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IS6110 Fingerprinting of *Mycobacterium tuberculosis* Strains Isolated from Northwest of Iran

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Abstract: IS6110-based DNA fingerprinting is currently the most widely used genetic marker for differentiating among *Mycobacterium tuberculosis* strains. To evaluate the DNA polymorphism among *Mycobacterium tuberculosis* strains and to determine if there is matching of IS6110 fingerprints representing recent transmission of tuberculosis. Totally one hundred and sixty five isolates of *M. tuberculosis* (53 from West Azarbaijan and 112 from East Azarbaijan) were analyzed by IS6110 restriction fragment length polymorphism fingerprinting. Isolates having identical RFLP patterns were considered a cluster. The average number of IS6110 copies per strain was 7.3 and ranged from 0 to 17 among the *M. tuberculosis* isolates. The IS6110-DNA patterns from these isolates were highly polymorphic. In conclusion 123 patterns were observed which 16 patterns were shared by 47 isolates (30.52%). Most strains (93.62%) had multicopy patterns and only 3 of clustered isolates had less than six IS6110 copies. In our study increased clustering was observed with isolates from male patients. RFLP analysis of 154 isolates of *M. tuberculosis* showed a considerable diversity, suggesting that most patients were infected with unique strains, probably resulted from reactivation of the latent infection.

Key words: *Mycobacterium tuberculosis*, DNA fingerprinting, RFLP, transmission

INTRODUCTION

Mycobacterium tuberculosis is one of the most harmful human pathogens worldwide, causing about 8 million new tuberculosis cases and 2-3 million deaths yearly (de Boer *et al*., 2002; Singh *et al*., 2007). Tuberculosis (TB) remains a major health problem worldwide, but it is more prevalent in underdeveloped and developing countries, in which over 95% of cases occur (Vukovié *et al*., 2003).

It is estimated that between 19-43% of the world's population is infected with *Mycobacterium tuberculosis*, but a few (5-10%) will develop active TB (ATS, 2000; Malik and Godfrey-Faussett, 2005). The key for controlling the spread of tuberculosis include proper case finding, rapid diagnosis of tuberculosis and prompt initiation of effective chemotherapy (Farnia *et al*., 2001; Asgharzadeh *et al*., 2007). It was estimated by conventional epidemiologic methods that 90% of the active cases of tuberculosis in developed countries resulted from reactivation during adulthood of an infection contracted years before and that recently transmitted disease had a minor role (Gutiérrez *et al*., 1998). The typing of *Mycobacterium tuberculosis* strains is important for case tracing, distinguishing between relapse and reinfection by an exogenous strain and identifying nosocomial, institutional and community outbreaks (Durmaz *et al*., 2003). DNA fingerprinting using the insertion

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sequence IS6110 as a probe, IS6110 restriction fragment length polymorphism, has become the standard technique for the comparison of *M. tuberculosis* isolates on the strain level (Niemann *et al*., 2000; Kam *et al*., 1999; Braden *et al*., 1997).

It is generally assumed that the level of clustering among *M. tuberculosis* isolates from a certain region is associated with the level of recent transmission. On the contrary, non-clustered cases are considered to indicate TB resulting from reactivation of latent infection (Vukovié *et al*., 2003). Patients with TB whose isolates cannot be grouped into clusters, i.e., those with unique DNA fingerprints, are assumed to have disease that results from the reactivation of latent infection acquired in the past (Murray, 2002). Finally IS6110 fingerprinting can be applied to confirm recent transmission of tuberculosis in a linked population or laboratory cross-contamination (de Boer *et al*., 2002).

In the present study, we have investigated DNA polymorphism and epidemiological relationships among *M. tuberculosis* strains isolated from tuberculosis patients residing in Northwest (East and West Azarbaijan) of Iran.

MATERIALS AND METHODS

Bacterial Strains

All isolates of *M. tuberculosis* were collected from patients who referred to central TB laboratory of Orumieh and Tabriz Tuberculosis and Lung Disease Research Centers from March 2004 to March 2005. Finally the RFLP patterns of 165 *M. tuberculosis* isolates (53 from West Azarbaijan and 112 from East Azarbaijan) were determined. The isolates were identified as *M. tuberculosis* by standard biochemical tests, including production of niacin, catalase activity, nitrate reduction, pigment production and growth rate (Asgharzadeh *et al*., 2006).

IS6110- RFLP

Extraction of DNA from *M. tuberculosis* isolates were performed by the standard protocol described by Van Soolingen *et al*. (994). The IS6110 probe was prepared by digoxigenin labeling of 245 bp amplification by the polymerase chain reaction (PCR). Briefly, the oligonucleotides INS-1 (5′CGT GAG GGC ATC GAG GTG GC) and INS-2 (5′ GCG TAG GCG TCG GTG ACA AA) [Tib-Molbiol, Germany] were used to amplify a 245 bp fragment from purified chromosomal *M. bovis* BCG DNA by PCR (van Embden *et al*., 1993). This fragment was purified and after solubilization, the DNA was labeled.

DNA fingerprinting was performed as described by van Soolingen *et al*. (1994) and van Embden *et al*. (1993). The extracted mycobacterial DNA was digested with *Pvu*II enzyme (Cinagen, Iran) and restriction fragments were separated in 0.8% agarose gel electrophoresis at 20 V for 18 h. Then, the fragments were transferred, from the gels to positively charged nylon membranes.

Hybridization was performed by using a 245 bp probe of insertion sequence IS6110 and detected by colorimetric system. A mixture of *Pvu*II-digested supercoiled DNA ladder (Sigma, USA) and *Hae*III-digested φX174 DNA (Fermentas, Lithuania) was used as an internal marker. The internal marker was added to the wells together with the cleaved *M. tuberculosis* DNA and visualized by reprobing the blots with DIG DNA labeling and detection kit (Roche, Germany). In addition to internal size marker, *Pvu*II-digested genomic DNA of reference strain M.tl 4323 was used in each southern blot experiment as an external size marker. RFLP patterns of the isolates were compared by visual examination (van Soolingen *et al*., 1994). A cluster was defined as a group of two or more isolates from different patients whose RFLP fingerprints were identical with respect to both the number and molecular size of all bands (Asgharzadeh *et al*., 2006, 2007).

Statistical Analysis

All patients included were classified into two groups, clustered and non-clustered. Categorical data were compared by Chi-square test (or Fishers exact test). p-values below 0.05 were considered significant.

RESULTS

From March 2004 to March 2005, one hundred and sixty five culture-confirmed cases of TB were subjected to IS6110-RFLP analyses, that 89 (53.94%) were isolated from male patients and 76 (46.06%) belonged to female ones. The age of patients ranged from 2.5 to 88 years.

As shown in Fig. 1, RFLP typing revealed a variable numbers of hybridizing bands that ranged from 0-17, with the majority of strains (93.3%) having at least five copies. The average copy number of IS6110 per strain was 7.3. Only 11(6.7%) isolates had less than five IS6110 copies: One isolates had four, one isolates had two and one isolates contained a single copy of IS6110 element, 8 isolates showed no copies of IS6110 element. RFLP analysis was performed on 154 isolates that contained five or more copies of IS6110 (Fig. 2). The two similar RFLP patterns showed in number 1 and 2 samples of Fig. 1.

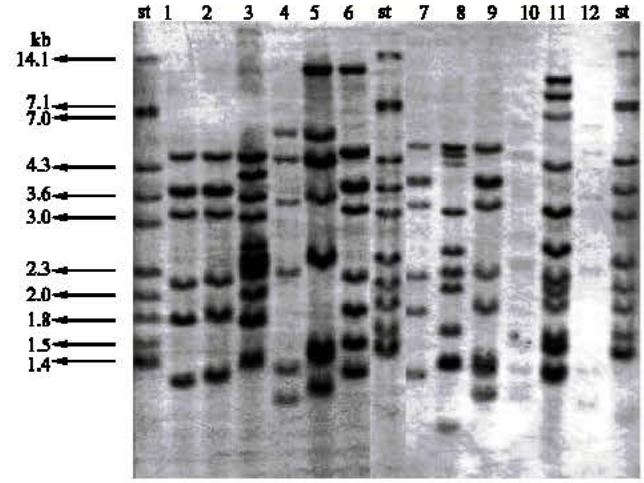

Fig. 1: Restriction fragment length polymorphism patterns of *Mycobacterium tuberculosis* isolates obtained by IS6110 probe. Lane 1-12 RFLP patterns of *Mycobacterium tuberculosis* isolates, St: RFLP pattern of *Mycobacterium tuberculosis* reference strain (M.tl 4323).

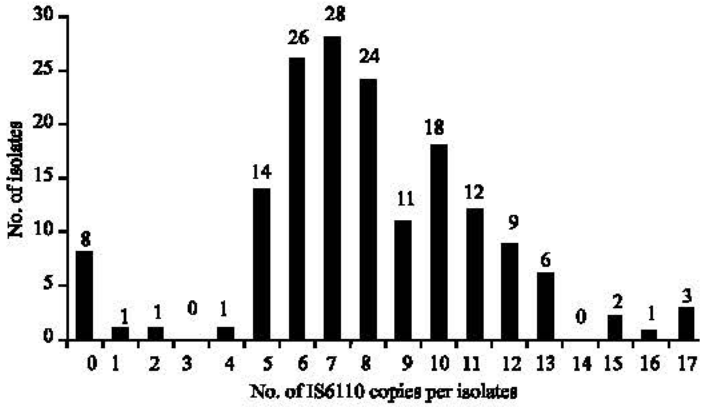

Fig. 2: No. of IS6110 elements in *M. tuberculosis* isolate from East and West Azarbaijan provinces of Iran

Table 1: Risk factors for clustering of tuberculosis in East and West Azarbaijan provinces of Iran

Risk factor	No. of clustered patients (%)	No. of non-clustered patients (%)	All patients (%)	p-value
Sex				
Male	27(57.4)	53(49.5)	80(51.9)	0.365
Female	20(42.6)	54(50.5)	74(48.1)	
Age group (year)				
≤30	3(6.4)	25(23.4)	28(18.2)	
31-40	5(10.6)	10(9.3)	15(9.7)	
41-55	11(23.4)	31(29.0)	42(27.3)	
≥56	28(59.6)	41(38.3)	69(44.8)	0.031
Site of TB				
Pulmonary	40(85.1)	94(87.9)	134(87.0)	0.641
Extra-pulmonary	7(14.9)	13(12.1)	20(13.0)	
Previous TB treatment	1(2.1)	7(6.7)	8(5.3)	0.247
Previous BCG vaccination	17(36.2)	50(48.4)	67(44.7)	0.157
History of family TB	7(14.9)	18(17.3)	25(16.6)	0.712
History of contact tracing	9(19.1)	19(19.8)	28(19.6)	0.928
PPD (+) test	26(59.1)	58(64.4)	84(62.7)	0.574
Previous hospitalization (during last year)				
Yes	15(33.3)	47(45.6)	62(41.9)	0.163
No	30(66.7)	56(54.4)	86(58.1)	
Smoking or alcohol abuse	20(42.6)	33(33.0)	53(36.1)	0.261
Metabolic diseases	9(19.6)	20(19.8)	29(19.7)	0.973

Among these isolates analyzed 123 different patterns were observed, 16 of these were shared by two or more patient's isolates and were detected in 47 strains (clustered isolates). The remaining 107 patterns were found only once (unique isolates). During the study period we identified two isolates from Nakhichevan-born TB patients, but they didn't share in clustering.

The clustered isolates comprised 30.52% of the total isolates and they formed 16 clustered consisting of 2 to 10 cases each. 69.48% of patients had a unique RFLP patterns. Although cases among male patients were more clustered (51.9%) than cases among female patients (48.1%), this was not significant ($p>0.05$). In this study patients with 56 or older age were strongly associated with clustering (59.6%), patients within this age group belonged to cluster more frequently than younger patients ($p<0.05$). Other risk factors associated with recent transmission of tuberculosis in clustered patients in comparison to non-clustered patients are shown in Table 1.

RFLP analysis confirmed the suspicion of laboratory cross contamination for two strains isolated in Orumieh central TB laboratory. One of these isolates was smear negative and had been processed with a smear positive isolate at the same time. We didn't find any contact tracing or epidemiological link between those patients.

DISCUSSION

The discovery of repetitive DNA elements in *M. tuberculosis* complex strains and the establishment of DNA fingerprinting techniques for *M. tuberculosis* with different genetic markers, especially with the IS6110 probe, have made it possible to study the epidemiology of TB at the molecular level and to detect the infectious source of the disease on the basis of clonal differentiation of *M. tuberculosis* isolates (Yang *et al*., 1994). Of one hundred and sixty isolates in this study, 11 (6.7%) isolates showed fewer than 5 copies of IS6110, but Farina *et al*. (2000) have reported that 5.4% of their isolates from Tehran had low banding patterns and 3.2% of isolates from Fars province had low copies of IS6110 (Doroudchi *et al*., 2000). Only 8 isolates did not contain the IS6110 element. Zero-copy strains have previously been described with slightly higher frequencies in patients from

Vietnam, China, Thailand and India (Rasolofo-Razanamparany *et al*., 2001). Present findings reinforced the hypothesis of van Soolingen *et al*. (1995) that certain genotypes of *M. tuberculosis* are associated with particular geographic region.

A majority of isolates from patients of East and West Azarbaijan exhibited unique RFLP patterns and only 30.52% of the patients were clustered in 16 clusters, suggesting that recent transmission accounted for 20.13% of the tuberculosis. Clustered isolates (30.52%) observed in our study is lower than that of Tehran (43%), the capital of the country (Farnia *et al*., 2004). The reason for differences found in clustering rates in other investigations may be the strict cluster definition of this study. The percentage of clustered isolates of 30.52% observed in our study is comparable with that described for other cities e.g., Paris (approximately 36%) (Gutiérrez *et al*., 1998) or New York (approximately 37%) (Alland *et al*., 1994), however it is remarkably lower than that observed in studies performed in the Netherlands (46%) (van Soolingen *et al*., 1997) and Botswana (42%) (Lockman *et al*., 2001). In contrast to other studies the difference between female and male patients-clustering were not statistically significant. The ratio of clustered versus non-clustered isolates were 51.6 and 48.4% for male and female TB patients, respectively. This might be due to low education, poverty and malnutrition of female patients. Unlike the other studies that younger age was strongly associated with clustering (Alland *et al*., 1994; Small *et al*., 1994; Diaz *et al*., 2001), in present study patients with older age (≥56 years) were strongly associated with clustering (59.6%), which reflects the likelihood of new infection in elderly people.

This might be due to unemployment and poor living condition and the low prevalence of AIDS syndrome or success of TB control in our area. The low rate of clustering indicates that tuberculosis among the study population results mainly from reactivation of latent infection or success of TB control programs in this region.

We conclude that the high average copy number of IS6110 per isolate in East and West Azarbaijan of Iran confirms the usefulness of this method for epidemiological studies without the necessity of using additional genetic markers. In order to increase the knowledge of epidemiology of the disease, other studies over long period of time and from different parts of the country is needed.

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