR-VNTR allelic distribution of tuberculosis isolates

Locus	Number of isolates with the specified ETR copy number							allelic diversity
	1	2	3	4	5	6	7	
A	1	41	88	59	4		1	0.65
B	54	128	2	5	5			0.48
C		22	15	124	23	8	2	0.55
D	4	41	145	4				0.39
E		17	158	18	1			0.32

Table 5. Risk factor for clustering of *M. tuberculosis* isolates in the northwest of Iran

Risk factor	No. of patients (%)	No. of clustered patients (%)	No. of non-clustered patients (%)	*p*-value*
Age (year)				0.827
20≥	14(7.22)	13(7.93)	1(3.33)	
21–40	41(21.13)	34(20.73)	7(23.33)	
41–59	60(30.93)	51(31.1)	9(30)	
≥60	79(40.72)	66(40.24)	13(43.34)	
Sex				0.054
Male	98(50.51)	78(47.56)	20(66.67)	
Female	96(49.49)	86(52.44)	10(33.33)	
Site of TB				0.012
Pulmonary	171(88.14)	149(90.85)	22(73.33)	
Extra Pulmonary	23(11.86)	15(9.15)	8(26.67)	
Occupation				
Farmer	16(8.25)	13(7.93)	3(10)	
Carpet weaver	8(4.12)	6(3.69)	2(6.67)	
Previous TB Hospitalization (During last year)	78(40.21)	66(40.24)	12(40)	0.980
Smoking	64(32.99)	52(31.71)	12(40)	0.374
History of family TB	34(17.53)	31(18.9)	3(10)	0.174
Previous TB Treatment	16(8.25)	11(6.71)	5(16.67)	0.079
Diabetic Patients	26(13.40)	24(14.63)	2(6.67)	0.271
Asthma	6(3.09)	5(3.05)	1(3.33)	
Prisoner	7(3.61)	5(3.05)	3(10)	0.109

* *p*-value less than 0.05 was considered significant.

obtained samples from 11 different geographic origins (Sola *et al.*, 2003). In the case of Hong Kong, since more strains were Beijing, more clustering was achieved, while in the study of Sola, samples were taken from different places, so the variation are more and clustering are less than our results. By this method, most TB cases in this area are due to recent transmission, rather than because of reactivation. The recent transformation of TB could be caused from general poverty and lower income for people due to economic problems, meanwhile, considering that the amount of clustering is dependent on typing method, strain type (Jonsson *et al.*, 2014), age of patients, and number of samples and duration of study, therefore, it seems that only the percentage of clustering cannot directly determine the transmission rate of tuberculosis and other factors must be taken into account.

There are many variations in the ally diversity of ETR-VNTR are seen in different geographical origins, in this study, Locus E had the lowest diversity (h= 0.32) and Locus B had the highest variety, While in Chongqing, China (Liu *et al.*, 2013), Locus C (h= 0.54) and in Samarra, Russian (Nikolayevskyy *et al.*, 2006) Locus E (h= 0.52) had the highest diversity, and similar to our research in Kerala India (Joseph *et al.*, 2013), Locus A (h= 0.74), and in Hong Kong (Kam *et al.*, 2006), Lucas A (h= 0.68), had the highest variety. Wide variety of Ally diversity which are different from each other due to the heterogeneity of isolates present in different parts of the world. In this research, the discriminatory power of ETR-VNTR was determined to be 0.9322 for all samples and 55 patterns were determined, While in Hong Kong (Kam *et al.*, 2006), HGDI was obtained 0.9654 on 337 isolates, and sola (Sola *et al.*,

2003) were obtained the discriminatory power 0.959 on 116 isolates, and in Kerala, India, with three Locus A, B, C, the HGDI were obtained 0.5523 on 168 isolates (Joseph *et al.*, 2013). In the study of Hong Kong, the number of specimens collected from samples are more than our research, therefore, the discriminatory power is high. In the study of Sola, although the number of samples was low, but because of the obtaining the samples were from different countries and regions, so the discriminatory power is higher than our research. But in India, due to the high prevalence of tuberculosis, and the lack of use of two locus D and E to calculate the discriminatory power and the relatively fewer of samples number, So the discriminatory power is obtained low. Therefore, the ETR-VNTR method in countries like Iran can be used to initial study of the dynamics of TB transmission and cross-contamination (Asgharzadeh *et al.*, 2016; Bialvaei *et al.*, 2017) .

In cases of extra-pulmonary tuberculosis, in contrast to Tehran's study (Farnia *et al.*, 2004), they were less likely to be inside the cluster. Considering the study of infection spreading sources of people with acute untreated pulmonary tuberculosis, which is likely to infect healthy people with sneezing, coughing and mouth water, so it is conceivable that people with pulmonary tuberculosis are more likely to be present within the cluster. In this research gender was considered as risk factor, and about 48% of the internal clusters were male (52 female) and about 67% (33% female) of people out of the cluster were men. Although, it was not statistically significant ($p>0.05$), they were more female in the cluster, While in the Tehran study male gender was a risk factor for clustering, and about 73% inside the clusters were male (Farnia *et al.*, 2004), so this difference is due to the low employment of women in the northwest of Iran, the low level of literacy in women, and the poverty of women and the relatively low prevalence of HIV in the region, which is commonly seen in men.

In recent study, from 25 clusters, East and West Azerbaijan provinces shared 13 clusters. As a result, the transfer of tuberculosis occurs between the two provinces. In this transition, the adjacency of the two provinces and the traffic between the two provinces is effective, so, many people from the West Azerbaijan province come to Tabriz for treatment in the center of East Azerbaijan. Therefore, there are likely to be strains of *M. tuberculosis* in the region that just infect people.

CONCLUSION

According to the results from this study and several previous studies, it can be concluded that ETR-VNTR with 0.9322 discriminatory power can detect strains located in the area. This method has an acceptable capability to examine the genetic diversity of isolates when a sufficient number of samples are examined. Therefore, it can be used as a primary method to examine the dynamics of TB transmission. There are strains of *Mycobacterium tuberculosis* located in the northwest of Iran that infect people and their circulations between patients are in cluster based manner. These results indicate lower entrance of isolates from other regions and it helps us for better control of infection.

Conflict of interest

The authors declare that they have no conflict of interest.

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