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Molecular Genotyping of *Mycobacterium tuberculosis* Isolates from Turkey

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Abstract: To survey the circulating strains of *Mycobacterium tuberculosis* in Turkey, all clinical isolates (381 patients) recovered in laboratories in six cities during one-month periods were collected and typed by IS6110-DNA fingerprinting and spoligotyping. Drug susceptibilities were also determined. About 23% of the isolates were resistant to one or more drugs and about 4% were multidrug resistant (i.e., resistant to at least isoniazid and rifampin). IS6110-DNA fingerprints and spoligotypes were obtained from 368 and 374 strains, respectively. Of the 374 isolates spoligotyped, 87 (23%) displayed unique spoligotypes and 287 (77%) displayed one of 34 spoligotypes (2-77 isolates per pattern). The clustered spoligotypes included ones that matched spoligotypes of the T (37% of isolates), LAM (20%), Haarlem (8%) and Beijing (2%) families. Of the 368 isolates IS6110-typed, 232 (63%) displayed unique IS6110-fingerprint patterns and 136 (37%) displayed one of 35 patterns (2-34 isolates per pattern). When IS6110 fingerprinting and spoligotyping information were combined for the 381 isolates tested, 273 isolates (72%) displayed unique genotypes and 108 isolates (28%) displayed one of 34 genotypes (2-24 isolates per genotype). In summary, many different strains are circulating in Turkey with no single strain appearing to be dominant as has been observed in other areas of the world with high tuberculosis incidence.

Key words: Tuberculosis, molecular epidemiology, drug resistance

INTRODUCTION

Tuberculosis remains one of the most significant infectious causes of death, annually causing ~2 million deaths worldwide[1]. In Turkey, the incidence of tuberculosis is approximately 27 cases per 100,000 population and about 20,000 new tuberculosis cases are reported each year[2]. Drug-resistant tuberculosis is increasing and is a significant threat to tuberculosis control because there are few drugs effective against *M. tuberculosis*. In Turkey, 20-26% of new cases of tuberculosis are resistant to at least one anti-tuberculosis drug and 3-10% of new cases are multidrug resistant[3-6].

Molecular typing of *M. tuberculosis* isolates can be useful in elucidating the natural history of the tuberculosis epidemic and evaluating tuberculosis control efforts. In the most widely used method, IS6110 restriction fragment length polymorphism (RFLP), variability in both the number of copies and the location of the IS6110 insertion elements generate variation in the RFLP patterns[7]. The molecular clock of IS6110 pattern variation is slow enough to be useful for outbreak investigation and yet fast enough for this to be the most discriminatory of the available typing techniques[8]. IS6110-based genotyping has been used successfully to trace transmission in outbreaks, confirm laboratory cross-contamination, identify risk factors for disease among populations of patients with TB and investigate transmission dynamics in populations[9].

One limitation of IS6110 genotyping is that isolates containing six or fewer IS6110 copies are poorly differentiated and a secondary typing method is needed for reliable discrimination of strains[1,17]. Spacer oligonucleotide typing (spoligotyping) is a secondary typing method that is useful for isolates with low-copy numbers of IS6110[10-14]. Spoligotyping is a PCR-based technique which detects the presence or absence of 43 spacers in the direct-repeat locus[15].

When molecular genotyping is applied at the population level, the clustering of isolates can provide important clues about the patterns and dynamics of transmission in the population[16]. In this study, we determined the relative frequency of *M. tuberculosis* strains in specific geographic areas to better define the spectrum of circulating strains in Turkey and provide clues as to transmission dynamics. To do this, all clinical isolates (381 patients) recovered from laboratories in six cities during one-month periods were collected and genotyped using a combination of IS6110-DNA fingerprinting and direct-repeat-based spoligotyping.

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MATERIALS AND METHODS

Bacterial strains: Three hundred eighty-one isolates of *M. tuberculosis* were obtained by six regional tuberculosis laboratories as part of routine medical care. These laboratories represent different geographical areas in six cities: Ankara (183 isolates), Antalya (50 isolates), Kayseri (34 isolates), Trabzon (88 isolates), Samsun (17 isolates) and Van (9 isolates). All isolates recovered in each laboratory during one or two 1-month periods in 2001 and 2002 were collected. None of these were "repeat" isolates from the same patient. Isolates were identified by standard methods, subcultured onto Lowenstein-Jensen medium and provided to the Refik Saydam Hygiene Center, Tuberculosis Reference Laboratory, Ankara, Turkey after removing patient identifiers. Confirmatory identification and drug susceptibility testing were done in the Centers for Disease Control and Prevention Mycobacteriology laboratory according to standard procedures[17].

IS6110 RFLP and cluster analysis: RFLP analysis (IS6110-DNA fingerprinting) was performed according to standard methods[7,18,19]. A cluster was defined as a group of two or more isolates with identical DNA fingerprint patterns if the isolates had more than six IS6110 bands or as a group of two of more isolates having the same IS6110 pattern and spoligotype if the isolates had six or fewer IS6110 bands.

Spoligotyping: Spoligotyping was performed using a commercial kit (Isogen Bioscience BV, Maarssen, The Netherlands) following the method of Molhuizen[15,19].

RESULTS

Drug susceptibility: Drug susceptibility test results were available for 367 of the 381 isolates (Table 1). Of the 367 isolates, 281 (77%) were drug susceptible and 86 (23%) were resistant to one or more drugs. Resistance to at least isoniazid and rifampin (i.e., multidrug-resistant strains) was found in 16 isolates (4.4%). Five isolates (1.3%) were resistant to all four drugs tested.

Spoligotyping: Spoligotypes were obtained for 374 isolates (Table 2): 87 isolates (23%) displayed unique spoligotypes and 287 isolates (77%) displayed one of 34 spoligotypes (2-77 isolates per pattern). Ten of the shared spoligotypes were related to the ancestral T clade (absence of spacers 33-36 and occasionally one or two other spacers; reviewed in[20]). The spoligotype of the largest cluster of strains (77 isolates, cluster A) matched that of the T1 subclade. The spoligotypes of five clusters (F, 8 isolates; H, 7 isolates; O, 4 isolates; T, 3 isolates; Q, 3 isolates) differed from the T1 spoligotype by the absence of a single spacer. The spoligotype of Cluster L (5 isolates) matched that of the T2 subclade. The spoligotypes of three clusters (C, 26 isolates; P, 3 isolates; S, 3 isolates) also shared the key features of the ancestral T cluster.

Table 1: Drug resistances of the *M. tuberculosis* isolates

	Isolates[a]	
Drug Susceptibility	number	%
Pan-susceptible	281	77
Resistant to one or more drugs	86	23
Resistant to one drug only	55	15
Isoniazid (INH)	12	3
Rifampicin (RIF)	15	4
Ethambutol (EMB)	4	1
Streptomycin (SM)	24	6
Resistant to two drugs only	13	3.5
INH+RIF	7	2
RIF+SM	1	0.2
RIF+EMB	3	0.8
SM+EMB	1	0.2
INH+SM	1	0.2
Resistant to three drugs only	7	2
INH+RIF+EMB	1	0.2
INH+RIF+SM	3	0.8
INH+EMB+SM	1	0.2
RIF+EMB+SM	2	0.5
Resistant to all four drugs	5	1
INH+RIF+EMB+SM	5	1
Isolates with no results available	14	

[a]Resistance information was available for 367 of the 381 isolates in this study. The 14 isolates without results were not included in percentage calculation

A second group of isolates (70 isolates in 4 clusters) displayed spoligotypes related to the spoligotypes of the LAM (Latino-American and Mediterranean) family (absence of spacers at positions 21-24 and 33-36; reviewed in[20]). Cluster B (54 isolates) displayed the LAM 7 spoligotype and Cluster K (5 isolates) displayed the LAM 9 spoligotype. The spoligotypes of Clusters G (8 isolates) and R (3 isolates) also shared the key features of the LAM family.

A third group of isolates (33 isolates in 3 clusters) displayed spoligotypes related to the spoligotypes of the Haarlem family of isolates (absence of spacers 31 and 33-36 and presence of spacer 32). Cluster D (17 isolates) displayed the Haarlem 3 spoligotype and Cluster E (12 isolates) displayed the Haarlem 1 spoligotype. The spoligotype of Cluster M (4 isolates) also shared the key features of the Haarlem family.

Seven strains (cluster I) displayed the spoligotyping pattern (absence of spacers 1-34, presence of 35-43) characteristic of the Beijing family.

IS6110 genotyping: IS6110-genotypes were obtained for 368 isolates (Table 3): 232 isolates (63%) displayed unique fingerprint patterns and 136 isolates (37%) fell into one of 35 clusters (2-34 isolates per pattern).

Table 2: Clustering of isolates by spoligotype

Cluster designation	No. of Clusters	Cluster size	% of isolates[a]	Spoligotype pattern	Spoligotype Family[b]
A	1	77	20.6	777777777760771	T 1
B	1	54	14.4	777777404760771	LAM 7[c]
C	1	26	6.9	037637777760771	T
D	1	17	4.5	777777777720771	Haarlem 3
E	1	12	3.2	777777774020771	Haarlem 1
F	1	8	2.1	377777777760771	T
G	1	8	2.1	000000007760771	LAM
H	1	7	1.8	677777777760771	T
I	1	7	1.8	000000000003771	Beijing
J	1	6	1.6	777777770000771	
K	1	5	1.3	777777607760771	LAM 9
L	1	5	1.3	777777777760731	T 2
M	1	4	1.1	777737777720771	Haarlem
N	1	4	1.1	776377777760771	S
O	1	4	1.1	777777737760771	T
P	1	3	0.8	777777776360771	T
Q	1	3	0.8	777737777760771	T 3
R	1	3	0.8	776017404760771	LAM
S	1	3	0.8	777777777740771	T
T	1	3	0.8	777777677760771	T
a-t	14	2	7.5		
	87	1	23		
	7	-	-	No Data[d]	

[a]The % of isolates was calculated as a percentage of the 374 isolates with available spoligotype information
[b]Spoligotype families are defined as described in Filliol *et al*.[20]
[c]LAM: Latino-American and Mediterranean family
[d]Seven isolates could not be typed by spoligotyping. Five of them have RFLP patterns containing more than 6 bands

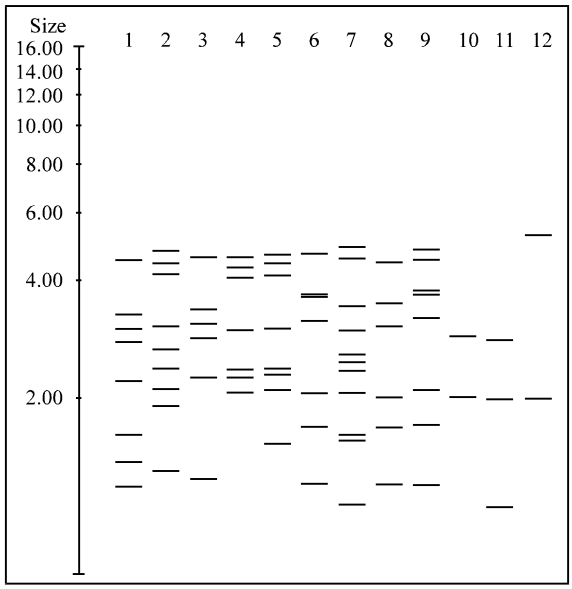

Fig. 1: IS6110-fingerprint patterns for each cluster with 3 or more isolates

IS6110-fingerprints of the clusters with 3 or more strains are shown in Fig. 1.

The numbers of IS6110 elements per strain consistently fall into a bimodal distribution and isolates are separated into two groups: low-copy-number isolates with six or fewer copies and high-copy-number isolates with more than six copies[18,21]. A similar

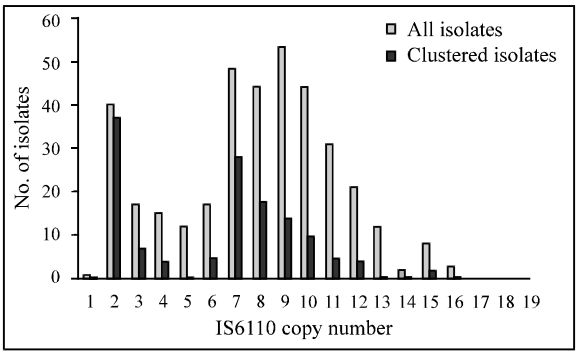

Fig. 2: Distribution of all isolates and clustered isolates by number of copies of IS6110

bimodal distribution was seen for the strains isolated in Turkey (Fig. 2). The greatest number of isolates had 7-12 IS6610 copies. There are also a large number of strains (40 isolates) with just 2 copies of IS6110. Within the copy number groups, the greatest amount of clustering of matching fingerprint patterns was seen in isolates with 2, 7 or 8 IS6110 copies.

Of the 102 low copy number isolates in this study, 44 displayed unique IS6110 genotypes and 58 fell into 9 clusters (2-34 strains per cluster). For low-copy-number isolates, IS6110 genotyping often overestimates the degree of clustering (strain relatedness) and a second typing method, such as spoligotyping, is combined with IS6110 genotyping to provide a more reliable measure of strain relatedness. By IS6110 genotyping, the 40 2-copy isolates included three

Table 3: Isolates clustered by IS6110 genotype

Pattern Number	No. of Clusters	Cluster size	Spoligotyping
239	1	34	B(24), R(3), A(1), D(1), H(1), 4 unique
60	1	14	A(3), C(2), F(5), T(1), 3 unique
150	1	7	C(5), b(2)
152	1	7	C(6), 1 unique
6	1	4	G(4)
43	1	4	J(3), n(1)
63	1	4	A(2), F(1), ND[a](1)
153	1	4	C(4)
16	1	3	G(2), 1 unique
90	1	3	O(3)
240	1	3	B(2), 1 unique
246	1	3	B(2), 1 unique
	23	2	[b]Data not shown
	232	1	Unique

[a]ND: Not done
[b]Spoligotyping data are not shown for the 23 clusters with only two isolates. Five of these clusters had isolates with different spoligotypes

Table 4: The degree of discrimination obtained with two typing methods individually and combined

Method	No. of different patterns	No. of unique isolates	No. of clustered isolates	No. of clusters
IS6110 RFLP alone	267(70%)	232(63%)	136(37%)	35
Spoligotyping alone	120(31 %)	87(23%)	287(77%)	34
IS6110+Spoligotyping	307(81%)	273(72%)	108(28%)	34

Spoligotyping data were obtained for 374 isolates and IS6110 genotyping data were obtained for 368 isolates. In total, 381 isolates were typed. Because isolates without typing information were not included in the percentage calculations, percentages are calculated based on 368 (IS6110-typing), 374 (spoligotyping alone), or 381 (both methods) isolates

strains with unique fingerprints and two clusters of 34 and 3 members. Spoligotyping of members of the 34-isolate IS6110 cluster differentiated these strains into three clusters of matching genotypes(24, 3, 2, isolates) and 5 unique genotypes (Table 3).

Secondary typing using spoligotyping can also improve strain discrimination among isolates with a high copy number of IS6110 elements. The combination of IS6110 fingerprinting and spoligotyping for the 381 isolates revealed that 273 (72%) isolates had unique genotypes and 108 (28%) isolates fell into one of 34 genotypes (2-24 strains per genotype) (Table 4).

The isolates clustered by genotype were in general not clustered by geographic region. Members of 16 of the 34 clusters were recovered from more than one of the regions. For example, members of the cluster of 24 isolates were found in four of the six regions sampled: Trabzon (9 isolates), Ankara (8 isolates), Antalya (6 isolates) and Van (1 isolate). None of the four 3-isolate clusters were found in only one city and about half (14 of 24) of the 2-isolate clusters were found only in one city.

DISCUSSION

Molecular characterization of *M. tuberculosis* strains has been used for more than a decade to study the epidemiology of tuberculosis and has proven to be a useful tool in many public health settings[22]. For example, the clustering of IS6110 fingerprints has been used to estimate the amount of transmission occurring in a population[23]. In the collection of 368 strains from Turkey that were genotyped using IS6110, the fact that 37% of isolates were grouped into 35 clusters of 2-34 isolates per cluster suggests that about a third of new cases were due to recent transmission. To better define the clusters of related strains, especially for the strains with six or fewer copies of IS6110, spoligotyping was used as a second genotyping method. Combining the two genotyping methods reduced the fraction of clustered strains and of suggested recent transmission to 28%. The fraction of strains recovered in the six cities in Turkey that fall into clusters is somewhat more than found in Switzerland (17%)[24] or Norway (20%)[25] and less than that found in studies in San Francisco (40%)[23], New York City (40%)[26], the United States as a whole (48%)[27], the Netherlands (50%)[1] and Denmark (50%)[28]. The percent of clustering determined in this study using IS6110 genotyping and spoligotyping is similar to recently reported studies (34%, 50%) that use a combination of IS6110 genotyping and pTBN12 fingerprinting to characterize clustering (29, 30)

Most clusters contained only 2 (24 clusters), 3 (4 clusters), 4 (2 clusters), 5 (2 clusters), or 6 (1 cluster) isolates. There was one large cluster (24 isolates) with a two-band IS6110-RFLP pattern (FP#239) and the LAM 7 clade spoligotype. A similar 2-band pattern also accounted for large clusters in the previously published studies (29, 30). These isolates were recovered in four cities (Trabzon, 9; Ankara, 8; Antalya, 6; Van, 1).

However, IS6110 insertion sites and hence RFLP patterns, are highly conserved among strains with only one to four IS6110 elements[31] and in these strains, spoligotyping improves confidence in the clustering, but may still not identify true clusters[12,13,31]. For example, in the 24-isolate cluster, the strains could be broken into 2 clusters of 8 strains and 4 unique strains based on drug susceptibility patterns. Overall, these limitations in strain differentiation may lead to an overestimation of the amount of clustering in the population.

Previous analyses of the SPoIDB3 database, which contains 11,708 spoligotype patterns from as many clinical isolates originating from more than 90 countries, identified several large families of *M. tuberculosis* strains including the Beijing, T, Haarlem, LAM and East African Indian families (reviewed in[20]). Members of several of these families were found in Turkey: about 37% of isolates were members of the T family, 20% were members of the LAM family, 8% were members of the Haarlem family and 2% were members of the Beijing family. This distribution is similar to what has been reported for European countries[20] although with a somewhat higher proportion of T and LAM family isolates than in Northern European countries. These data also suggest that there is no dominant *M. tuberculosis* clade in Turkey such as has been observed in Asia and former USSR republics, where more than half of all isolates are members of the Beijing family.

Most *M. tuberculosis* isolates recovered in Turkey are susceptible to all first-line anti-tuberculosis drugs. In our study, about 23% of isolates were resistant to one or more drugs and about 4% of isolates were multidrug resistant (i.e., resistant to at least isoniazid and rifampin). This amount of drug resistance is consistent with published surveys[3-6,29] of drug-resistant tuberculosis in Turkey which reported that about one-third of all cases (20-26% of new cases and 40-50% of previously treated cases) were resistant to at least one anti-tuberculosis drug and 5-10% of all cases were multidrug resistant. Patient information was not collected in our study and future studies will be needed to collect such information to further define the pattern of drug-resistant tuberculosis in the areas surveyed.

Overall, this study highlights the diversity of *M. tuberculosis* strains in Turkey. The observation of 307 different genotypes in the 381 isolates suggests that the genetic diversity of *M. tuberculosis* strains in Turkey is high with many different strains circulating in Turkey. Also, because many of the clustered isolates were recovered from more than one region, there does not appear to be a geographically restricted distribution of these strains. Further studies are needed to follow the spectrum of circulating strains to evaluate ongoing transmission and to evaluate the effectiveness of tuberculosis control strategies

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