

Comparison of the genetic convergence between mycobacterium strains by three RFLP-based methods in central province of Iran

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ABSTRACT

Objective(s): The utilization of molecular techniques in the epidemiology of tuberculosis have provided an opportunity for using effective markers to trace the transmission of the disease. The purpose of this study was to compare the genetic patterns of *Mycobacterium tuberculosis* by three methods of RFLP technique.

Materials and Methods: In a cross-sectional and prospective study, 95 strains of *M. tuberculosis* isolates were selected for DNA fingerprinting. Extraction of DNA from *Mycobacterium* strains and DNA fingerprinting with IS-6110, PGRS and DR probe were performed by standard protocols.

Results: Overall, the diversity of RFLP among 95 tuberculosis patients were 48, 50 and 45 on the basis of IS6110, PGRS and DR patterns, respectively. Twenty of these patterns (21.1%) with IS6110-RFLP, twenty-two (23.2%) with PGRS-RFLP and seventeen (17.9%) with DR-RFLP occurred with unique RFLP patterns, whereas the remaining 28 patterns were communal. The risk factors of clustering among tuberculosis patients were age < 45 years, new cases, degree of sputum smear $\geq 2+$, and close contact.

Conclusion: Our study demonstrated that IS6110-RFLP, PGRS-RFLP and DR-RFLP genotyping could roughly identify similar proportions of clustered (secondary) cases as well as the same risk factors for clustering.

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Introduction

Tuberculosis remains a major public health problem worldwide. The world prevalence rate is about 178 per 100000 individuals and causes approximately 15 deaths per year among 100000 populations (1). Epidemiological studies have been hampered by lack of markers that are able to differentiate various *Mycobacterium tuberculosis* strains. The capacity to differentiate *M. tuberculosis* strain patterns by DNA fingerprinting has shown promises in tuberculosis (TB) control since this tool was first applied to outbreak investigations and population-based studies in the early 1990s (2). One of the significances of DNA fingerprinting of *M. tuberculosis* is its capability to measure the discrepancy of *M. tuberculosis* strain patterns. Before the progression of molecular methods, the majority of epidemiological investigations were based on

phage typing. The appearance of molecular techniques in the epidemiology of tuberculosis provided an opportunity to use effective markers for tracing the phenomenon of transmission of the disease in the human environment, and identification of the phylogenetic characteristics of *M. tuberculosis* strains (3). Pattern recognition of *M. tuberculosis* strains complex, based on the analysis of nucleic acids, allows the indication of differences within specific sequences, frequency determinations, and the location of these successions in the genome. Usually, the initial phase of such analysis is the digestion of bacilli chromosome with specific restriction enzymes, and subsequent separation of the fragments obtained using agarose gel electrophoresis as well as the determination of their size.

Recent developments in restriction fragment length polymorphism (RFLP) analysis have made it

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feasible to evaluate molecular epidemiology of Mycobacteria. Variable numbers of RFLP appear to be scattered in the *M. tuberculosis* genome, where they are arranged differently from one strain to another. Determination of these patterns has, in turn, the potential to be an extremely useful tool for epidemiological studies of *M. tuberculosis*. For example, it has been demonstrated that strains isolated from patients not involved in the same tuberculosis outbreak exhibit different RFLP patterns, whereas strains isolated from patients involved in the same tuberculosis outbreak have identical RFLP patterns (4).

Molecular Methods

IS6110 sequence

Due to high polymorphism of the IS6110-RFLP element, methods based on this sequence are characterized by a considerable discriminative potential, and are commonly used in molecular epidemiology. Despite many advantages, the IS6110-RFLP method has certain disadvantages and limitations. The starting material for analysis must be an abundant culture of bacilli, from which large amounts of genomic DNA of high quality and purity can be isolated. The procedure is effort consuming and the results are obtained after several days. In addition, the method has a considerable limitation when applied for the typing of strains which, in the genome, possess 0-5 copies of IS6110 sequences. In such cases, a combination of bands is obtained which are poorly polymorphic in relation to other strains with a low number of IS6110 copies, which, in turn, decreases discriminative potential of the method and decreases its value in typing of such strains (5).

Polymorphic GC-rich repetitive sequences (PGRS)

Very abundant repetitive sequences in the genome *M. tuberculosis* complex are the Polymorphic GC-rich Repetitive Sequences (PGRS). PGRS elements are present at 26-30 sites on chromosome and consist of many tandem repetitions, 96 bp in length. Polymorphism of these sequences is used in typing, where GC-rich repetitive sequence contained the recombinant plasmid pTBN12 is used as the probe. The method is a supplement for analysis of the *M. tuberculosis* complex strains possessing a small number of copies of sequence IS6110, or those that are devoid of this sequence. It is noteworthy that PGRS sequences were also identified in the genome of atypical bacilli. It is approximated that this method has a high discriminative potential, comparable to the IS6110-RFLP technique. (6)

Region Direct Repeat (DR)

Apart from the above-mentioned sequences, other short sequences were identified in the *M.*

tuberculosis complex genome, which are used as typing markers. These sequences belong chromosomal DR (Direct Repeat) regions, positioned in variable numbers of repetitions, so-called hot spot regions for integration of the IS6110. DR regions consist of short 36 nucleotides DR fragments divided by unique spacers, 35-41 bp in length. Both the number of copies of DR fragments and the presence of specified spacers are changeable features and constitute a basis for indicating differences between the strains (7, 8).

In this report, we compare three methods of RFLP, IS6110, PGRS and DR patterns of 95 strains isolated from patients living in central province of Iran.

Materials and Methods

Study population and data collection

In this cross-sectional prospective study, 103 strains of *M. tuberculosis* isolated in Central province of Iran between March 2011 and September 2012 were selected for DNA fingerprinting. Isolates were grown from smear-positive TB patients. After getting informed consent, data were collected by trained technicians using standard questionnaires. Information was obtained on gender, age, nationality, close contact, previous history of TB, present residence, human immunodeficiency virus (HIV) infection confirmed by Western blot, tuberculin skin test, and degree of sputum smear.

Collection of *M. tuberculosis* isolates and RFLP analysis

Sputum samples were collected in sterilized containers, processed by modified Petroff's method using 4% NaOH and cultured on Lowenstein Jensen (LJ) media. During the observation of growth in 103 culture media, a series of biochemical tests including niacin, nitrate reduction, catalase at 68°C, tween-80 hydrolysis and aryl-sulphatase were performed to confirm *M. tuberculosis*. Of the 103 isolates, 95 were confirmed as *M. tuberculosis*.

Extraction of DNA from *Mycobacterium* strains and DNA fingerprinting with IS6110, PGRS and DR probes were performed by standard protocols (9). For IS6110-RFLP, a 245-bp probe was amplified by PCR using primers INS-1 (631-650) (5'CGT GAG GGC ATC GAG GTG GC3') and INS-2 (856-875) (5'GCG TAG GCG TCG GTG ACA AA3') probes. For PGRS- RFLP and DR- RFLP, we used a probe of (5' CGG CCG TTG CCG CCG TTG CCG CCG TTG CCG CCG3') in which 3'end was labeled with non-radioactive digoxigenin by random primed DNA labeling technique using DIG DNA labeling and detection kit (Roche Diagnostics, Germany). DNA was digested separately by Pvu II enzyme, and after gel electrophoresis, the DNA fragments were transferred to positively charged nylon membrane by Southern blotting method. DNA of a reference strain (ATCC 38808) was used in each

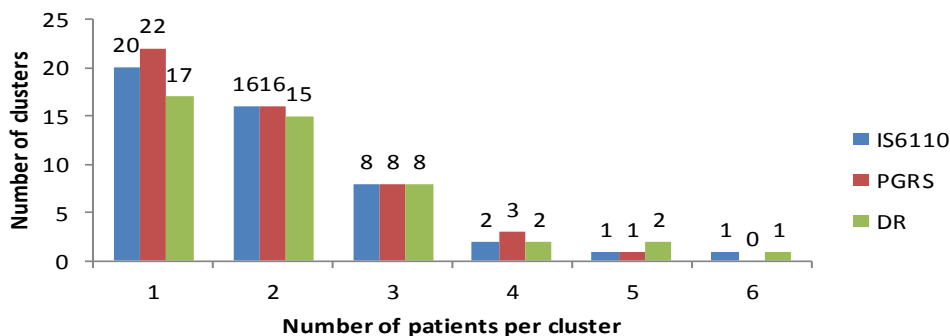


Figure 1. Sizes and frequencies of clusters consisting of 95 patients with tuberculosis by three methods or RFLP

Southern blot experiment as an external quality control of RFLP method. A detailed analysis of the resulting image of hybridization based on the number and size of bands was performed by specialized Gel Pro software (Media cybernetics, Italy) to determine genetic types (9). All patients were classified under two groups: individuals with clustered and non-clustered *M. tuberculosis* strains. A 'conventional' cluster was defined as two or more patients whose isolates were identified as related by RFLP.

Statistical analysis

Categorical data were compared by chi-square test or Fisher's exact test. The paired t-test was performed to determine whether the continuous variable differed between the two groups and P-Value below 0.05 was significant.

Results

Patients' characteristics

Of the initial 103 patients with pulmonary tuberculosis (TB), eight patients were excluded because the patients were infected with *M. bovis* and non-tuberculosis mycobacteria. Therefore, 95 individuals who were determined as MTB were concerned in final

analysis.

Most of them (92.5%) were Iranian. The mean age was 64.5 ± 20.7 years and the gender of 46 (48.5%) patients was male and 49 (51.5%) was female. Among them, 85 (89.5%) were new patients and 10 (10.5%) were those previously treated. 9 (9.5%) patients had close contact with tuberculosis patient, 3 (3.2%) were HIV-positive, and 53 (55.8%) were urban. Smear from 10 (10.5%) patients were paucibacillary, 26 (27.4%) were 1+, 30 (31.6%) were 2+ and 29 (30.5%) were 3+ in microscopic examination.

RFLP analysis and cluster characteristics

Overall, the diversity of RFLP of 95 tuberculosis patients, were 48, 50 and 45 on the basis of IS6110, PGRS and DR patterns, respectively. Of these patterns, 20 (21.1%) with IS6110-RFLP, 22 (23.2%) with PGRS-RFLP, and 17 (17.9%) with DR-RFLP occurred with unique RFLP patterns. The remaining 28 patterns were communal in 75 (78.9%) patients by IS6110-RFLP, 73 (76.8%) by PGRS-RFLP and 78 (82.1%) by DR-RFLP in clusters. The cluster sizes ranged from 2 to 6 isolates (Figure 1). Our study found no strains belonging to the Beijing family.

Table 1. Risk factors associated with cluster and non-cluster cases among 95 patients with tuberculosis in Iran (PGRS-RFLP)

Variable	Cluster group 73 (76.8%)	Non-cluster group 22 (23.2%)	Odd ratio (95% CI)	P-Value
Age				
Age < 45 yr	43 (45.3%)	5 (5.3%)	4.87	0.0035
Age ≥ 45 yr	30 (31.6%)	17 (17.9%)	(1.62-14.65)	
Gender				
Male	35 (36.8%)	10 (10.5%)	0.90	0.5162
Female	38 (40%)	12 (12.6%)	(0.35-2.36)	
Type of patient				
New case	68 (71.6%)	15 (15.8%)	6.35	0.0054
Previous treatment	5 (5.3%)	7 (7.4%)	(1.78-22.75)	
Nationality				
Iranian	67 (70.5%)	21 (22.1%)	0.53	0.4864
Immigrant (Afghans)	6 (6.3%)	1 (1.1%)	(0.06-4.67)	
Inhabitant				
Urban	42 (44.3%)	14 (14.7%)	0.77	0.8052
Rural	31 (32.6%)	8 (8.4%)	(0.29-2.07)	
Close contact with TB patient				
Yes	15 (15.7%)	1 (1.1%)	5.43	0.0394
No	58 (61.1%)	21 (22.1%)	(2.68-43.68)	
HIV infection				
Yes	1 (1.1%)	2 (2.1%)	0.14	0.1330
No	72 (75.7%)	20 (21.1%)	(0.01-1.60)	
Sizes of mantoux test				
<15mm	34 (35.8%)	9 (9.5%)	0.79	0.8075
≥15mm	39 (41.1%)	13 (13.7%)	(0.30-2.08)	
Degree of sputum smear				
≤1+	27 (28.4%)	14 (14.7%)	2.85	0.0139
≥2+	46 (48.5%)	8 (8.4%)	(1.02-7.98)	

Risk factors for clustering and unique RFLP patterns

In our analysis, several risk factors for clustering became apparent that might have important implications for tuberculosis control and practicing physicians. The analysis revealed that for patients with tuberculosis, age below 45 years ($P = 0.0035$), new cases ($P = 0.0054$), degree of sputum smear $\geq 2+$ ($P = 0.0139$) and close contact ($P = 0.0394$) were associated with an increased risk of being clustered. Table 1 shows the variables that were significantly associated with clustering among the patients.

Discussion

Tuberculosis is still endemic in Iran, despite continuous efforts to control the disease through mass BCG vaccination at birth, active case finding, and multidrug therapies. These efforts have resulted in some changes in the epidemiological parameters of the disease. In this context, the recent description of RFLP patterns as a reliable tool for differentiation of *M. tuberculosis* strains is of great interest. In the present study, with DNA fingerprinting, different hybridization patterns were observed for *M. tuberculosis* isolates from Central Province of Iran, suggesting differences in copy number and genomic location of the element. The results of this study indicated that PGRS-RFLP identified more genetic pattern (50 types) than IS6110-RFLP (48 types) and DR-RFLP (45 types). Our study demonstrated that IS6110-RFLP, PGRS-RFLP and DR-RFLP genotyping identified roughly similar proportions of clustered (secondary) cases as well as the same risk factors for clustering. Our study also revealed that for patients with tuberculosis, ages younger than 45 years, new cases, degree of sputum smear $\geq 2+$ and close contact were associated with an increased risk of being clustered.

A molecular fingerprinting study of clinical isolates of *M. tuberculosis* on a Caribbean island with IS6110 and DR probes produced similar patterns of bands (10). In an earlier study, *M. tuberculosis* strains from the People's Republic of China and Mongolia formed a remarkably homogeneous family of strains, evolved from recent clonal expansion based on IS6110 and other genetic markers (DR and PGRS) (11). In another study carried out in French Polynesia, strains exhibiting similar IS6110 RFLP types also exhibited identical DRRFLP patterns, suggesting linkage between IS6110 and DR sequences (12).

Van Soolingen *et al* studied 16 strains originating from six different countries differed in their PGRS and DR restriction fragment patterns, although a majority of these strains contained a single copy of IS6110 on a 1.5 Kb Pvu II restriction fragment (13). Sahadevan *et al* at the Tuberculosis Research Center in Madras, using *M. tuberculosis* isolates from South-

India, have reported 30 different patterns from 96 individual isolates of *M. tuberculosis* by DR-RFLP analysis (14). In study of Farnia *et al* on 292 *M. tuberculosis* in Tehran, 232 (79.4%) belonged to clusters while 60 (21.6%) did not, and concluded that tuberculosis among the study population in Tehran mainly from reactivation of latent infection (15). Genotyping of the *M. tuberculosis* isolates by PGRS-RFLP with Pvu II and Alu I in Markazi province of Iran by Rafiee *et al*, displayed a wide range of genetic diversity suggestive of the great diversity of PGRS in *M. tuberculosis* strains. They concluded that the majority of the patients had tuberculosis with different etiologies (16). In study of Asgharzadeh *et al* in East Azarbaijan Province of Iran, RFLP typing was performed on 119 culture-positive specimens. Using IS6110 as a probe, *M. tuberculosis* strains were assigned to clusters based on identical DNA fingerprints. Ninety-three distinct IS6110 patterns were revealed. Eighty-one of these patterns were unique and 12 were shared by 2 to 8 strains, therefore they concluded that RFLP typing is a useful instrument for gaining more knowledge about transmission, occurrence of micro-epidemics, and tracing the international sources (17). The use of these markers in population-based studies can describe tuberculosis either resulted from reactivation of a latent infection or because of recent transmission with rapid progression to active tuberculosis (18). Molecular genotyping has an important role in contact investigations, and public health personnel may use the genotyping data to give notice the direction and intensity of the contact investigation to search for epidemiological links and to find other contacts that may be mycobacterial infection or mycobacterial disease (19, 20).

Conclusion

Molecular epidemiology has added a new dimension to the classical epidemiology of tuberculosis and greatly improved our knowledge of the transmission dynamics of *M. tuberculosis* within different populations. DNA fingerprinting technology identifies risk factors for acquiring infection and the features of the secondary cases. Our study showed that discriminatory powers of the three methods are approximately similar in our population, and therefore, all methods resembled in making deduction in population-based studies.

Clustering, by itself, is not always equivalent to recent transmission however; it may be part of it. The important limitation related to RFLP is the time required for obtaining the results. It is known that the population under study, the length of observation, and the specificity of the clustering case-definition factors play important roles in interpreting this measure. Additional investigation into this area is necessary.

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Conflict of interests

The authors declare no competing interests.

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