Beijing/w and major spoligotype families of *Mycobacterium tuberculosis* strains isolated from tuberculosis patients in Eastern Turkey

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SUMMARY

The aim of this study was to determine the Beijing/W family and major phylogenetic clades of *Mycobacterium tuberculosis* strains of tuberculosis patients in a city with a tuberculosis incidence higher than the country average. A total of 220 *M. tuberculosis* strains isolated over a period of more than four years were typed by spoligotyping. Spoligotyping resulted in 64 different patterns, 38 (17.3%) of which were unique, and 26 were clusters including 182 (82.7%) strains. The major shared types were ST 53 (n=55, 25%), ST 41 (LAM7-TUR; n=19, 8.6%), and ST 284 (n=15, 6.8%). The major clades observed ranked in the following order: ill-defined T superfamily (n=112, 50.9%); Latino-American-Mediterranean (LAM; n=33, 15%); Haarlem (n=24, 10.9%); and the S family (n=9, 4.1%). Three strains were in the Beijing family. A high number of strains (33 strains) showed patterns that did not fall within any of the major clades described. *M. tuberculosis* strains in Malatya have both STs showing a widespread distribution over the world and those restricted to this city, confirming the highly diverse nature of tuberculosis. Our results suggest that the Beijing clade, which is more prevalent among the strains with MDR and isoniazid resistance, is currently not a problem in Eastern Turkey.

KEY WORDS: Tuberculosis, Turkey, Molecular epidemiology, Spoligotyping, Beijing genotype

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INTRODUCTION

Although it is a historical disease, we are still far from achieving success in the fight against tuberculosis.

About one third of the overall population throughout the world is infected by *M. tuberculosis* and each year 2 million individuals die from tuberculosis (WHO, 2005). Accurate diagnosis, treatment and epidemiology of tuberculosis are essential to control the disease. Development of novel molecular typing methods contributes to the classical knowledge on the epidemiology of tuberculosis (Qian *et al.,* 1999; Sola *et al.,* 2001; van Soolingen *et al.,* 1995).

There are a number of methods for the molecular typing of tuberculosis bacilli, such as IS*6110* restriction fragment length polymorphism (RFLP), spoligotyping, pTBN12 fingerprinting, and mycobacterial interspersed repetitive unites-variable numbers of tandem repeat (MIRU-VNRT) typing (Cavusoglu *et al.,* 2006; Mokrousov *et al.,* 2004; Sola *et al.,* 2003). The spoligotyping method is based on displaying the heterogeneity caused by spacers and repetitive sequences in the polymorphic chromosomal DR (direct repeat) locus (Kamerbeek *et al.,* 1997). Researches have shown that spoligotyping is a practical and rapid method in both clinical and molecular epidemiology. Databanks including spoligotyping results are formed with an aim to investigate the worldwide distribution of the detected clades and to compare them with each other. The SpolDB4,

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which was established to serve this goal, includes information on more than 45,000 isolates (Brudey *et al.,* 2006).

Initial spoligotyping studies showed that one of the *M. tuberculosis* spoligotypes, Beijing, was predominant in Beijing, China and other studies followed suggesting that it was also widely prevalent throughout Asia and in the West Pacific (Caminero *et al.*, 2001). Many reports from Germany, Italy, Russia, Estonia, South Africa and Columbia documented that the isolates identified as Beijing genotype were associated with multiple drug resistance (Caminero *et al.,* 2001; Drobniewski *et al.,* 2002; Filliol *et al.,* 2002; Filliol *et al.,* 2003; Glynn *et al.,* 2002; Lari *et al.,* 2004; Toungoussova *et al.,* 2002; van Soolingen *et al.,* 1995). Similarly, the multi-drug resistant "W" genotype isolated from a tuberculosis epidemic affecting more than 350 individuals in New York was a member of the Beijing genotype clade (Bifani *et al.,* 1996). Two additional studies from Vietnam found that the Beijing genotype was predominant particularly among very young patients (Anh *et al.,* 2000; Le *et al.,* 2000). Overall, these reports suggest that the Beijing/W genotype has become widespread throughout the world, and that it has implications on drug resistance.

Studies on the molecular epidemiology of tuberculosis in Turkey are primarily based on IS*6110* RFLP and pTBN12 typing methods (Cavusoglu *et al.,* 2006; Durmaz *et al.,* 2003b; Durmaz *et al.*, 2003a). Such reports contribute to our understanding of the clonal relationship between *M. tuberculosis* isolates in our city and throughout Turkey. However, our knowledge on the major spoligotype profiles and particularly the Beijing/W genotype is limited. There is only one published study conducted on 147 *M. tuberculosis* strains collected from Malatya (Zozio *et al.,* 2005). The study showed that three shared types (ST); ST41 (LAM7-TUR family), ST53 (ill-defined T1 superfamily), and ST50 (Haarlem 3 family) were the prevalent STs encountered in more than half the typed strains. The study described a new phylogeographically-specific clone called LAM7-TUR, which was a specific for Turkey, and suggested further studies be conducted on more strains to understand the real situation about the distribution of this STs in our city and country.

The aim of this study was to obtain information on the status of Beijing and other spoligotype clades among *M. tuberculosis* isolates collected within a period of more than four years in Malatya, a city in east Anatolia, which has a population of 850,000, with 250-300 new tuberculosis cases each year, which exceeds the average tuberculosis incidence of the country (32 vs. 26 per 100 000 persons, respectively).

MATERIAL AND METHODS

M. tuberculosis strains: A total of 220 strains isolated from patients diagnosed with *M. tuberculosis* primary infection attending Inonu University Turgut Ozal Medical Center, two tuberculosis dispensaries, and Governor Hospital in the period between January 2001 and July 2005 were included in the study. The total number of *M. tuberculosis* strains isolated in the city throughout

TABLE 1 - *Distribution of the 220 strains in according to study period and multi-drug resistance statue.*

Year	*No of the strains isolated/year*	*No of the strains analyzed*	*Sampling rate (%)*	*No of MDR strains*
2001	85	60	70.5	3
2002	126	52	41.3	4
2003	70	39	55.7	2
2004	95	55	57.9	4
2005 (first six months)	50	14	28.0	-
Total	426	220	51.6	13

this period was 426. Thus, 220 strains included in the study represented 51.6% of the overall isolates. All of the 220 patients were characterized by pulmonary TB and originated from Malatya (there were no foreign-born cases). The mean age was 33.2 years (range: 3-89), and the male-to-female ratio was 1.4. The strains analyzed in this study included the isolates for which stock cultures were available and which were not analyzed in our published study (Zozio *et al.,* 2005). Only one isolate of each patient was typed. The strains were identified based on their growth characteristics, biochemical tests such as nitrate reduction and niacin accumulation, and by the LCD-Array Chipron Myco-ID-Basic 1.2 (Chipron GmbH, Germany) or GenoType Mycobacterium CM (Hain Lifescience GmbH, Germany) kits, according to the manufacturer's recommendations. The distribution of strains according to years and the percentage of sampling are shown in Table 1.

Resistance tests for antimicrobial drugs

The resistance of strains to isoniazid (INH), rifampicin (RIF), ethambutol (ETB) and streptomycin (SM) were evaluated using either Bactec 460 radiometric or Bactec MGIT 960 (Becton Dickinson and Company, Sparks, MD, USA) systems, according to the manufacturer's recommendations. Drug resistance was defined as greater than 1% growth in the presence of 0.1 mg of isoniazid per ml, 2 mg of rifampicin per ml, 2.5 mg of ethambutol per ml, or 2 mg of streptomycin per ml. Strains resistant to INH and RIF or additionally to one or more of the other drugs were considered multi-drug resistant (MDR) (Sharma and Mohan, 2004).

Spoligotyping

DNA was extracted from *M. tuberculosis* strains, grown in Lowenstein-Jensen medium, by the CTAB method (van Soolingen *et al.,* 1991). *M. tuberculosis* DNA was amplified using DRa (biotinylated 5') and DRb primers as described by Kamerbeek *et al.*, (Kamerbeek *et al.,* 1997). The amplification product was hybridized with the membrane carrying probes (produced by Immunetics, Boston, USA) specific to the 43-spacer region and the product was visualized by radiation on x-ray film using chemiluminescence. The data obtained were analyzed using the SpolDB4 (http://www.pasteur-guadeloupe.fr:8081/SITVITDemo/outilsConsultation.jsp) database, and clusters of "shared-type (ST)" and clades were determined.

IS*6110 RFLP typing* IS*6110*-RFLP of *Pvu*II-digested DNA was performed as previously described (Van Embden *et al.,* 1993). After electrophoresis, the restriction fragments on the gel were denatured and blotted onto nylon membrane by the alkaline transfer procedure, and hybridization was carried out with a chemiluminescent 521 bp IS*6110* fragment produced by PCR. *M. tuberculosis* H37Rv was used as an international standard. Banding patterns of the strains were analyzed with GelCompar II software (Version 3.5 Applied Math, Ghent, Belgium).

Statistical analysis

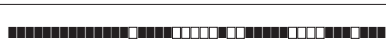
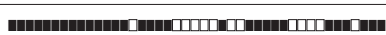
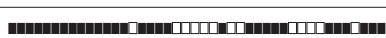
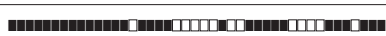
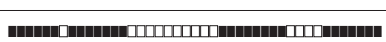
Molecular typing results were analyzed using Taxotron (Institut Pasteur, P. Grimont) and Bionumerics (v 3.1, Applied Maths, Sint Maarten Latem, Belgium). The pairwise distance between clinical isolates was computed using the 1-Jaccard index (Saitou and Nei 1987). The UPGMA (unweighted pair-group method using arithmetic averages) was used for clustering of the isolates (Sneat and Sokal, 1973).

RESULTS AND DISCUSSION

Antibiotic resistance testing revealed that 13 out of 220 strains (5.9%) were multi-drug resistant (MDR), and 54 (24.5%) strains showed resistance to at least one drug. The rates of monoresistance to INH, RIF, ETB, and SM were 5.5% (12/220 strains), 2.7% (6/220 strains), 2.3% (5/220 strains), and 5.5% (12/220 strains), respectively. The remaining 153 strains (69.5%) were susceptible to all drugs tested. Resistance testing has become critical for the control of tuberculosis due to increasing multi-drug resistance. Although many countries undertake resistance studies, the actual rate of resistance is still unknown. The rates of at least one drug resistance and multi-drug resistance in Turkey are 14.3-41% and 2.2-3% respectively (Durmaz *et al.,* 2003b; Durmaz *et al.,* 2003a).

Spoligotyping, which is rapid and convenient, ensures the identification of ST and clades of strains (Sola *et al.,* 2003; Yang *et al.,* 1998). In the present study, typing of 220 strains resulted in 64 dif-

TABLE 2 - *Orphan and newly-defined clusters in this study.*

Strain	*Spoligotyping pattern*	*Octal number*	*Drug resistance*	*STs*	*No. of strain in this study*	
					n	*(%)*
N531		777767404760731	Susceptible			
N533		777767404760731	Susceptible			
N534		777767404760731	Susceptible	Unknown (new cluster)	6	2.7
N547		777767404760731	Susceptible			
N566		777767404760731	Susceptible			
N571		777767404760731	Susceptible			
N433		777377777760601	Susceptible			
N441		777377777760601	Non-MDR[a]	Unknown (new cluster)	3	1.4
N598		777377777760601	Susceptible			
N410		777777637760771	Susceptible	Orphan	1	0.5
N411		777777677760771	Susceptible	Orphan	1	0.5
N426		777737777700171	Susceptible	Orphan	1	0.5
N537		773760007760771	Non-MDR[b]	Orphan	1	0.5
N543		377777763760771	Susceptible	Orphan	1	0.5
N553		777737777700171	Susceptible	Orphan	1	0.5
N561		037637477720771	Susceptible	Orphan	1	0.5
N576		740617777760771	Susceptible	Orphan	1	0.5
N579		777777777740171	Susceptible	Orphan	1	0.5
N581		700377777760771	Susceptible	Orphan	1	0.5
N595		377777763760771	Susceptible	Orphan	1	0.5
N596		617777774020760	Susceptible	Orphan	1	0.5
N606		773760007760771	Susceptible	Orphan	1	0.5
N614		037637477720771	Susceptible	Orphan	1	0.5
N964		377777763760771	Susceptible	Orphan	1	0.5
N969		777771777760771	Susceptible	Orphan	1	0.5
N971		716376777760771	Susceptible	Orphan	1	0.5
N974		000000007740771	Non-MDR[c]	Orphan	1	0.5
N170		037637477720771	Susceptible	Orphan	1	0.5
N239		777000277740071	Non-MDR[d]	Orphan	1	0.5
N241		037636377760771	Susceptible	Orphan	1	0.5
N274		074002007760771	Susceptible	Orphan	1	0.5
N291		377777763760771	Susceptible	Orphan	1	0.5
N297		736377700000000	Susceptible	Orphan	1	0.5

[a]Resistance to isoniazid and streptomycin; [b]resistance to isoniazid; [c]resistance to rifampicin; [d]resistance to streptomycin.

ferent spoligotype patterns. While 182 strains were in the 26 clusters including 2 new clusters with 9 strains in this study, 38 (17.3%) strains displayed unique profiles. Hence, the clustering rate of Malatya's strains was 82.7%. Comparison of the spoligotyping results obtained in our study with those in the SpolDB4 databank (http://www.pasteur-guadeloupe.fr:8081/SITVIT Demo) revealed that 24 of the 38 unique patterns (10.9 %) yielded actual orphan profiles that did

TABLE 3 - *Distribution of the 24 clusters with previously described STs.*

Spoligotyping pattern	*Octal number*	*ST*	*Ratio in the 220 strains (%)*		*Family*
			N	*%*	
■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□■■■■■■■	777777777760771	53	55	25.0	T1
■■■■■■■■■■■■■■■■■■■□□□□■□□■■■■■□□□■■■■■■■	777777404760771	41	19	8.6	LAM7-TUR
□□□□■■■■■■■□□■■■■■■■■■■■■■■■■■■□□□■■■■■■■	037637777760771	284	15	6.8	T1
□■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□■■■■■■■	377777777760771	7	9	4.1	T
■■■■■■■■■■■■■■■■■■■■□■■■■■■■■■□□□■■■■■■■	777777737760771	86	8	3.6	T
■■■■■■■■■■■■■■■■■■■■■■■□□□□□■□□□■■■■■■■	777777774020771	47	7	3.2	H1
■■■■■■■■■■■■■■■■■■■■■■■□□□□□■□□□■■■□■■■	777777774020731	62	6	2.7	H1
■■■■■■■■■■■■■■■■■■■■■■■■■■■□■□□□■■■■■■■	777777777720771	50	5	2.3	H3
■■■■■■■■■■■□□■■■■■■■■■■■■■■■■□□□■■■■■■■	777717777760771	131	5	2.3	T3
■■■■■■■■■■■■■■■□□■■■■■■■■■■■■□□□■■■■■■■	777777177760771	1252	5	2.3	X
■■■■■■■■■■■■■■■□■■■■■■■■■■■■■□□□■■■■■■■	777777377760771	40	4	1.8	T
■■■■■■■■□□■■■■■■■■■■■■■■■■■■■□□□■■■□■■■	776377777760731	784	4	1.8	S
□□□□□□□□□□□□□□□□□□□□□■■■■■■■■□□□■■■■■■■	000000007760771	4	4	1.8	S
□□□□□□□□□□□□□□□□□□□□□■□□□□□■□□□■■■■■■■	000000004020771	2	3	1.4	H2
■■■■■■■■■■■■■■■■■■■□□□□■□□■■■■□□□■■■■■□□	777777404760760	1937	3	1.4	LAM7-TUR
■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■■□□□■■■■■■■	777777607760771	42	3	1.4	LAM9
□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■	000000000003771	1	3	1.4	Beijing
■■■■■■■■■■■■■■■■■■■■■■■□□■■■■□□□■■■■■■■	777777776360771	123	3	1.4	T1
■■■■■■■■■■■■■■■■■□□□□□■□□■■■■□□□■■■□■■■	777777404760731	1261	2	0.9	LAM7-TUR
■□■■■■■■■■■□■■■■■■■■■■■■■□□□■□□□■■■■■■■	577737777420771	361	2	0.9	H4
□□■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□■■■■■■■	177777777760771	191	2	0.9	T1
■■■■■■■■■■■■■■□□□■□□□□□□■■■■■■■■□□□■■■■■■■	777761007760771	766	2	0.9	LAM
□■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□■■■□■■■	377777777760731	853	2	0.9	T2
■■■■■■■■■□■■■■■■■■■■■■■■■■■■■■□□□■■■■■■■	777377777760771	1166	2	0.9	T1

Abbreviations, ST: Shared types, T: ill-defined T families, LAM: Latin-American-Mediterranean TUR: Turkey, H: Haarlem, X: X family, S: S family

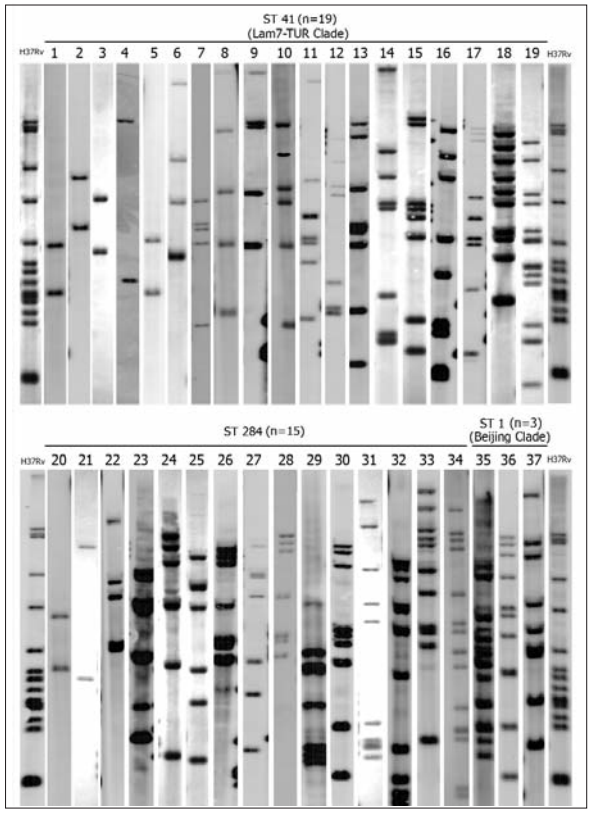


FIGURE 1 - *IS6110 RFLP profiles of the strains classified in ST41 (LAM7-TUR), ST284, and ST 1(Beijing). H37Rv; Mycobacterium tuberculosis H37Rv strain used as a reference strain. Digits on top of the figure indicated the strains numbers in each ST.*

not cluster with any strain (Table 2). The remaining 14 strains constructed a cluster with the strains in the SpolDB4 database.

The distribution of 24 clusters with known ST numbers is shown in Table 3. The most common STs were ST 53 (n=55, 25%), ST 41 (LAM7-TUR; n=19, 8.6%), and ST 284 (n=15, 6.8%). The distribution of spoligotyping profiles varies according to countries and regions. In our neighboring country Iran, the most common STs, ST 47, ST 11, ST 26, and ST 1, differed from the predominant STs in Malatya, which is close to this country (Farnia *et al.*, 2006). In another study in Turkey, ST41, ST53 and ST50 genotypes were the most predominant strains in decreasing order in two different cities, Malatya and Ankara (Zozio *et al.,* 2005). The predominant ST 53 strain in our study is also widespread throughout the world, particularly in European countries (Puustinen *et al.*, 2003). It was interesting to find that the ST 41 (LAM7-TUR), which is phylogeographically specific to Anatolia (Zozio *et al.,* 2005), was the second most prevalent type in Malatya. The percentage of ST 284, a globally rare (0.1%) type, was high (6.8%) in our city. Similar results were obtained in a previous study in Turkey with a 5.7% prevalence of ST 284 (Zozio *et al.,* 2005). A few reports suggested that this genotype, which was also detected in Bulgaria and Saudi Arabia, may be phylogeographically specific to the Middle East (Zozio *et al.,* 2005). To analyze the ST 41 (LAM7-TUR) and ST 284, which were the two prevalent types found in our study population, in detail IS*6110* RFLP typing was performed. Of the 19 LAM7-TUR strains, 9 had fewer than 6 copies of IS*6110* (low copy number strains), 10 had IS*6110* copy numbers ranging from 6 to 13. Only one strain yielded a similar IS*6110* RFLP profile with a strain identified as ST284, the remaining 18 strains had unique IS*6110* RFLP profiles. Four of the 15 strains classified as ST284 had low copy and 11 had 6-14 copies of IS*6110*. Only one strain having 6 copies had a similar IS6110 RFLP profile, the other strains were unique (Figure 1).

The most common clades in our study were ill-defined T clade (n=112, 50.9%), LAM7-TUR (n=26, 11.8%) (specific to our country), followed by Haarlem (n=24, 10.9%), S (n=9, 4.1%), X (n=5, 2.3%), LAM9 (n=3, 1.4%), and LAM (n=3, 1.4%). The number of strains from the Beijing/W family was 3 (1.4%). In addition, one strain, each from CAS1- Delhi was detected. The clade names of 33 strains could not be identified (Table 4). The predominant *M. tuberculosis* clades vary across populations. For example, about 50% of Beijing and Beijing-like strains are in the Far East, 25% of Haarlem clade is in Europe, and approximately 50% of LAM clade is in South America (Brudey *et al.,* 2006). Haarlem, LAM and T clades were often transiently detected in Africa, Central America, Europe and South America (Brudey *et al.*, 2006; Zozio *et al.,* 2005). T clade is detected in all continents and accounts for about 30% of strains in the databank. A study from Iran showed that the east African-Indian (24%), central Asian (20.8%), T clade (20.7%), Haarlem I (4.4%), and Beijing (3.2%) were the major *M. tuberculosis* superfamilies (Velayati *et al.,* 2006). The results of another study from Turkey (Gencer B

TABLE 4 - *Major spoligotyping families found in this study.*

Family	*No. of strains*	*Percentage in this study*
T lineage	112	50.9
T	24	10.9
T1	81	36.8
T2	2	0.9
T3	5	2.3
LAM lineage	33	15.0
LAM	3	1.4
LAM7-TUR	26	11.8
LAM9	3	1.4
LAM5	1	0.5
Haarlem	24	10.9
S	9	4.1
X	5	2.3
Beijing	3	1.4
CAS1-Delhi	1	0.5
Unknown	33	15.0

and Shinnick TM, 2005) indicating the predominance of T (37%), LAM (20%) and Haarlem (8%) clades, are in agreement with our findings. However, while the Beijing clade ranked fourth in that study, we detected only three Beijing strains (1.4%). Two of the three Beijing strains had 10 copies of IS*6110*, the other had 9 copies of IS*6110* and these strains represented unique RFLP profiles (Figure 1). Our results regarding the rate of Beijing genotype were similar to the study performed in which more than 4000 strains were collected from Istanbul/Turkey. This study showed that 1.1% of the strains were Beijing genotypes and the strains having Beijing genotype were originated from the former Soviet Union (Koksalan *et al.,* 2006). In reports from neighboring countries including different groups, the percentages of Beijing strains were 44.5% in Russia, 29.2% in Estonia, and 70.8% in Azerbaijan (Pfyffer *et al.,* 2001; Sola *et al.,* 2003). Many studies from New York, Estonia, Cuba and Russia demonstrate a strong correlation between MDR and Beijing strains and indicate that this strain has a high virulence (Caminero *et al.,* 2001; van Soolingen *et al.,* 1995). A study from China reported that the Beijing clade accounted for 77.8% of multi-drug resistant strains (Le *et al.,* 2000), another study from Azerbaijan detected a rate of 52.3% (Pfyffer *et al.,* 2001). A study from Istanbul showed that Beijing genotype among at least rifampicin resistant strains was significantly more prevalent than among rifampicin-susceptible strains (Koksalan *et al.,* 2006). However an association with drug resistance could not be detected for the Beijing strain in reports from Southeast Asia (Baker *et al.,* 2005). Another important finding is that INH resistance alone was higher among Beijing/W and Beijing-like strains compared to others (Streicher *et al.,* 2004). Of the three Beijing isolates in this study, two showed multi-drug resistance and one showed resistance to the combination of isoniazid and streptomycin. When 13 strains with MDR were analyzed, two (15.4%) were of Beijing genotype. Although the number of Beijing strains is limited, our data are in agreement with the results of other reports suggesting an association of the Beijing genotype with MDR.

We detected only one Delhi isolate of the Central Asian (CAS) clade. In a study from Pakistan, the CAS clade was the predominant genotype with a rate of 39% (Hasan *et al.*, 2006). Despite reports indicating that the CAS1-Delhi strain was strongly associated with isoniazid resistance (Baker *et al.,* 2005), our strain was sensitive to all drugs. The X clade is among the well-defined genotypes. To date, three subclades, X1, X2 and X3 have been determined. X clade is the predominant genotype particularly in South America (14%) and United Kingdom (Brudey *et al.,* 2006; Sola *et al.,* 2003). We detected 5 strains (2.3%) from the X clade.

Similar to the results of the previous study in Turkey (Zozio e*t al.,* 2005), of the 220 strains analyzed, 33 (15%) did not correlate with any strain in the global databank and thus they had profiles specific to Malatya. This indicates that *M. tuberculosis* strains in our region have high genetic heterogeneity. The philogenetic heterogeneity of tuberculosis, which is a major public health problem in Turkey, may be attributed to the large population (69.6 million according to the 2002 census) residing in a wide area (780,576 km^2) extending from Asia to Europe.

In conclusion, despite the predominance of

spoligotypes such as Beijing originating from Asian countries, Malatya, which is close to the neighboring countries in Asia, has a very limited number strains identified as Beijing type and has a heterogeneous *M. tuberculosis* population comprising globally distributed types and country-specific types.

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