NEW MICROBIOLOGICA, 30, 229-240, 2007

# Molecular characterization of *Mycobacterium tuberculosis* isolates from Izmir, Turkey

**Can Bicmen[1], Nuran Esen[2], Edward A. Graviss[3], Natalie Williams-Bouyer[3], Srinivas V. Ramaswamy[3], Nuran Yulug[2]**

[1]*Microbiology and Clinical Microbiology Laboratory, Research and Training Hospital For Chest Diseases and Chest Surgery, Yenisehir, Izmir, Turkey;*
[2]*Department of Microbiology and Clinical Microbiology, Dokuz Eylul University Faculty of Medicine, Inciralti, Izmir, Turkey;*
[3]*Department of Pathology, Baylor College of Medicine, Houston, USA*

**SUMMARY**

In recent years, molecular typing methods have been used in epidemiologic studies of *Mycobacterium tuberculosis* isolates in various areas of the world. However, there have been few data on this issue in Turkey. We describe the molecular characterization of 56 *Mycobacterium tuberculosis* isolates recovered from individual patients in Izmir and the surrounding area by three different molecular methods. Isolated *M. tuberculosis* strains were characterized by IS*6110* RFLP, spoligotyping and major genetic group designation. In total, 51 RFLP and 35 spoligopatterns were identified. Fourteen (25%) isolates were indicated as low copy number. Based on three genotypic characterization methods together, five clusters with two isolates each were identified. Most of the isolates (98.2%) were assigned as genetic groups 2 or 3. Only one isolate was identified as Beijing family strain (principal genetic group 1). The shared international clades were found to be Beijing-family, var T1 (ST 37), LAM (Latin-American-Mediterranean) 7 (ST 41), LAM 9 (ST 42), Haarlem 1 (ST 47), Haarlem 3 (ST 50) and T1 (ST 53). In this study, IS*6110* RFLP, spoligotyping and major genetic group designation were found to be useful methods for molecular epidemiologic studies.

*KEY WORDS: Mycobacterium tuberculosis*, Molecular characterization

*Received* February 12, 2007 *Accepted* February 26, 2007

## INTRODUCTION

Tuberculosis (TB) continues to be an emerging infectious disease spreading from person-to-person in the population through socialization. In spite of effective anti-TB drugs and preventive efforts, one-third of the world's population is infected with *Mycobacterium tuberculosis* and carries the risk of clinical TB presentation. In Turkey, the Ministry of Health has reported the incidence of TB in 1965, 1985 and 2003 to be 172/100,000, 62/100,000 and 24/100,000 respectively. In addition, TB incidence in the seven regions of Turkey in 2003 has been reported as: 35/100,000 (Marmara Region), 26/100,000 (Black Sea Region), 25/100,000 (South Eastern Anatolian Region), 25/100,000 (Aegean Region), 19/100,000 (Mediterranean Region), 17/100,000 (Eastern Anatolian Region) and 14/100,000 (Central Anatolian Region) (Ministry of Health of Turkey, Tuberculosis Control Department, 2004; World Health Organization, 2005). A few of the most important factors which affect TB control in Turkey are the high population increase, migration patterns and social and cultural differences among the regions. Emergence of increasing numbers of infections due to drug-resistant *M. tuberculosis* indicates the need for

*Corresponding author*
Can Bicmen
Microbiology and Clinical Microbiology Laboratory
Research and Training Hospital for Chest Diseases
and Chest Surgery, Yenisehir
Izmir 35110, Turkey
E-mail: cbicmen@yahoo.com

follow-up strategies, programmatic evaluation and appropriate treatment regimens against TB (World Health Organization, 1997). To clarify the characteristics and transmission dynamics of the global dissemination of TB, molecular characterization of *M. tuberculosis* isolates and investigative association studies among patients have been useful tools for epidemiologic studies.

IS*6110*-RFLP (Restriction Fragment Length Polymorphism) is an internationally standardized method for molecular epidemiologic characterization of *M. tuberculosis* isolates. This genotyping method is the "gold" standard on which *M. tuberculosis* characterization is based (Van Embden *et al.*, 1993; Friedman *et al.*, 1995; Chaves *et al.*, 1996; Kamerbeek *et al.*, 1997; Kremer *et al.*, 1999). Spacer oligonucleotide typing (spoligotyping) is a PCR-based checker-board hybridization method which analyzes the polymorphisms in the *M. tuberculosis* complex direct repeat (DR) chromosomal region consisting of identical 36-bp DRs alternating with 35- to 41-bp unique spacer sequences and is faster and easier to perform than IS*6110*-RFLP (Kamerbeek *et al.*, 1997). Additionally, spoligotyping has been shown to be more sensitive than the standardized IS*6110*-RFLP technique in characterization of TB isolates with low copy numbers (<6) (Kremer *et al.*, 1999; Bauer *et al.*, 1999; Soini *et al.*, 2000; Soini *et al.*, 2001). In additional studies, isolates have also been assigned one of three major genetic groups based on nucleotide polymorphism at codons 463 and 95 of the genes encoding catalase-peroxidase and A subunit of DNA gyrase, respectively (Sreevatsan *et al.*, 1997). The aim of the present study was to assess IS*6110*-RFLP, spoligotyping and major genetic group designation to identify meaningful fingerprint clusters among *M. tuberculosis* isolates recovered from pulmonary TB patients located in Izmir and surrounding area (Aegean Region).

## MATERIALS AND METHODS

### Patients

The population was composed of TB patients among residents of Izmir and the surrounding area. The samples were selected from patients according to their application to the hospitals. The period between the selected patients was at least 2 weeks. All patients were evaluated as pulmonary TB according to the clinical and laboratory aspects of WHO guidelines (World Health Organization, 1997) and were culture positive. Demographic data were collected retrospectively from the patients' records using standard forms.

### *Mycobacterium tuberculosis* strains

Susceptible, resistant and multidrug resistant (MDR) (resistant to at least isoniazid (I) and rifampicin (R) at the same time) strains were included in our study. MDR isolates were specifically selected more than the percentage of our population because MDR TB is an ascending problem in the world, and our country carries a potential risk. Between June 2000 and January 2001, 1002 (51 MDR, 591 pansusceptible and 360 resistant except MDR) isolates from 671 patients were identified from respiratory specimens of pulmonary TB patients in the Microbiology Laboratories of the Training Hospital for Chest Diseases and Chest Surgery and Dokuz Eylul University Hospital in Izmir, Turkey. In this period of time, *M. tuberculosis* isolates recovered and identified from 56 patients (10 mono- or poly-drug resistant, 15 MDR and 31 pansusceptible) were analyzed in this study. As phenotypical features of the isolates are also important in addition to the molecular characterization, drug susceptibility testing of four major anti-TB agents [streptomycin (S), I, R and ethambutol (E)] was performed using the BACTEC 460 TB system (BD Biosciences, Sparks, MD, USA).

### Drug susceptibility

Among 15 MDR isolates, 12 were resistant to four major anti-TB drugs (SIRE), two isolates were resistant to I, R and E and one isolate was resistant to S, I and R. Besides these susceptibility patterns, three isolates were resistant to S and R, R and E, S and E, respectively, whereas five isolates were resistant only to isoniazid, one isolate was rifampicin resistant and another was ethambutol-resistant. Thirty-one isolates were susceptible to all four anti-TB agents.

### Molecular characterization

Chromosomal DNA extraction and IS*6110*-RFLP were performed by an internationally standardized protocol (Van Embden *et al.*, 1993).

Extracted DNA was also used for spoligotyping. The IS*6110* patterns were analyzed with the BioImage (Ann Arbor, Mich.,USA) Whole Band Analyzer, version 3.4.2. Spoligotyping was performed with a commercially available kit (Isogen Bioscience BV, Maarssen, The Netherlands) in accordance with the manufacturer's recommendations. Spoligotyping results were compared with the data of the World Spoligotyping Database (SpolDB3) (Sola *et al.*, 2001) and (SpolDB4) (Brudey *et al.*, 2006) of Institut Pasteur de Guadeloupe and Houston Database (HD) (Soini *et al.*, 2000). If the isolates possessed common spoligotype patterns with the international databases, SpoDB3 and 4 (SpolDB3/4) and HD, isolates were identified as "ST" and "S", respectively. Molecular characterization by IS*6110* RFLP, spoligotyping, and major genetic group designation was performed by Can Bicmen at the Houston Tuberculosis Initiative laboratory at Baylor College of Medicine, Houston, Texas. Finally, the isolates were assigned to one of three principal genetic groups on the basis of nucleotide polymorphism at codons 463 and 95 of the genes encoding catalase-peroxidase and the A subunit of DNA gyrase, respectively. Group 1 had the allele combination *katG* codon 463 CTG (Leu) and *gyrA* codon 95 ACC (Thr); group 2 had *katG* 463 CGG (Arg) and *gyrA* codon 95 ACC (Thr), and group 3 organisms had *katG* 463 CGG (Arg) and *gyrA* codon 95 AGC (Ser) (Sreevatsan *et al.*, 1997). Nucleotide sequencing was performed with an ABI Prism™ 377 DNA sequencer (Applied Biosystems, USA).

**Statistical analysis**

Discrimination ability of each characterization method was analyzed by the Hunter Gaston Index (HGI) (Hunter and Gaston, 1988). This index applied to a direct comparison of the discriminating power of typing methods and combined typing schemes.

## RESULTS

**IS*6110* RFLP**

IS*6110* profiling identified 51 fingerprint patterns ranging from 2 to 21 copies. Among 56 isolates fingerprinted by IS*6110* RFLP, fourteen isolates (25%) were identified as low-copy number (≤5) isolates. Forty-six isolates (82.1%) had unique fingerprint patterns. The remaining 10 isolates (17.9%) fell into 5 print clusters with two isolates each (isolate numbers for each cluster: 4-43, 6-53, 17-32, 22-45, 27-41) (Table 1). In these five clusters with identical RFLP patterns, the corresponding IS*6110* band copies were found to be 2, 4, 5, 7 and 7, respectively. Drug resistance patterns, number of IS*6110* copies and similarity features of IS*6110* RFLP patterns are summarized in Table 1. Representative IS*6110*

TABLE 1 - *Phenotypical resistance and molecular characterization features of* Mycobacterium tuberculosis *isolates.*

| *Isolate No* | *Drug Resistance Pattern[1]* | *IS6110 Copy number* | *IS6110 RFLP similarities[2]* | *IS6110 Fingerprint (T)[3]* | *Spoligotype No (TS)[4]* | *Spoligotyping similarities[5]* | *Spoligotype code* | *Major Genetic Group[6]* |
|---|---|---|---|---|---|---|---|---|
| 1 | SIRE | 9 | U | T 44 | TS 35 | 13, 16, 20, 34, 46, 52, 55, 56 | 7777 7777 7760 771 | 2 |
| 2 | I | 2 | U | T 26 | TS 22 | 4, 25, 43, 47 | 7777 7740 4760 771 | 2 |
| 3 | SR | 9 | U | T 40 | TS 31 | U | 7777 7777 4360 771 | 2 |
| 4 | Pansusceptible | 4 | 43 | T 27 | TS 22 | 2, 25, 43, 47 | 7777 7740 4760 771 | 2 |
| 5 | Pansusceptible | 10 | U | T 41 | TS 33 | U | 7777 7777 6360 771 | 3 |
| 6 | Pansusceptible | 7 | 53 | T 4 | TS 4 | 53 | 0376 3777 7760 771 | 2 |

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| *Isolate No* | *Drug Resistance Pattern*[1] | *IS6110 Copy number* | *IS6110 RFLP similarities*[2] | *IS6110 Fingerprint (T)*[3] | *Spoligotype No (TS)*[4] | *Spoligotyping similarities*[5] | *Spoligotype code* | *Major Genetic Group*[6] |
|---|---|---|---|---|---|---|---|---|
| 7 | Pansusceptible | 10 | U | T 38 | TS 30 | 14 | 7777 7777 4060 771 | 2 |
| 8 | Pansusceptible | 10 | U | T 9 | TS 9 | U | 7776 7737 7760 771 | 3 |
| 9 | Pansusceptible | 11 | U | T 33 | TS 32 | U | 7777 7777 4760 771 | 3 |
| 10 | RE | 13 | U | T 34 | TS 26 | U | 7777 7770 7760 771 | 3 |
| 11 | SE | 12 | U | T 20 | TS 17 | U | 7777 6767 7760 771 | 2 |
| 12 | Pansusceptible | 15 | U | T 8 | TS 8 | U | 7677 7777 7760 771 | 3 |
| 13 | SIRE | 9 | U | T 45 | TS 35 | 1, 16, 20, 34, 46, 52, 55, 56 | 7777 7777 7760 771 | 3 |
| 14 | Pansusceptible | 11 | U | T 39 | TS 30 | 7 | 7777 7777 4060 771 | 2 |
| 15 | I | 14 | U | T 42 | TS 34 | U | 7777 7777 7720 771 | 2 |
| 16 | Pansusceptible | 10 | U | T 47 | TS 35 | 1, 13, 20, 34, 46, 52, 55, 56 | 7777 7777 7760 771 | 2 |
| 17 | Pansusceptible | 7 | 32 | T 2 | TS 2 | 32 | 0000 0000 7760 771 | 2 |
| 18 | Pansusceptible | 6 | U | T 31 | TS 24 | U | 7777 7747 4760 771 | 2 |
| 19 | Pansusceptible | 9 | U | T 32 | TS 25 | U | 7777 7760 7760 771 | 3 |
| 20 | Pansusceptible | 12 | U | T 43 | TS 35 | 1, 13, 16, 34, 46, 52, 55, 56 | 7777 7777 7760 771 | 3 |
| 21 | I | 13 | U | T 3 | TS 3 | U | 0037 7777 4020 771 | 2 |
| 22 | SIRE | 5 | 45 | T 16 | TS 14 | 45 | 7777 6177 7760 771 | 2 |
| 23 | I | 9 | U | T 15 | TS 13 | 35 | 7777 6000 7760 771 | 2 |
| 24 | I | 2 | U | T 13 | TS 12 | U | 7777 4740 4740 771 | 2 |
| 25 | SIRE | 4 | U | T 25 | TS 22 | 2, 4, 43, 47 | 7777 7740 4760 771 | 2 |
| 26 | SIRE | 4 | U | T 24 | TS 21 | U | 7777 7740 4760 731 | 2 |
| 27 | Pansusceptible | 2 | 41 | T 17 | TS 15 | 39, 41 | 7777 6740 4760 771 | 2 |
| 28 | Pansusceptible | 2 | U | T 10 | TS 10 | U | 7777 2740 4760 771 | 2 |
| 29 | SIRE | 16 | U | T 1 | TS 1 | U | 0000 0000 0003 771 | 1 |
| 30 | Pansusceptible | 11 | U | T 21 | TS 18 | U | 7777 6777 4020 771 | 2 |
| 31 | Pansusceptible | 7 | U | T 36 | TS 28 | U | 7777 7777 4020 701 | 2 |
| 32 | SIRE | 7 | 17 | T 2 | TS 2 | 17 | 0000 0000 7760 771 | 2 |

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→ TABLE 1 - *Phenotypical resistance and molecular characterization features of* Mycobacterium tuberculosis *isolates.*

| *Isolate No* | *Drug Resistance Pattern[1]* | *IS6110 Copy number* | *IS6110 RFLP similarities[2]* | *IS6110 Fingerprint (T)[3]* | *Spoligotype No (TS)[4]* | *Spoligotyping similarities[5]* | *Spoligotype code* | *Major Genetic Group[6]* |
|---|---|---|---|---|---|---|---|---|
| 33 | IRE | 8 | U | T 6 | TS 6 | U | 3777 6767 7760 771 | 3 |
| 34 | SIRE | 10 | U | T 46 | TS 35 | 1, 13, 16, 20, 46, 52, 55, 56 | 7777 7777 7760 771 | 3 |
| 35 | R | 15 | U | T 14 | TS 13 | 23 | 7777 6000 7760 771 | 2 |
| 36 | SIRE | 9 | U | T 22 | TS 19 | U | 7777 6777 7720 771 | 2 |
| 37 | SIRE | 9 | U | T 23 | TS 20 | U | 7777 6777 7760 771 | 2 |
| 38 | Pansusceptible | 12 | U | T 5 | TS 5 | U | 3763 7777 7760 771 | 3 |
| 39 | Pansusceptible | 5 | U | T 18 | TS 15 | 27, 41 | 7777 6740 4760 771 | 2 |
| 40 | Pansusceptible | 12 | U | T 19 | TS 16 | U | 7777 6760 7760 771 | 2 |
| 41 | Pansusceptible | 2 | 27 | T 17 | TS 15 | 27, 39 | 7777 6740 4760 771 | 2 |
| 42 | Pansusceptible | 11 | U | T 37 | TS 29 | U | 7777 7777 4020 771 | 2 |
| 43 | IRE | 4 | 4 | T 27 | TS 22 | 2, 4, 25, 47 | 7777 7740 4760 771 | 2 |
| 44 | SIRE | 8 | U | T 30 | TS 23 | 49 | 7777 7743 4760 771 | 2 |
| 45 | SIRE | 5 | 22 | T 16 | TS 14 | 22 | 7777 6177 7760 771 | 2 |
| 46 | SIR | 21 | U | T 51 | TS 35 | 1, 13, 16, 20, 34, 52, 55, 56 | 7777 7777 7760 771 | 2 |
| 47 | Pansusceptible | 2 | U | T 28 | TS 22 | 2, 4, 25, 43 | 7777 7740 4760 771 | 2 |
| 48 | Pansusceptible | 10 | U | T 7 | TS 7 | U | 3777 7777 7760 771 | 3 |
| 49 | Pansusceptible | 5 | U | T 29 | TS 23 | 44 | 7777 7743 4760 771 | 2 |
| 50 | E | 14 | U | T 11 | TS 11 | 54 | 7777 3777 7760 771 | 3 |
| 51 | Pansusceptible | 11 | U | T 35 | TS 27 | U | 7777 7776 4020 771 | 2 |
| 52 | Pansusceptible | 13 | U | T 48 | TS 35 | 1, 13, 16, 20, 34, 46, 55, 56 | 7777 7777 7760 771 | 3 |
| 53 | Pansusceptible | 7 | 6 | T 4 | TS 4 | 6 | 0376 3777 7760 771 | 2 |
| 54 | Pansusceptible | 9 | U | T 12 | TS 11 | 50 | 7777 3777 7760 771 | 2 |
| 55 | Pansusceptible | 14 | U | T 50 | TS 35 | 1, 13, 16, 20, 34, 46, 52, 56 | 7777 7777 7760 771 | 3 |
| 56 | Pansusceptible | 13 | U | T 49 | TS 35 | 1, 13, 16, 20, 34, 46, 52, 55 | 7777 7777 7760 771 | 3 |

[1]I: resistant to isoniazid, S: resistant to streptomycin, R: resistant to rifampicin, E: resistant to ethambutol; [2]U: Unique pattern, isolates with the same RFLP patterns were determined with their isolate numbers; [3]T: Turkish RFLP pattern according to the IS*6110* fingerprinting; [4]TS: Turkish spoligotyping pattern; [5]Isolates with the same spoligopatterns were determined with their isolate numbers; [6]Three principal genetic groups were identified on the basis of nucleotide polymorphism at codons 463 and 95 of the genes encoding catalase-peroxidase and the A subunit of DNA gyrase, respectively (Sreevatsan *et al.*, 1997).

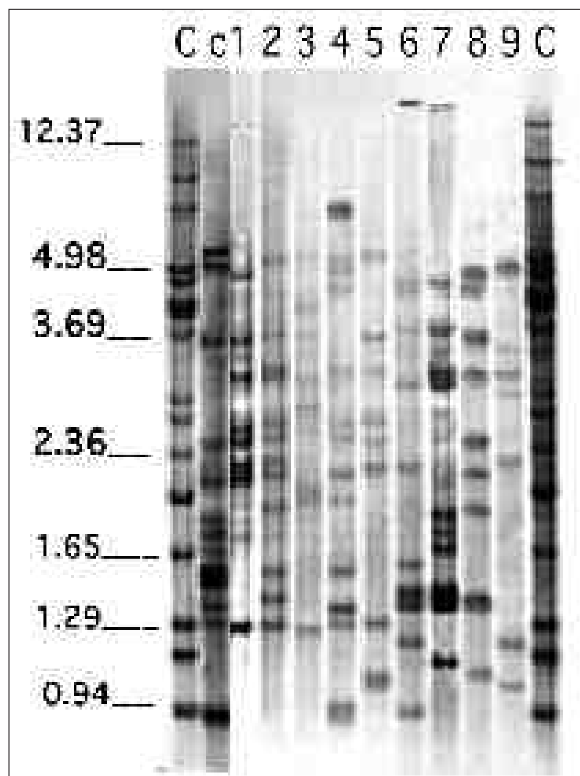


FIGURE 1 - *Representative IS6110 RFLP patterns of M. tuberculosis isolates.*
*C: Standard strain NY 2650, c: Standard strain H37Rv 1) Isolate no: 9 (copy number: 11), 2) Isolate no:10 (copy number: 13), 3) Isolate no: 11 (copy number: 12), 4) Isolate no: 12 (copy number: 15), 5) Isolate no: 13 (copy number: 9), 6) Isolate no: 14 (copy number: 11), 7) Isolate no: 15 (copy number: 14), 8) Isolate no: 16 (copy number: 10), 9) Isolate no: 17 (copy number: 7).*

RFLP patterns of *M. tuberculosis* isolates are shown in Figure 1.

**Spoligotyping**

A total of 35 spoligotype patterns were identified (Table 2). In contrast to IS*6110* RFLP clustering, 31 isolates (55.4%) were clustered into 10 spoligotype patterns (2 to 9 isolates/pattern). The remaining 25 isolates (44.6%) had unique spoligotype patterns. The most frequent spoligotype patterns were ST 53 (S 29) (16.1%), ST 41 (8.9%), ST 186 (5.4%), ST 4 (S 34) (3.6%), ST 37 (S 77) (3.6%) and ST 254 (3.6%). A MDR isolate resistant to four major anti-TB drugs in Tables 2 and 3 was assigned as Beijing Type (isolate no: 29) (ST 1) (S 1). Spoligopatterns and the isolates which had similarity with the international databases are shown in Table 2. According to the spoligotyping results; isolates with numbers in each cluster were; isolate nos: 17 and 32 are ST 4 (S 34), isolate no: 50 and 54 are ST 37 (S 77), isolate nos: 2, 4, 25, 43 and 47 are ST 41, isolate nos: 1, 13, 16, 20, 34, 46, 52, 55 and 56 are ST 53 (S 29), isolate nos: 27, 39 and 41 are ST 186, isolate nos: 23 and 35 are ST 254, isolate nos: 6 and 53 are ST 284, isolate nos: 7 and 14 are ST 1872, isolate nos: 22 and 45 are orphan, and isolate nos: 44 and 49 are orphan. (Table 2). Seven spoligotyping patterns (isolate nos: 2-4-25-43-47 are ST 41, isolate nos: 27-39-41 are ST 186, isolate no: 26 is ST 1261, and isolate nos: 22-45, 24, 28, 49 are orphan) were associated with low-copy number isolates. Of the 14 low-copy number isolates (25 %), six isolates (clusters: 4-43, 22-45, 27-41) had the same RFLP and spoligotype patterns, three isolates (2, 25, 47) had unique IS*6110* profiles but the same spoligotype patterns. Three of the remaining five isolates (24, 26, 28) had unique RFLP and spoligotyping patterns and two of them (39 and 49) had unique IS*6110* profiles but shared spoligotype patterns. Spoligotyping is thus useful in the discrimination of some of similar IS*6110* low-copy number isolates. However, two isolates (2 and 47) with two copies showing different RFLP patterns also had the same spoligotype pattern. A potential limitation of our study is the relatively small number of isolates. Although the samples representing spread of DNA fingerprinting patterns were taken at two major hospitals in Izmir and Aegean Region, this may be another limitation of the study.

The isolates which also had the same IS*6110* RFLP profile had the same spoligopatterns in five clusters with two isolates each (isolate numbers for each cluster: 4-43, 6-53, 17-32, 22-45, 27-41) (Table 3). However, drug susceptibility patterns of the isolates within two of these five clusters (clusters: 4-43 and 17-32) were different. Data regarding patients having the same molecular features of DNA fingerprinting characterization and phenotypical resistance findings are summarized in Table 3. Among 46 isolates which showed unique patterns by RFLP shown in Table 1, 25 isolates (54.3%) were also found to be unique by spoligotyping. The results of phenotypical resistance, molecular characterization features of RFLP and spoligotyping patterns are summarized in Table 1.

TABLE 2 - *Spoligotyping patterns of Mycobacterium tuberculosis isolates.*

| *Isolate No* | *ST*[1] | *Number of isolates (%)* | *Octal Code* | *Spoligotyping pattern*[2] | *% in SpolDB4*[3] | *Clade*[4] |
|---|---|---|---|---|---|---|
| 29 | 1 (S 1) | 1(1,8) | 000000000003771 | □□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■■ | 7,90 | Beijing |
| 1, 13, 16, 20, 34, 46, 52, 55, 56 | 53 (S 29) | 9 (16,1) | 777777777760771 | ■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■ | 6,46 | T 1 |
| 48 | 7 | 1 (1,8) | 377777777760771 | □■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■ | 0,09 | var T 1 |
| 12 | 1122 | 1 (1,8) | 767777777760771 | ■■■■■□■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■ | 0,06 | var T 1 |
| 50, 54 | 37 | 2 (3,6) | 777737777760771 | ■■■■■■■■■■■■□■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■ | 0,43 | var T 1 |
| 11 | 173 | 1 (1,8) | 777767677760771 | ■■■■■■■■■■■■■■□■■■■■□■■■■■■■■■■■□□□□■■■■■■■ | 0,01 | var T 1 |
| 37 | 118 (S 145) | 1 (1,8) | 777767777760771 | ■■■■■■■■■■■■■■□■■■■■■■■■■■■■■■■■□□□□■■■■■■■ | 0,20 | var T 1 |
| 5 | 123 | 1 (1,8) | 777777776360771 | ■■■■■■■■■■■■■■■■■■■■■■■■■■□□■■■■■■□□□□■■■■■■■ | 0,04 | var T 1 |
| 23, 35 | 254 | 2 (3,6) | 777760007760771 | ■■■■■■■■■■■■■■□□□□□□□□□□■■■■■■■■□□□□■■■■■■■ | 0,23 | T 5 rus |
| 17, 32 | 4 (S 34) | 2 (3,6) | 000000007760771 | □□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■□□□□■■■■■■■ | 0,35 | var T 5 rus |
| 6, 53 | 284 | 2 (3,6) | 037637777760771 | □□□□■■■■■■■■□□■■■■■■■■■■■■■■■■■■□□□□■■■■■■■ | 0,17 | T undefined |
| 33 | 0 | 1 (1,8) | 377767677760771 | □■■■■■■■■■■■■■□■■■■■□■■■■■■■■■■■□□□□■■■■■■■ | 0,00 | T undefined |
| 8 | 0 | 1 (1,8) | 777677377760771 | ■■■■■■■■■■■□■■■■■■□■■■■■■■■■■■■■□□□□■■■■■■■ | 0,00 | T undefined |
| 22, 45 | 0 | 2 (3,6) | 777761777760771 | ■■■■■■■■■■■■■■□□□■■■■■■■■■■■■■■■□□□□■■■■■■■ | 0,00 | T undefined |
| 18 | 0 | 1 (1,8) | 777777474760771 | ■■■■■■■■■■■■■■■■■■■□□■■■■□□■■■■■□□□□■■■■■■■ | 0,00 | T undefined |
| 7, 14 | 1872 | 2 (3,6) | 777777774060771 | ■■■■■■■■■■■■■■■■■■■■■■■■■□□□□□■■□□□□■■■■■■■ | 0,01 | T undefined |
| 3 | 2037 | 1 (1,8) | 777777774360771 | ■■■■■■■■■■■■■■■■■■■■■■■■■□□□■■■■■■□□□□■■■■■■■ | 0,01 | T undefined |
| 9 | 353 | 1 (1,8) | 777777774760771 | ■■■■■■■■■■■■■■■■■■■■■■■■■□□■■■■■■■□□□□■■■■■■■ | 0,04 | T undefined |
| 42 | 47 (S 13) | 1 (1,8) | 777777774020771 | ■■■■■■■■■■■■■■■■■■■■■■■■■□□□□□□■□□□□■■■■■■■ | 2,03 | H 1 |
| 15 | 50 (S 28) | 1 (1,8) | 777777777720771 | ■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□■□□□□■■■■■■■ | 3,15 | H 3 |
| 21 | 0 | 1(1,8) | 003777774020771 | □□□□□□□■■■■■■■■■■■■■■■■■■□□□□□□■□□□□■■■■■■■ | 0,00 | var H 1 |
| 30 | 151 | 1 (1,8) | 777767774020771 | ■■■■■■■■■■■■■■□■■■■■■■■■■□□□□□□■□□□□■■■■■■■ | 0,04 | var H 1 |
| 51 | 45 (S 152) | 1 (1,8) | 777777764020771 | ■■■■■■■■■■■■■■■■■■■■■■■□■□□□□□□■□□□□■■■■■■■ | 0,17 | var H 1 |
| 31 | 0 | 1 (1,8) | 777777774020701 | ■■■■■■■■■■■■■■■■■■■■■■■■■□□□□□□■□□□□■■■□□□■ | 0,00 | var H 1 |
| 36 | 75 | 1 (1,8) | 777767777720771 | ■■■■■■■■■■■■■■□■■■■■■■■■■■■■■■□■□□□□■■■■■■■ | 0,08 | var H 3 |
| 2, 4, 25, 43, 47 | 41 | 5 (8,9) | 777777404760771 | ■■■■■■■■■■■■■■■■■■■□□□□□■□□■■■■■■■□□□□■■■■■■■ | 0,43 | LAM 7 tur |

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→ TABLE 2 - *Spoligotyping patterns of Mycobacterium tuberculosis isolates.*

| *Isolate No* | *ST*[1] | *Number of isolates (%)* | *Octal Code* | *Spoligotyping pattern*[2] | *% in SpolDB4*[3] | *Clade*[4] |
|---|---|---|---|---|---|---|
| 19 | 42 (S 260) | 1 (1,8) | 777777607760771 | ■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■■□□□□■■■■■■■ | 2,71 | LAM 9 |
| 10 | 535 | 1 (1,8) | 777777707760771 | ■■■■■■■■■■■■■■■■■■■■■□□□■■■■■■■■□□□□■■■■■■■ | 0,08 | LAM |
| 28 | 0 | 1 (1,8) | 777727404760771 | ■■■■■■■■■■■■□■□■■■■□□□□□■□□■■■■■□□□□■■■■■■■ | 0,00 | var LAM 7 tur |
| 24 | 0 | 1 (1,8) | 777747404740771 | ■■■■■■■■■■■■■□□■■■■□□□□□■□□■■■■□□□□□■■■■■■■ | 0,00 | var LAM 7 tur |
| 27, 39, 41 | 186 | 3 (5,4) | 777767404760771 | ■■■■■■■■■■■■■■□■■■■□□□□□■□□■■■■■□□□□■■■■■■■ | 0,02 | var LAM 7tur |
| 26 | 1261 | 1 (1,8) | 777777404760731 | ■■■■■■■■■■■■■■■■■■■□□□□□■□□■■■■■□□□□■■■□■■■ | 0,03 | var LAM 7tur |
| 40 | 150 | 1 (1,8) | 777767607760771 | ■■■■■■■■■■■■■■□■■■■■□□□□■■■■■■■■□□□□■■■■■■■ | 0,15 | var LAM 9 |
| 44, 49 | 0 | 2 (3,6) | 777777434760771 | ■■■■■■■■■■■■■■■■■■■□□□■■■□□■■■■■□□□□■■■■■■■ | 0,00 | var LAM |
| 38 | 884 (S 271) | 1 (1,8) | 376377777760771 | □■■■■■■■□□■■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■ | 0,01 | S |

[1]Spoligotypes were indicated as "ST" and "S" according to the SpolDB and HD databases (in brackets), respectively; [2]■ and □ indicate the presence and absence, respectively, of the specific spacer at positions 1 to 43 in the DR locus (Kamerbeek et al.,1997); [3]According to the worldwide spoligotyping database provided by Institut Pasteur de Guadeloupe (SpolDB4); [4]According to the international clades defined by Filliol et al (Filliol *et al.*, 2002) and SpolDB4. LAM: Latin-American-Mediterranean clade, H: Haarlem family, O: Orphan, var: designates a variant of a given clade.

**Major genetic group designation**

Thirty-nine isolates belonged to major genetic group 2, 16 isolates belonged to genetic group 3 and one isolate identified in group 1 as Beijing Type (isolate no: 29) (ST 1) (S 1). IS*6110* copy numbers, spoligotype patterns and principal

TABLE 3 - *DNA fingerprinting characterization with the drug resistance pattern.*

| *Isolate No* | *Number of copies* | *Spoligopattern* | *Major Genetic Group* | *Drug Resistance* |
|---|---|---|---|---|
| 4 | 4 | ST 41 | 2 | pansusceptible |
| 43 | 4 | ST 41 | 2 | IRE |
| 22 | 5 | orphan | 2 | SIRE |
| 45 | 5 | orphan | 2 | SIRE |
| 27 | 2 | ST 186 | 2 | pansusceptible |
| 41 | 2 | ST 186 | 2 | pansusceptible |
| 6 | 7 | ST 284 | 2 | pansusceptible |
| 53 | 7 | ST 284 | 2 | pansusceptible |
| 17 | 7 | ST 4 | 2 | pansusceptible |
| 32 | 7 | ST 4 | 2 | SIRE |

I: resistant to isoniazid, S: resistant to streptomycin, R: resistant to rifampicin, E: resistant to ethambutol

genetic groups for the isolates are shown in Table 2. Five clusters of Turkish TB isolates discriminated by three methods are shown in Table 3.

**Patients' data**

Searching demographic data of the patients with identical RFLP and spoligotyping patterns, we found that the residencies of these patients were in different geographic areas. Moreover, in two MDR patients in the same cluster, collection dates of the isolates differed by four months. Drug resistance patterns of the patients were different in two clusters with two patients in each also having different locations of residencies. When we searched the demographic data of the patient whose specimen was a Beijing type, this 28-year-old male patient who was born in Uzbekistan was an immigrant from Azerbaijan (a former Russian State) who moved to Turkey in 2000.

**Statistical analysis**

HGI was found as 0.997, 0.964 and 0.441 for IS*6110* RFLP, spoligotyping and genetic typing, respectively. Evaluating the RFLP, spoligotyping and genetic typing together, did not increase the HGI. The acceptable HGI for a molecular epidemiologic method is >0.90 (Hunter and Gaston, 1988). Therefore, IS*6110* and spoligotyping were accepted as available methods for molecular fingerprinting studies in contrast to major genetic group designation.

## DISCUSSION

Various molecular methods have been used by researchers in epidemiologic studies for characterizing *M. tuberculosis* isolates (Small *et al.*1994; Van Soolingen and Hermans, 1995; Torrea *et al.*, 1995; Montoro *et al.*, 1998; Park *et al.*, 2000). IS*6110* RFLP has been universally accepted as a standard reference method (Kremer *et al.*,1999). However, this method is not sufficient in characterizing isolates with low-copy numbers (<6) (Torrea *et al.*, 1995; Chaves *et al.*,1996; Bauer *et al.*, 1999; Soini *et al.*, 2001). Therefore, a secondary typing method is required for further discrimination of these isolates. Recently, "spacer oligonucleotide typing" (spoligotyping) defined by Kamerbeek *et al.* has been used for characterization of *M. tuberculosis* complex isolates (Goguet de la Salmoniere *et al.*, 1997; Goyal *et al.*, 1997; Sola *et al.*, 1998; Zumarraga *et al.*1999; Soini *et al.*, 2000). In comparison with IS*6110* RFLP, the sensitivity and discrimination power of spoligotyping is better than IS*6110* profiling for isolates with low-copy numbers (Kamerbeek *et al.*, 1997; Bauer *et al.*, 1999; Soini *et al.*, 2000; Goyal *et al.*, 1997). In a previous study from Turkey (Durmaz *et al.*, 2003), Durmaz and coworkers reported low copy number isolates ranging up to 25 %. These values are somewhere between the values found in Asia and Europe (Bauer *et al.*, 1999; Torrea *et al.*, 1995; Park *et al.*, 2000; Doroudchi *et al.*, 2000).

In the present study, five clusters with two isolates each were identified by IS*6110* RFLP whereas 10 clusters with multiple isolates (2 to 9 isolates/pattern) were identified by spoligotyping. Although these isolates were recovered from patients having different locations of residencies, they were presumed to be clonal as they were all from the same geographic region (Aegean Region).

ST 53 (S 29) and ST 41 (16.1% and 8.9%, respectively) were found as the most frequent spoligotypes in our study. According to the SpolDB3/4 (Sola *et al.*, 2001; Brudey *et al.*, 2006). ST 53 (S 29) is the most frequent spoligopattern widespread around the world isolated in China, France, Romania, Great Britain, Holland, Cuba, Italy, Denmark, Senegal, U.S.A. and Russia in accordance with our findings. ST 41 is another strain which is widespread and found in France, Holland, Denmark and Romania (Sola *et al.*, 2001). Recently, Zozio *et al.* described a new phylogeographically specific clone of *M. tuberculosis* which was defined as ST 41 in SpolDB3 and designated LAM 7 tur because of its high frequency within Turkish isolates (Zozio *et al.*, 2005). In the present study, the shared international clades which were defined by Filliol *et al.* (Filliol *et al.*, 2002) and SpolDB4 were identified as Beijing (ST 1) (*n*=1; 1.8%), var T1 (ST 37) (*n*=2; 3.6%), LAM 7 (ST 41) (*n*=5; 8.9%), LAM 9 (ST 42) (*n*=1; 1.8%), Haarlem 1 (ST 47) (*n*=1; 1.8%), Haarlem 3 (ST 50) (*n*=1; 1.8%) and T1 (ST 53) (*n*=9; 16.1%) (Table 2). T group of families as described by Filliol *et al.* likely represented old genotypes disseminated in Europe. In addition, a Beijing type isolate resistant to four major anti-

TB agents was identified in a patient who was born in Uzbekistan and found to be an immigrant from Azerbaijan in 2000. This finding has recently been supported by an ongoing research project comprising a large number of isolates carried out by Koksalan and coworkers regarding Beijing-family isolates in the Istanbul area, recovered from citizens of the former Soviet Union, most frequently being Azerbaijanians (Koksalan *et al*., 2005). As these patients were generally infected by MDR strains and known to have some difficulties in their treatments, high risk of transmission of these strains has to be taken into account for further TB control follow-up strategies of TB in Turkey.

*M. tuberculosis* isolates with different genetic groups are prevalent in different areas of the world. Soini *et al.* (Soini *et al*., 2001) reported that major genetic group 1 isolates were significantly more prevalent among patients of Asian origin, whereas Mexican and U.S.-born patients had been infected with isolates belonging to major genetic group 2. In another study, nearly all *M. tuberculosis* isolates with <6 IS*6110* elements collected in Cape Town, South Africa, were members of a lineage of the principal genetic group 2 (Warren *et al*., 2004). Only one isolate from this setting was identified as being a member of a distinct low-IS*6110*-copy-number lineage of the principal genetic group 1, which corresponds to group I and had been primarily associated with patients from East Africa and Asia (Soini *et al*., 2001; Warren *et al*., 2004). Examination of the SpolD database indicated that principal genetic group 2 isolates with the characteristic direct-variable-repeat (DVR) 33-to-36 and DVR 18 deletions had been isolated in 27 different countries (Warren *et al*., 2004). In Italy, most of the isolates (85.5%) belonged to genotypic groups 2 and 3, which included most isolates from Italian-born patients (Lari *et al*., 2005). The remaining isolates were genotypic group 1 organisms, which were prevalent among foreign-born patients. Data from these studies indicate that group 1 organisms are evolutionarily old whereas they might have evolved further to group 2 and 3 (Soini *et al*., 2001, Lari *et al*., 2005). In the present study, major genetic group 2 was found most frequently among the isolates from Turkish TB patients. In addition, 16 isolates were found to be genetic group 3 and only one (Beijing type isolate) was defined as genetic group 1. It was thought that there was strict correlation pointed out by Soini *et al.* (Soini *et al*., 2000) and Brosch *et al.* (Brosch *et al*., 2002) that strains of genetic group 2 or 3 characteristically lack spacers 33-36 while spacers 32 and 37 were normally present. The same situation can also be seen for the Turkish isolates represented in Tables 1 and 2, indicating that in almost all cases spoligotype information can predict the major genetic groupings. However, major group designation can provide supporting information for molecular and evolutionary characteristics of the *M. tuberculosis* isolates (Sreevatsan *et al*., 1997). Although the differentiation into genetic groups 1, 2 and 3 is a useful tool for evaluating the evolutionary position of a given strain, the differentiation by this grouping is a rather rough subdivision [the genetic group 1 contains very diverse strains of the *M. tuberculosis* complex as shown by Sreevatsan *et al.* (Sreevatsan *et al*., 1997) and Brosch *et al.* (Brosch *et al*., 2002)]. According to our results the discrimination capacity of this method for a fingerprinting method was low (HGI: 0,441).

In conclusion, the present study provides useful data on the molecular epidemiologic features of tuberculosis in Turkey, where there are limited data regarding spoligotyping and IS*6110* RFLP patterns and no data on major genetic groups. Molecular characterization of *M. tuberculosis* isolates has disclosed a large number of patterns in different geographic regions all over the world. IS*6110* profiling and spoligotyping might provide increased discrimination among TB isolates. We recommend the step-wise scheme of molecular typing in the Turkish setting, using spoligotyping as a first method, with further subtyping of clustered isolates by IS*6110*-RFLP. Although IS*6110*-RFLP is the gold standard except for low copy number isolates, spoligotyping is an easy to use, cost-effective, acceptable method for fingerprinting, and has comparable objective results according to worldwide databases. Thus, creation of a database in Turkey may help to address transmission links and aid in the implementation of public health services for TB control in Izmir, Turkey.

*ACKNOWLEDGEMENT*

*We sincerely thank Thierry Zozio, Christophe Sola and Nalin Rastogi from Institut Pasteur de*

*Guadeloupe for sharing their spoligotyping database (SpolDB4) with us.*

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