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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

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Abstract - In recent years in spite of medical advancement, tuberculosis remains as a worldwide health problem. Therefore, identifying the source of transmission of infection is necessary for decreasing of tuberculosis (TB), also determining the varieties of TB strains by DNA fingerprinting helps to achieve this objective. The aim of present study was to determine tuberculosis transmission dynamics in Northwest of Iran with MIRU-VNTR and IS6110-RFLP methods. MIRU-VNTR performed for analysis of 125 strains and restriction fragment length polymorphism (RFLP) typing was performed on 119 culture-positive specimens during a period of September 2002 to March 2003 in tuberculosis centres of the region. We found 93 distinct MIRU-VNTR patterns, including in 21 clustered patterns and 72 unique patterns from isolated strains. The discriminatory power of MIRU-VNTR typing in our study was high (Hunter-Gaston discriminatory index, HGDI = 0.9932) for isolates. Ninety-three distinct IS6110 patterns were revealed. Twelve clusters were found among total of 38 strains. The clusters included 26 patients who infected by 12 another's. HGDI for our IS6110-RFLP method was 0.9928. The minimum estimate for the proportion of tuberculosis that was due to transmission with IS6110-RFLP was 21.9% and with MIRU-VNTR was 26.4%.In clusters the same patterns of Nakhichevanees patients and Iranian patients were revealed in three clusters with MIRU-VNTR and one cluster in IS6110 which showed that Nakhichevanees patients referred to tuberculosis centres of province could be a source of tuberculosis transmission. RFLP typing has more discriminatory power and it can be concluded that this method is a useful instrument for the better understanding of transmission and the occurrence of micro-epidemics and source tracing.

Key words: Mycobacterium tuberculosis, MIRU-VNTR, IS6110-RFLP, transmission.

INTRODUCTION

Mycobacterium tuberculosis is justly considered one of the most successful human pathogens; it infects one-third of the human population and kills about 3 million people every year (Dye *et al*., 1999). DNA fingerprinting of *Mycobacterium tuberculosis* strains is an important tool in differentiating strains and studying the epidemiology of tuberculosis (Alland *et al*., 1994; Small *et al*., 1994; Braden *et al*., 1997), IS6110 restriction fragment length polymorphism (RFLP) typing of *Mycobacterium tuberculosis* has been used extensively in studies on tuberculosis transmission and is one of the most widely applied and standardized molecular typing methods (Bock *et al*., 1998; Valway *et al*., 1998; van Soolingen *et al*., 2001). *Mycobacterium tuberculosis* outbreak isolates often show identical IS6110-RFLP patterns, and clustering by identical patterns is associated with well identified risk factors for transmission, whereas unrelated patients often have isolates with different IS6110 fingerprintings (van Soolingen, 1999; Diel *et al*., 2002; Oelemann *et al*., 2007), but this method is labour intensive and require weeks for culturing *M. tuberculosis*, which limited prospective use of this method (Barnes and Cave, 2003). Also IS6110-RFLP is not always reliable indicator of epidemiological linkages among tuberculosis patients (van Deutekom *et al*., 2005). Furthermore, because in many setting tuberculosis often results from casual contacts, majority of the transmissions can be identified only after combining the genotyping of the *M. tuberculosis* isolates with intensive epidemiological investigation (van Deutekom *et al*., 2005). These problems resulted in new trends of molecular epidemiology markers.

Variable number tandem repeat (VNTR) typing is an invaluable tool for genotyping and provides data in a simple and format based on the number of repetitive sequences in so-called polymorphic micro- or mini-satellite region (Magdalena *et al*.,

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1998). Mazars *et al.* (2001) introduced VNTR for *M. tuberculosis* which named maycobacterial interspersed repetitive unit-variable number tandem repeat (MIRU-VNTR). This method is highly reproducible and much faster than IS6110-RFLP typing and displays a discriminatory power close to the IS6110-RFLP method (Cowan *et al*., 2002; Maguire *et al*., 2002). A system based on 12 loci is now the most widely used in clinical mycobacteriology (Supply *et al*., 2001a; Allix *et al*., 2004), some new studies showed genotypes based on these 12 loci are highly stable among epidemiologically linked isolates but sufficiently diverse to generate a resolution approaching that of IS6110-RFLP in some sets of isolates from low-incidence setting or diverse geographic regions (Mazars *et al*., 2001; Supply *et al*., 2001b; Blackwood *et al*., 2004).

The estimated rate of TB in Iran in 2004 was 27 in 100000 (WHO, 2006) but in our studied region the estimated rate was lower; which can be due to low case finding or low prevalence of TB in this region.

In present study we aimed to perform two methods for isolates from Northwest of Iran for finding probable epidemiological linkage and comparing these two methods in identifying epidemiological links.

MATERIAL AND METHODS

Study population and data collection. The study included patients with confirmed TB that were reported to central TB laboratory of Tuberculosis and Lung disease research centre in Tabriz from September 2002 to March 2003. Tabriz is capital of East Azerbaijan province of Iran which located in Northwest of Iran, in neighbour-hood of Nakhichevan state of Republic of Azerbaijan. The estimated population of the province is 3,500,000 of which about two-fifth are inhabitants of Tabriz (Fig. 1). Seventy one of our samples were from Tabriz, 14 from Nakhichevan, 6 from Marand, 5 from Shabestar, 5 from Azarshahr, 4 from Sarab, 3 from Maraghe, 3 from Jolfa, 3 from Ahar, 3 from Moghan, 2 from Ajabshir, 2 from Heris, 2 from Bostan Abad, 1 from Bonab and one patient from Khoy. Information about age, sex, geographical origin and history of tuberculosis were collected by staffs of TB centre.

Bacterial strains and drug susceptibility test. The species identification of isolates was based on polymerase chain reaction (PCR) method and standard microbiological tests (Rieder *et al.,* 2000). From 127 isolates, two isolates were identified *Mycobacterium bovis*. Therefore, 125 isolates used for MIRU-VNTR typing, six isolates had not enough DNA for RFLP typing, therefore, 119 isolates used for IS6110-RFLP typing. These clinical isolates were recovered from sputum (n = 99), bronchial fluids (n = 19), abscess aspirates (n = 2), urine (n = 2), cerebrospinal fluids (n = 2), pleural fluid (n = 1), endometrial biopsy (n = 1) and neckmass biopsy (n = 1).

The susceptibilities of the isolates to isoniazid (INH), rifampin (RF), streptomycin (SM) and ethambutol (ETB) were determined by the proportional method (Rieder *et al.,* 2000).

DNA extraction. Two loopfuls of cultured *Mycobacterium* were suspended in 400 ml of TE buffer (10 mM Tris-HCL, 1 mM EDTA, pH 8.0) and placed at 80 ºC for 20 min to kill the bacteria, DNA was extracted by lysozyme, SDS, proteinase K and CTAB, extracted DNA after sedimentation with isopropanol and washing with ethanol 70% was resolved in 100 µl TE buffer (van Embden *et al*., 1993).

IS6110-RFLP. Analysis was performed as described by van Soolingen *et al.* (1994). Extracted mycobacterial DNA was digested with *Pvu*II, subjected to electrophoresis and hybridised with a 245 bp PCR-amplified probe directed against the right arm of IS6110. After hybridisation, the insertion sequence was visualised with a colorimetric system, the DIG DNA labelling and detection kit (Roche, Germany) by following the manufacturer's instruction. A mixture of *Pvu*II-digested supercoiled DNA ladder (Sigma) and *Hae*III - digested Fx174 DNA (Fermentas) was used as an internal marker. *Pvu*II - digested genomic DNA of *M. tuberculosis* reference strain Mt14323 was used as external marker in each gel. The IS6110 fingerprinting pattern was compared by visual examination (van Soolingen *et al*., 1994). A cluster of *M. tuberculosis* was defined

FIG. 1 - Map of the Northwest region of Iran.

as two or more isolates with exhibited the same number of copies (six or more) of the IS6110 fragment with identical molecular sizes (van Soolingen *et al*., 1999). All strains were compared to detect probable transmission of infection between patients.

MIRU-VNTR. PCR was performed in 20 µl volume that contained 5 to 50 ng of DNA, 0.5 µM of specific primers (Supply *et al*., 2001a) in the presence of 1.5 mM $MgCl_2$, 100 µM of each dNTP, 50 mM KCl, 20 mM Tris-HCl, pH 8.4, and 1.25 U recombinant DNA polymerase (Cinnagen co., Iran). DNA was amplified by general PCR. All PCR were initiated by a 7-min denaturising step at 94 ºC and completed by a 7-min extension step at 72 ºC. The temperature cycles for different types of PCRs were as follow, 35 cycles of 45 s at 94 ºC, annealing temperature for 45 s and 72 ºC for 55 s. Annealing temperatures were used as follow: 65, 63, 68, 65, 59, 67, 59, 65, 64, 63, 68, and 65 ºC for MIRU loci 2, 4, 10, 16, 20, 23, 24, 26, 27, 31, 39, and 40 respectively.

Negative controls consisted of the PCR components on reaction mixtures lacking *Mycobacterium* DNA. PCR products were electrophoreses in 1.5% agarose gel and after staining with 0.5 µg/ml ethidium bromide visualised under UV light. The size of fragments was determined in comparing with 100 bp DNA ladder plus size marker (Fermentas) (Supply *et al*., 2001a).

Statistical analysis. The Hunter-Gaston discriminatory index (HGDI) described by Hunter and Gaston (2003) was used as a numerical index for MIRU-VNTR discriminatory power. HGDI was calculated by using the following formula:

$$HGDI = 1 - \left[\frac{1}{N(N-1)}\sum_{j=1}^{s} n_j(n_j - 1)\right]$$

where N is the total number of strains in the typing scheme, s is the total number of different MIRU-VNTR patterns, and n_j is the number of strains belonging to the jth pattern. Dendrogram was created by MVSP (Multi Variable Statistical Package) software and statistical analyses were performed by x^2 test (chi-square test). P values below 0.05 were considered statistically significant. The minimum estimate for the proportion of tuberculosis that was due to transmission with IS6110-RFLP was calculated (by number of clustered samples minus number of clusters divided by the number of all samples).

RESULTS

A total of 125 isolates of *M. tuberculosis* from the reference laboratory of tuberculosis and lung disease research centre in Northwest of Iran were collected. Six of isolates could not be used for RFLP analysis, therefore, the RFLP pattern of 119 isolates from 125 isolates were determined. The age of the patients ranged from 12 to 90 years which young patients (≤ 45 years old) represented 43% of study population. The male and female ratio was 1.3:1. Eighty eight (70.2%) of the 125 strains were susceptible to INH, RF, SM and ETB; 29.8% of the strains were found to be resistant to at least one drug, with 23.2% of the strains resistant to SM, 13.6% resistant to INH, 8% resistant to RF, and four strains resistant to ETB. Among Iranian patients only two strains were multi-drug resistance (MDR). In patients from Nakhichevan state of Azerbaijan 28.5% were MDR and two isolates were extended drug resistance (XDR).

IS6110-RFLP typing

The RFLP patterns of 119 isolates were determined. The copy number of IS6110 in each of the isolates varied from 0 to 18. Ninety three distinct IS6110-RFLP patterns were recognised. Eighty one of these patterns were unique and 12 were shared by 2 to 8 strains. Thirty eight strains (31.9%) belonged to one of 12 clusters that were found among the total of 119 strains. The largest cluster comprised eight patients, all were susceptible to antibiotics and all were from Tabriz. Next cluster was made of five patients, three of them were from Tabriz and others from Maraghe. Another cluster comprised patients from Tabriz and one from Sarab. The last large cluster comprised four patients with different origins (Ajabshir, Heris and Tabriz).

Other clusters included 3 and 2 patients. In conventional epidemiological investigation, in cluster 1, patients both were from Tabriz and one of them was resistance to rifampin, similar condition was revealed in cluster 8, in clusters 2, 3, 6, 10 and 12 patients had similar origin and susceptibility to antibiotics. We couldn't find any other epidemiological links in clustered patients.

In cluster 9, one Nakhichevanees patient who was 35 years old and referred to our centre for treatment in several time showed similar RFLP pattern with two Iranian patients. However, he was XDR, but both Iranian patients were susceptible to all antibiotics. Strongly, he was the source of infection for Iranian patients and did not follow his treatment process completely. Patients of this cluster had similar MIRU-VNTR profile and included in cluster 15 of MIRU-VNTR clusters (Fig. 2).

No similar MIRU-VNTR profile was found for patients with lowers than five copy number of IS6110.

All patterns were compared with other 192 patterns that were previously obtained from various parts of country (which 54 patterns were from Northwest of Iran) and only one (1.1% of isolates) was found to have the same as an isolates that was previously observed in Northwest of Iran. We didn't

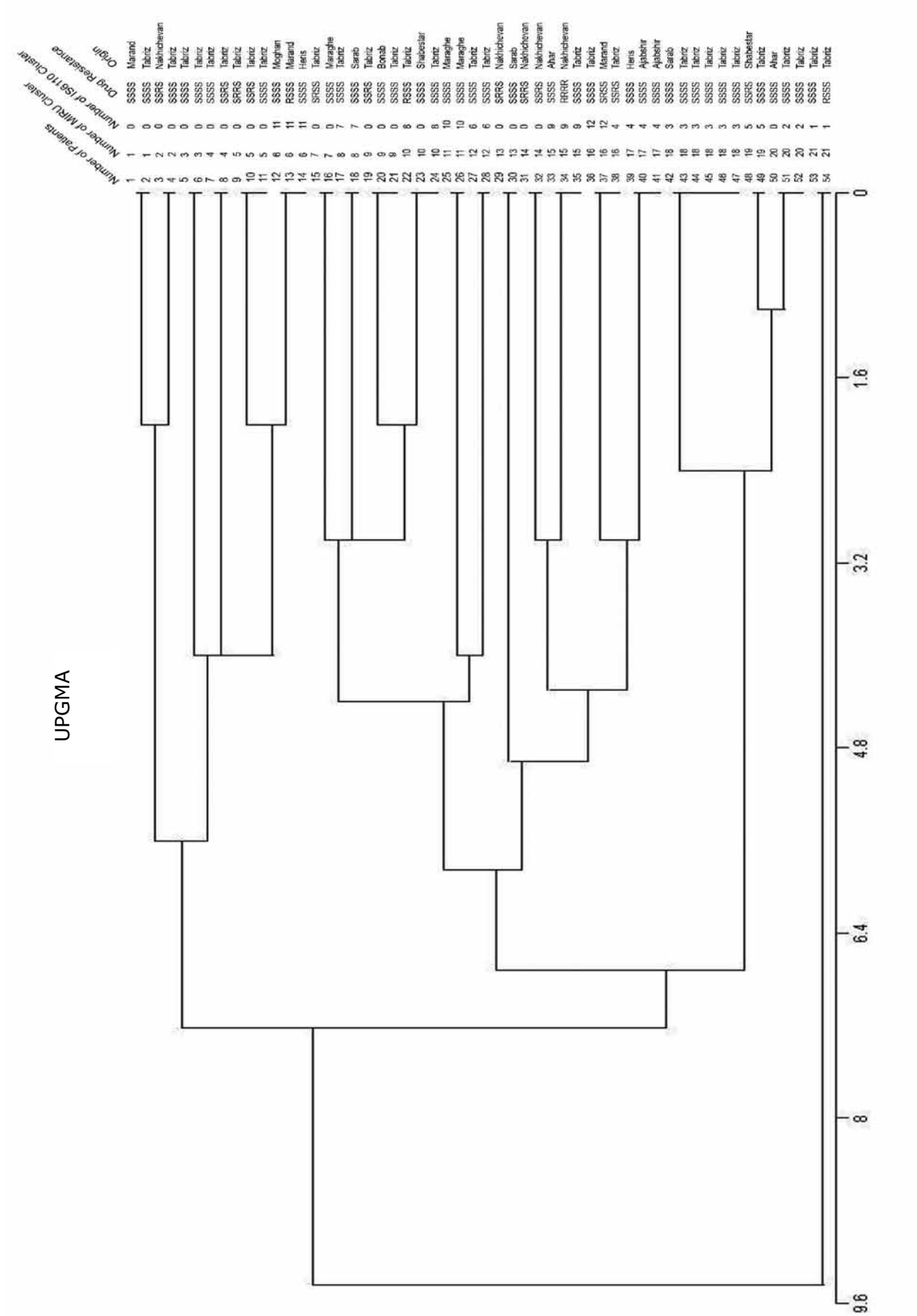

FIG. 2 - Dendrogram based on MIRU-VNTR. Column 1: number of patients included in study; column 2: MIRU-VNTR cluster number of patients; column 3: IS6110-RFLP cluster number of patients; column 4: drug resistant pattern of patients included rifampin, isoniazid, streptomycin and ethambutol respectively; column 5: origin of patients.

observed any statistically significant difference in the rate of clustering for age, sex, and site of TB ($P > 0.05$), but a significant difference was found for resistance in clustered and non-clustered patients ($P < 0.05$) and clustered patients were more susceptible to antibiotics.

MIRU-VNTR typing

Ninety three distinct profiles were identified, including 21 clustered profiles and 72 unique patterns. Fifty four isolates (43.2%) included in clusters.

In clusters, the largest cluster comprised six patients, four female and two male (cluster 18) (Fig. 2). Also, these patients had similar RFLP pattern and all of them were suffering from poor living condition. Eight clusters were composed three patients and twelve clusters composed of two patients. In MIRU-VNTR typing three clusters found to shared similar profile with Nakhichevanees patients. The first cluster has been described on IS6110-RFLP section of result (cluster 15). The second cluster (cluster 2) composed one Iranian and one Nakhichevanees patients who had resistance to Streptomycin and the last cluster included two Nakhichevanees patients (cluster 14) which both were resistance to streptomycin and one of them was resistant to isoniazid.

HGDI for our IS6110-RFLP method was 0.9928 and for MIRU-VNTR was 0.9932. The minimum estimate for the proportion of tuberculosis that was due to transmission with IS6110-RFLP was 21.9% [(38-12)/119] and with MIRU-VNTR was 26.4% [(54-21)/125].

DISCUSSION

MIRU-VNTR is a PCR based method for the typing of *M. tuberculosis* isolates that require amplification followed by size analysis of 12 independent loci; it can be highly effective in detecting the source case of infection and providing data for improving the control programs of tuberculosis. In addition, molecular typing using IS6110-RFLP has generally been used as the standard method in the studies of the transmission of tuberculosis; on the assumption that IS6110-RFLP based clustering of cases is the result of recent transmission. In present study we used IS6110-RFLP and MIRU-VNTR typing methods for study transmission of tuberculosis in Northwest of Iran and comparing reliability of these two methods in molecular epidemiology investigations. In MIRU-VNTR typing fifty four isolates (43.2%) included in one of the twenty one clusters, which showed transmission condition of tuberculosis. It was lower than a previous study in Singapore with 55.3% (Sun *et al*., 2004). The minimum estimate of transmission of tuberculosis in Northwest of Iran with MIRU-VNTR was 26.4% and with IS6110-RFLP was 21.9%. There is 4,5% difference on detection of minimum estimate by these two methods. This difference can be due to low discriminatory power or false clustering of MIRU-VNTR. More evidence proves that it is due to false clustering in MIRU-VNTR, but generally transmission of tuberculosis in our region relatively is on good condition compared with the minimum estimate of transmission in San Francisco which was 24% (Borgdorff *et al*., 2000) and in London which was 14.4% (Maguire *et al*., 2002) and in South Africa which was 50% (Godfrey-Fausset *et al*., 2000). Our results suggest that the majority of the tuberculosis cases in Northwest of Iran were due to reactivation. Presence of clusters with two or three patients, suggest that tuberculosis in this region of Iran is likely due to micro-epidemics. Same origins of clustered patients showed presence of epidemiological links, but we couldn't find any identification or relation between patients.

In our study prevalence of tuberculosis was higher for male (58.27%) than female (41.73%). Also, no difference was observed between the younger or older age categories and most patients had generalised poor living condition and unemployment.

In IS6110-RFLP typing we had lower clustering rate than MIRU-VNTR and there were 12 clusters with 32 isolates which shared on eight to two patients in each cluster. Three clusters in MIRU-VNTR typing and one cluster in IS6110-RFLP typing had patients from Nakhichevan (neighbour state of Republic of Azerbaijan). In this study 11.2% of patients were from this region and 35.71% of them included in clusters in MIRU-VNTR typing. These patients have been referred to our centre for treatment, because tuberculosis treatment in our country is entirely free, so patients are referring from neighbour countries like Azerbaijan, Turkey and Iraq. This travelling can cause the transmission of tuberculosis to Iran. Especially in our region we have travelling from Nakhichevan, and multidrug resistance tuberculosis in Republic of Azerbaijan is prevalent (Pfyffer *et al*., 2001). We compared our IS6110-RFLP patterns with previous studies and we had seven isolates which had similar pattern with previous studies in other parts of country. This low rate of similarity was also reported by farina *et al* (Farnia *et al*., 2001). It can be due to the distance between the areas. However since RFLP analysis was not performed in all regions of the country, including neighbour provinces, the true extent of clustering among patients of this region with patients elsewhere in Iran is probably underestimated.

Our study confirmed suggestion that drug sensitive strains are more transmissible and the absence of drug resistance strains in the largest cluster supported this suggestion.

IS6110 fingerprinting is laborious and requires weeks of *M. tuberculosis* culturing, which limits the

possibilities to use typing prospectively for more efficient tuberculosis control (van Embden *et al*., 1993; Oelemann *et al*., 2007). Also some other problems complicate the determination of clustering rates in population-based studies and exchange of data (Oelemann *et al*., 2007). MIRU-VNTR offers a potential solution to these drawbacks, in present study MIRU-VNTR revealed more transmission and clusters; these results can be due to false clustering and lower discriminatory power of 12 loci MIRU-VNTR. Therefore, it provided a tool with low resolution power and as previously demonstrated MIRU-VNTR genotypes were apparently too frequently unstable among presumably related isolates (Scott *et al*., 2005). Our study showed instead of higher HGDI of 12 loci MIRU-VNTR than IS6110, IS6110 had more discriminatory power. In Northwest of Iran tuberculosis has a low prevalence; therefore, low transmission of tuberculosis is supposed.

In some recent studies recommended use of 15 loci MIRU-VNTR for more discriminatory power and combination of 15 loci MIRU-VNTR and spoligotyping as a standard and automatic able method for genotyping (Supply *et al*., 2006; Oelemann *et al*., 2007).

CONCLUSION

In conclusion sanitation of tuberculosis in Northwest of Iran is in a good condition and reactivation of latent infection and then transmission of infection were effective in prevalence of tuberculosis. In order to prevent transmission of drug resistant tuberculosis from neighbour countries, new tuberculosis centres should be establish near the border to prohibit travelling of patients and long attendance of them. In spite of the hardships of IS6110-RFLP, it has more discriminatory power than 12-loci MIRU-VNTR based method in our study. This study was performed in a six month period time and was limited to Northwest of Iran, probably number of cases in cluster would have been greater when considering in larger period of time and space.

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