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Determination of genotypic varieties and genotyping of multiple drug-resistant tuberculosis by the RFLP and spoligotyping methods

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Background/aim: The purpose of the present study was to determine the distribution and epidemiological features of mycobacteria with molecular methods.

Materials and methods: Fifty-five culture-positive samples were analyzed by polymerase chain reaction-restriction enzyme length polymorphism (PCR-RFLP) at species level, and their molecular typing was performed with spoligotyping. The IS6110 region and the locus of gene coding for Hsp65 were amplified. RFLP profiles were obtained by cutting the Hsp65 region with the *Hae* III and *BstE* II (*Eco*91I) enzymes. Spoligotyping was carried out by commercial kit. The H37Rv strain was used as the control.

Results: All samples showed the same cutting pattern with the H37Rv strain. The RFLP profiles of 9 strains identified as "mycobacteria other than tuberculosis" were compatible with the *M. tuberculosis* complex. Spoligotyping of 55 isolates detected 13 different genetic profiles. The Beijing genotype was not detected. One isolate was described as an orphan strain according to the SpolDB4 database. The most frequently detected family was T1 with 32 strains (64%), followed by 9 isolates (18%) belonging to the LAM7 TUR family.

Conclusion: PCR-RFLP is a specific, rapid, and effective method in routine diagnosis of mycobacteria. Spoligotyping is an ideal method in the determination of genotypic varieties of mycobacteria.

Key words: *Mycobacterium tuberculosis*, RFLP, spoligotyping

1. Introduction

Although tuberculosis is one of the oldest diseases in history, it is still a serious public health problem. Tuberculosis ranks first (25%) among the causes of preventable mortality worldwide (1). Today there are more than 100 species of mycobacteria other than tuberculosis (MOTT) in the genus *Mycobacterium* besides the members of the *Mycobacterium tuberculosis* complex (*M. tuberculosis*, *M. bovis*, *M. microti*, and *M. africanum*). Identification of the mycobacteria produced with various methods is an essential stage in the diagnosis of infection. The rapid and accurate identification of mycobacteria will allow both taking measures against transmission and determining appropriate treatment alternatives. Depending on the increase in immune-suppressed cases and the developments in diagnostic techniques, an increasing number, and more and more species, of MOTT bacteria are isolated as disease agents every passing day (2). Since conventional methods take a long time, molecular methods were put into use as supplementary methods for quick diagnosis (3).

The phylogenetic features of *M. tuberculosis* and its dynamics of spreading became more comprehensible upon the development of molecular techniques. The recurrence of tuberculosis, whether a secondary infection is a reinfection or reactivation, and the simultaneous presence of infections by two or more strains in a patient can be revealed through molecular typing. In this way, the efficiency of tuberculosis control and treatment protocols can be investigated. Great benefits are obtained in the investigation of epidemics and the identification of intralaboratory cross-transmission. With these methods, the transmission within a specific geographical area can be analyzed and the exogenous superinfections in patients with either a complete or an inadequate immune system can be determined. It can be revealed that there are infections by different strains in relapsing patients. The origin of multiple drug-resistant (MDR) strains and their ways of spreading can be shown with the methods of molecular typing. The typing of *M. tuberculosis* strains may provide the answers to important epidemiological questions such

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as the origin of an infection in a community or a family, its spreading, and early detection of those strains that have acquired resistance. Molecular epidemiology helps to monitor the distributions in the community of those isolates that are considered more virulent (4–8).

To specify the differences among the slowly reproducing *Mycobacterium* species, Plikaytis et al. (9) first developed a method with PCR and RFLP analyses in 1992. Later, Telenti et al. (10) investigated the genes that encoded 65-kDA heat-shock proteins by using PCR and restriction enzyme analyses. The RFLP method is a technique that can determine the single base mutations or the DNA fragments in different sizes. It is based on the examination of the DNA by dividing it into fragments in different sizes with restriction endonucleases (RE). Spoligotyping (spacer oligonucleotide typing) is a method of typing that depends on the DNA polymorphism occurring in a special chromosomal locus called 'direct repeat (DR)', which is only found in *M. tuberculosis* complex bacteria (11). The method is based on the amplification of the DNA sequence of the highly polymorphic DR locus in the chromosomes of the *M. tuberculosis* complex strains. This locus was first identified by Hermans et al. (11), who sequenced this region in the *M. bovis* BCG strain, which is used in vaccination against tuberculosis worldwide. As clinical isolates vary according to the presence or absence of these spacer sequences, mycobacterial strains can be differentiated by these different hybridization patterns or spoligotypes (11).

In our study, we aimed to investigate the diversity of genotypes and to type the strains in MDR tuberculosis induced by *M. tuberculosis* strains with the RFLP and spoligotyping methods.

2. Materials and methods

2.1. Culture-antibiotic sensitivity testing

To conduct this research, approval was obtained (Resolution No. 03-2008/38) from the Ethics Committee at Gaziantep University. Some 55 samples, sent from various clinics of the Medical Faculty Hospital of Gaziantep University, were investigated between January 2005 and January 2007 in the present study. Identification of species was performed by PCR-RFLP analysis, and the molecular types were investigated by spoligotyping in the samples in which acid-resistant bacillus (ARB) and growth in culture were detected with the Ehrlich–Ziehl–Neelsen (EZN) staining method. In this retrospective study, the samples tested consisted of stock bacterial cultures and did not directly involve any human subjects.

Of the samples, 47 (85.4%) were obtained from patients followed up in the clinic of thoracic diseases, 3 (5.5%) from patients followed up in the clinic of thoracic surgery, 3 (5.5%) from patients followed up in the clinic of pediatrics, and 2 (3.6%) from patients followed up in the clinic of general surgery; 16 of the patients (29.1%) were female and 39 (70.9%) were male. A sample of each patient was included in the research. The 55 samples sent to our laboratory consisted of 48 (87.3%) sputum samples, 3 (5.5%) bronchoalveolar lavage samples, 1 (1.8%) gastric lavage sample, 1 (1.8%) abscess material, 1 (1.8%) pleural fluid sample, and 1 (1.8%) lymphatic gland biopsy sample. After homogenization and decontamination with 3%–4% NaOH and N-acetyl-L-cysteine, the samples were cultured into the BACTEC 12B liquid medium. The ARBs were investigated with EZN staining. Differentiation between *M. tuberculosis* complex and nontuberculous mycobacteria was achieved by selective inhibition of the *M. tuberculosis* isolates in the presence of 5 mL/mL p-nitro-a-acetylamino-b-hydroxypropiophenone (NAP) according to the BACTEC manual. Drug susceptibility testing against rifampicin (R), isoniazid (I), streptomycin (S), and ethambutol (E) was performed by using the BACTEC 460-TB according to the manufacturer's instructions.

The samples included in the research were particularly selected from patients with MDR tuberculosis. *Mycobacterium tuberculosis* H37Rv (ATCC 27294) was used as the control strain.

2.2. Isolation of the *Mycobacterium* DNA

The Chelex method was used for the extraction of *Mycobacterium* DNA (12). During this procedure, the aim was to protect the nucleic acids against disintegration, to remove the amplification inhibitors from the medium, and to purify and concentrate the target nucleic acid. The samples were preserved at –70 °C in order to be used for PCR.

2.3. Amplification of the IS6110 region specific to the *M. tuberculosis* complex by PCR

Primers T4 and T5 of the IS6110 region specific to the *M. tuberculosis* complex were used in the study (T4: 5'-CCT GCG AGC GTA GGC GTC GG -3', T5: 5'-CTC GTC CAG CGC CGC TTC GG -3'). To amplify the DNA region, a PCR mix, amounting to 45 µL in total (distilled water: 30.75 µL; PCR buffer (10X): 5 µL; $MgCl_2$ (25 mM): 3 µL; dNTP (10 mM): 1 µL; primer 1 (T4, 10 pmol/µL): 2.5 µL; primer 2 (T5, 10 pmol/µL): 2.5 µL; and Taq polymerase (5 U/µL): 0.25 µL) was prepared in a 1.5 mL Eppendorf tube on ice; 5 µL of *Mycobacterium* DNA was added to it, and the mixture placed into the thermal cycler. Following the application of PCR at 94 °C for 5 min in a cycle in the thermal cycler, they were kept at 94 °C for 1 min, at 60 °C for 1 min, and at 72 °C for 2 min in 35 cycles. Later, they were kept at 72 °C for 8 min and the PCR products were obtained. The samples were run in 1.5% agarose gel at 125 V for 20 min. They were examined under a UV transilluminator. The samples that had the expected bands were considered positive.

2.4. Amplification of the Hsp 65 region specific to all *Mycobacteria* by PCR

To detect all mycobacteria, a classical PCR was performed using Primers TB11: 5'-ACC AAC GAT GGT GTG TCC AT-3' and TB12: 5'-CTT GTC GAA CCG CAT ACC CT-3' belonging to the Hsp 65 region. A PCR mix containing 30.75 µL of distilled water, 5 µL of PCR buffer (10X), 3 µL of $MgCl_2$ (25 mM), 1 µL of dNTP (10 mM), 2.5 µL of primer 1 (TB11, 10 pmol/µL), 2.5 µL of primer 2 (TB12, 10 pmol/µL), and 0.25 µL of Taq polymerase (5 U/µL) was prepared, and 5 µL of the sample's DNA was added to it. Following the application of a cycle at 94 °C for 5 min in the thermal cycler, 35 cycles were applied at 94 °C for 1 min, at 55 °C for 1 min, and at 72 °C for 2 min. The PCR was completed with a cycle at 72 °C for 8 min. Bands were obtained by running the products in 1.5% agarose gel. The samples that had the expected bands were considered positive.

To determine the *Mycobacterium* species by cutting the PCR product of the 65-kDa heat-shock protein region specific to all *Mycobacteria* with the restriction enzymes, the procedure described by Taylor et al. was applied (13,14). The sequence amplified by the primers of the HSP 65 region was investigated in the agarose gel. By cutting 11 µL of each sample observed to have the appropriate band (at the expected location and in adequate concentration) with *Hae*III (*Bsu*RI) (distilled water: 2.0 µL; buffer R + : 1.5 µL; *Hae*III: 0.5 µL (10 units/µL, Fermentas); PCR product: 11.0 µL; and total: 15.0 µL) and *Bst*EII (*Eco*91I) (distilled water: 2.0 µL; buffer O + : 1.5 µL; *Bst*EII: 0.5 µL (10 units/µL, Fermentas); PCR product: 11.0 µL; and total: 15.0 µL) enzymes, the *Mycobacterium* species were identified. The products cut by the restriction enzymes were run in 3% NuSieve GTG agarose (FMC BioProduct). DNA Molecular Weight Marker VIII (19–1114 bp) (Roche) was used as a marker. The *Mycobacterium* species were identified by evaluating the base sizes of the bands that appeared upon the cutting of the PCR product according to the table provided by Taylor and Telenti (10,14).

2.5. Spoligotyping

For the procedure of spoligotyping, studies were conducted according to the instructions by the manufacturing firm (Isogen Biosolutions Spoligotyping kit, Ocimum Biosolutions Company, the Netherlands). Primers DRa and DRb were used for amplification. DRa: 5'-ggT TTT ggg TCT gAC gAC-3' (marked with biotin at the 5'-end), DRb: 5'-CCg AgA ggg gAC ggA AAC-3'. The PCR master mix was prepared as follows: dH_2O: 8.5 µL; DMSO: 1 µL; master mix: 12.5 µL; DRa: 0.25 µL; DRb: 0.25 µL; and DNA: 2.5 µL. The evaluation was made according to the positivity of the spacers. Spoligotyping is expressed as a word that comprises 43 letters. This alphabet contains 3 letters: positive, negative, and indefinite. The spoligotype patterns were encoded in triplet codes, and the types were identified (Figure 1).

The obtained results were compared with the international spoligotyping database, and the spoligotypes were detected (15).

The distribution of spacers in the DR region is also used in the differentiation of species (Figure 2).

2.6. Statistical analyses

SPSS for Windows, version 11.5, was used and $P < 0.05$ was considered significant.

3. Results

The 55 samples included in the study were obtained from various clinics (Table 1). Attention was paid to the fact that the samples were MDR tuberculosis agents. In the culture and identification carried out previously, 46 samples had been evaluated as *M. tuberculosis* complex and 9 samples as MOTT.

3.1. Drug resistance

The susceptibility of MOTT bacteria to antituberculosis drugs was not studied. Of the strains in the 46 samples, 18 (39%) were susceptible to all antituberculosis drugs, whereas 11 (24%) were resistant to 1 drug, 11 (24%) to 2 drugs, 3 (6.5%) to 3 drugs (isoniazid, rifampicin, and ethambutol), and 3 (6.5%) agents to 4 drugs. Resistance patterns of the 28 drug-resistant isolates are shown in Table 2.

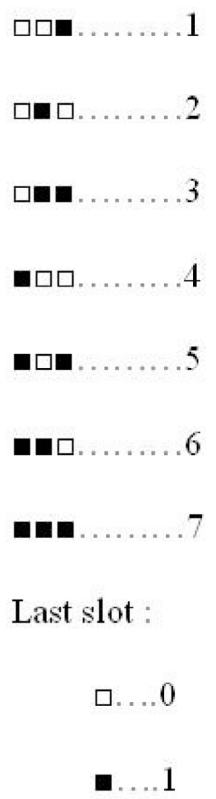

Figure 1. Interpretation of the spoligotyping images (43 slots).

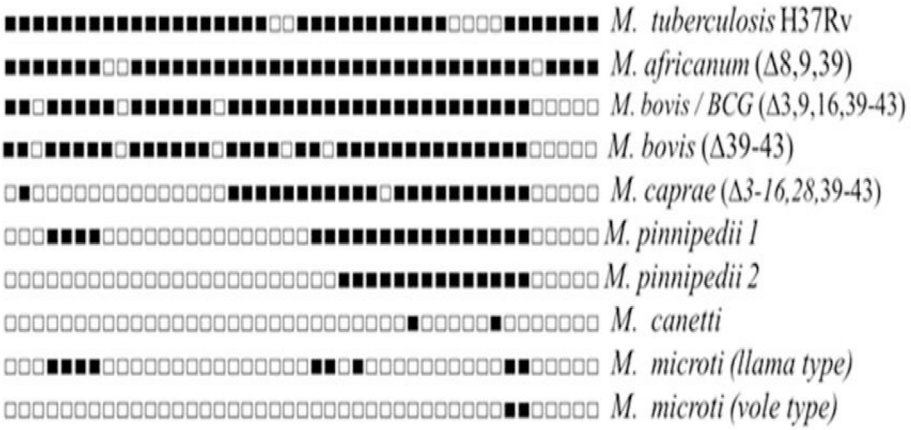

Figure 2. Differentiation of the species with spoligotyping (16).

Table 1. Distribution of the samples according to the departments from which they were obtained.

	Sputum n (%)	Bronchoalveolar lavage (BAL) n (%)	Gastric lavage (GL) n (%)	Abscess material n (%)	Pleural fluid n (%)	Lymphatic gland n (%)
Thoracic diseases	41 (85.4)	2 (66.7)	0 (0.0)	1 (100)	1 (100)	0 (0.0)
Thoracic surgery	2 (4.2)	1 (33.3)	0 (0.0)	0 (0.0)	0 (0.0)	1 (100)
Pediatrics	2 (4.2)	0 (0.0)	1(100)	0 (0.0)	0 (0.0)	0 (0.0)
Other	3 (6.2)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Total	48 (100)	3 (100)	1 (100)	1 (100)	1 (100)	1 (100)

Table 2. Resistance patterns of the drug-resistant isolates.

Drugs	Number of the resistant isolates n (%)
Streptomycin (S)	0 (0.0)
Isoniazid (I)	6 (21.4)
Rifampicin (R)	3 (10.7)
Ethambutol (E)	2 (7.1)
S + I	4 (14.3)
I + R	5 (17.9)
I + E	1 (3.6)
R + E	1 (3.6)
I + R + E	3 (10.7)
S + I + R + E	3 (10.7)
Total	28 (100)

3.2. PCR-RFLP

Following the isolation of all samples with the Chelex method, the IS6110 DNA region specific to the *M. tuberculosis* complex was multiplied with PCR using Primers T4 and T5 (Figure 3). All of the 55 samples formed a band. RFLP was performed following the multiplication of the Hsp 65 region specific to all *Mycobacteria* with PCR using Primers TB11 and TB12. H37Rv was used as the control strain. Bands were detected by running the cut products in 3% Nu-sieve agar. When the bands were evaluated according to the molecular weight marker, all of the 55 samples yielded the same RFLP images, and the results were identical with those of the H37Rv strain (Figure 4). The RFLP profiles of the 9 strains found to be MOTT with the NAP test were also detected to be identical. The samples cut by the BstEII and HaeIII enzymes were evaluated in the algorithm (10,14) according to the band sizes. All strains were congruent with the *M. tuberculosis* complex.

3.3. Spoligotyping

Some 13 different genetic profiles were obtained in 55 *M. tuberculosis* isolates by spoligotyping (Table 3). While 5 isolates formed a unique pattern, the remaining 50 isolates

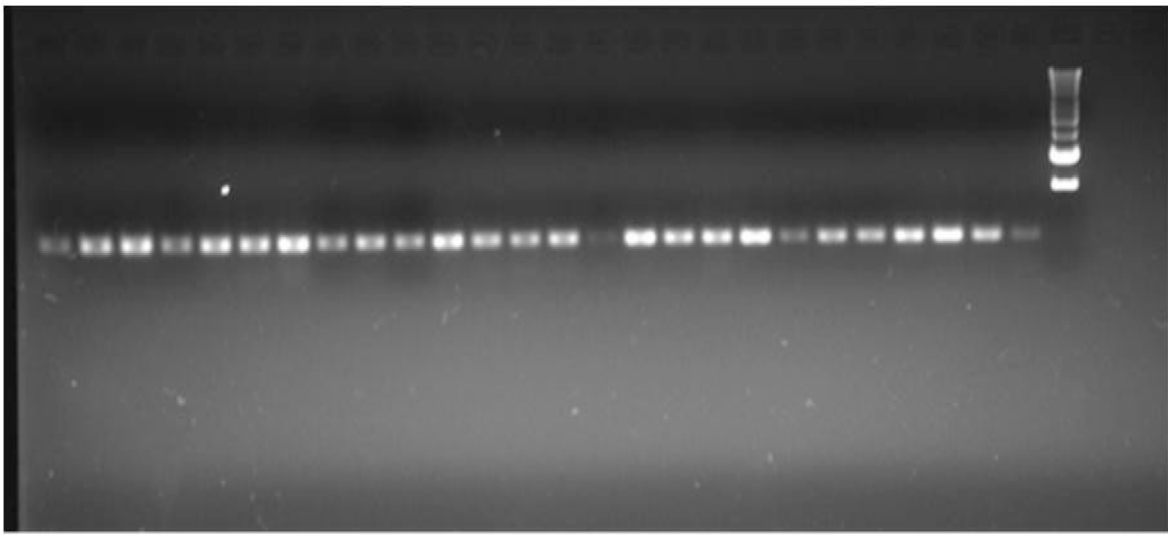

Figure 3. Images of the bands belonging to the 26 samples for the IS6110 DNA region specific to the *M. tuberculosis* complex in agarose gel.

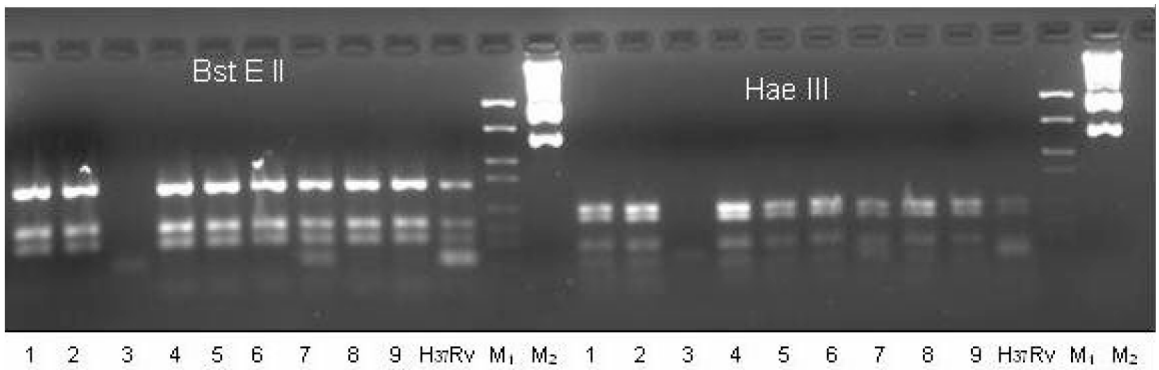

Figure 4. The RFLP profiles of the samples.

(90.9%) formed 8 clusters, with each of them containing 2 to 23 isolates. The Beijing genotype, determined to be associated with multiple drug resistance, was not encountered. Resistance of a total of 46 *M. tuberculosis* complex isolates to antituberculosis drugs and their familial characteristics are shown in Table 4. All of the 3 *M. tuberculosis* complex isolates resistant to all of the 4 antituberculosis drugs investigated in our study and 2 of the isolates resistant to 3 drugs were detected to be in the T1 family. No significant difference was found between the resistance detected in the *M. tuberculosis* complex isolates and the types of families (P = 0.983).

4. Discussion

Mycobacteria can lead to epidemics today as they have done throughout history and appear as new epidemiological types or drug-resistant microorganisms. According to calculations by the World Health Organization, the number of newly infected people will reach 1 billion in 2020 unless efficient control measures are developed (16,17). According to data from the Turkish Public Health Institution of the Ministry of Health, about 13,000 to 14,000 new tuberculosis patients are recorded in Turkey annually. In 2012, the total number of recorded tuberculosis cases was 14,691 (18).

Unlike many other diseases affecting developing countries, tuberculosis is a disease that can be controlled and treated by taking efficient measures. In order to take efficient control measures to prevent tuberculosis from spreading, knowledge of the clonal relationship between the isolates is required. The phylogenetic features of *M. tuberculosis* and its dynamics of spreading became more comprehensible with the development of molecular techniques. The recurrence of tuberculosis, whether the secondary infection is a reinfection or reactivation, and the simultaneous presence of infections by two or more strains

Table 3. The spoligotyping characteristics of the strains.

No.	Spoligotyping (Octal encoding)	Number of strains (n = 55)	%	Family
1	777777777760771	22	40	T1
2	777777737760771	5	9	T1
3	777777777560771	2	3.6	T1
4	777777677760771	1	1.8	T1
5	377777777760771	1	1.8	T1
6	777777777777771	1	1.8	T1
7	777777404760771	9	16.3	LAM 7 TUR
8	777777777773771	6	10.9	MANU 1
9	777777777763771	2	3.6	MANU 2
10	777777774020771	2	3.6	H1
11	777777777773771	2	3.6	H3
12	777777607760771	1	1.8	LAM 9
13	617777774020760	1	1.8	Orphan strain

Table 4. Resistance of 46 *M. tuberculosis* complex isolates to antituberculosis drugs and their familial characteristics.

		SIRE										Total n %
		RRRR n %	RRSS n %	SRRR n %	SRRS n %	SRSR n %	SRSS n %	SSRR n %	SSRS n %	SSSR n %	SSSS n %	
Family	H1	0 0	1 2.2	0 0	0 0	0 0	0 0	0 0	0 0	0 0	1 2.2	2 4.3
	H3	0 0	1 2.2	0 0	0 0	0 0	0 0	0 0	0 0	0 0	1 2.2	2 4.3
	LAM7 TUR	0 0	1 2.2	0 0	3 6.5	1 2.2	0 0	0 0	0 0	0 0	4 8.7	9 19.6
	LAM9	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	1 2.2	1 2.2
	MANU1	0 0	0 0	1 2.2	0 0	0 0	3 6.5	0 0	0 0	0 0	2 4.3	6 13
	MANU2	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	1 2.2	1 2.2
	T1	3 6.5	1 2.2	2 4.3	2 4.3	0 0	3 6.5	1 2.2	3 6.5	2 4.3	7 15.2	24 52.2
	Orphan strain	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	1 2.2	1 2.2
Total		3 6.5	4 8.7	3 6.5	5 10.9	1 2.2	6 13.0	1 2.2	3 6.5	2 4.3	18 39.1	46 100

R: resistant, S: susceptible.

used in the differentiation of the MOTT bacteria. However, a mixed culture composed of the TB complex and the MOTT bacillus can only show the presence of the MOTT bacillus by causing an increase in the growth index (GI). Furthermore, an erroneous increase in the GI can be seen as a result of use of the substrate by the contaminant in the event of contamination by bacteria other than mycobacteria. In some studies it is stated that the quantity of the inoculum is also essential (19,20). Thus, it is recommended to verify the NAP test with nucleic acid probes, chromatographic methods, or conventional methods (1).

The Hsp 65 gene is also present in mycobacteria other than the *M. tuberculosis* complex and displays polymorphism among the mycobacterium species. The identification of mycobacteria by investigating the Hsp65 gene with the RFLP method was first developed by Telenti et al. (10) and this method has been used in applications since 1993. Some 33 PCR-based restriction enzyme pattern (PRA) samples were identified as original in the study performed; 19 of them corresponded to single species, while the remaining 14 of them were associated with 5 species (*M. flavescens*, 2 types; *M. chelonae*, 2 subspecies; *M. kansasii*, 2 types; *M. gordonae*, 5 types; and *M. fortuitum*, 3 subspecies) (10). Taylor et al. (14) added 5 more PRA samples to the algorithm by Telenti (an additional species and 4 new subtypes of the previously identified species). Later Devallois et al. (21) further added 11 new PRA samples: 5 additional species and 6 subtypes of the previously identified species. In our study, a restriction enzyme analysis was conducted by multiplying the 65-kDA heat-shock protein region with 439 base pairs belonging to the 55 mycobacterium isolates produced in the BACTEC tubes by the PCR, and BstEII and HaeIII were used as enzymes. The isolates were run in 3% agarose gel (NuSieve GTG agar), and the obtained band patterns were evaluated. All patterns were found identical with H37Rv (*M. tuberculosis* complex). In their studies in the agarose gel, Brunello et al. (22) state that problems of differentiation are experienced in the images of bands below 100 bp and that there may be deviations of about 10 bp. Therefore, it is expressed that comparative studies with 10% PAGE will be appropriate. The evaluation of bands in the agarose gel, a method first applied by Telenti et al. (10), was later used by many researchers (14,21,23). Detection of the real dimensions of the obtained base fragments is rather difficult with the naked eye when devices (QIAxcel, Qiagen) or computer programs (e.g., Pharmacia Biotech ImageMaster VDS) developed to this end are unavailable. The results are obtained by evaluating the recorded data with computer programs, and safe reading is possible from 6 bases to 1000 bases by means of the devices for DNA sequence analysis (24). In laboratories where these devices and programs are unavailable, the result can be obtained by using and comparing the standard strains besides the DNA markers when determining the size of fragments. In our study, the fragments obtained by cutting with BstEII were evaluated as about 240, 120, and 80 (±10) base pairs, and the fragments obtained with the HaeIII enzyme were evaluated as 160, 140, and 70 (±10) base pairs. In the table created by Telenti et al. (10), these values show the *M. tuberculosis* complex group (10). The interpretation of the octal values obtained by spoligotyping in our study also confirms that the isolates belong to the *M. tuberculosis* complex group. In their study, Brunello et al. (22) investigated the Hsp65 gene regions of 54 *Mycobacterium* species with the RFLP method and identified the *Mycobacterium* species by using 3% agarose and 10% PAGE in order to determine the restriction fragments. They stated in their study that the real sizes of the fragments they had obtained with the PAGE-based method provided the computation more precisely than the agarose gel electrophoresis and that it was better than the agarose gel electrophoresis in the understanding of species. In their study on 47 *M. tuberculosis* complex isolates and 36 MOTT, Bahrmand et al. (25) expressed that species could be differentiated more easily with this method and that it might be employed in the rapid diagnosis of *Mycobacterium* species in the clinical laboratory. On the other hand, in a study by Varma-Basil et al. (26), it was stated that *M. tuberculosis* was identified in the Hsp65 region in 84.5% of the ARB-positive samples and in 11% of the ARB-negative samples among the direct sputum samples of 226 patients with the RFLP method. The researchers expressed that when compared with preparations stained with the EZN staining method, this method was more successful than all conventional methods in the rapid direct detection of *M. tuberculosis* from the samples despite its low susceptibility (76.6%, 149/167) (26).

The method of spoligotyping is based on the DNA sequence amplification of the highly polymorphic DR locus in the chromosomes of *M. tuberculosis* complex strains, and 43 clinical samples can be studied in one go (6,27). In our study, 13 different genetic profiles were obtained in 55 *M. tuberculosis* isolates with spoligotyping. While 5 isolates formed a unique pattern, the remaining 50 (90.9%) isolates formed 8 clusters, with each of them containing 2 to 23 isolates. It was determined that one of the isolates (1.8%) was an orphan strain that was not congruent with any clusters known as a result of the comparison with the strains in the SpolDB4 database. It was seen that the most prevalent family among the strains included in the study was the T1 family with 32 isolates (64%), followed by the LAM7 TUR family with 9 isolates (18%). In a study performed for a dissertation, the 450 *M. tuberculosis* strains isolated from Malatya and the Marmara, Mediterranean,

and Aegean regions were typed with the *IS6110* RFLP and spoligotyping methods, and 47 clusters (85.3%), including 384 strains and 66 unique (14.7%) profiles, were obtained with spoligotyping (28). In the same study, 49 (10.9%) orphan strains that did not match any strain were detected and it was stated that the T-superfamily and families LAM and Haarlem were the most prevalent families (28). In addition, Otkun et al. (29) found 41 different patterns (44.5%) in the 92 isoniazid- and rifampicin-resistant *M. tuberculosis* isolates they collected from seven regions of Turkey, and stated that the patterns of 3 spoligotyping families (18.5%, T; 16.3%, LAM; and 5.4%, Haarlem) out of them represented 40.2% of the isolates. They expressed that they thought the spoligotyping patterns of the isolates resembled the distribution of European origins, that MDR isolates originated from different sources, and that drug resistance developed independently in different patients for such reasons as treatment insufficiencies (29).

In a spoligotyping study with 71 isoniazid-resistant isolates in Poland by Augustynowicz-Kopec et al. (30), it was stated that about 80% of the isolates belonged to either the T-family or the Haarlem family. In the spoligotyping in 3319 isolates collected from various countries in the continents of Europe, America, Africa, and Asia it was indicated that 259 clusters were formed and that 540 orphan spoligotypes were obtained. It was stressed that 18% of the isolates were of the Beijing type and that the common types varied by continent (31). The culture and spoligotyping susceptibility and specificity of 350 ARB-positive samples were investigated in a study with a total of 107 samples (64 ARB-positive and 43 ARB-negative) and it was determined that the susceptibility and specificity of the spoligotyping method were 98.2% and 95.5%, respectively (32). In the same study, the susceptibility of the cultures in the liquid medium was provided as 91.4% and their specificity as 97.7% (32).

In a study performed in Bulgaria in 2008, the genotypes of 113 *M. tuberculosis* isolates were examined with the methods of spoligotyping and MIRU-VNTR; 15 spoligotyping clusters containing 2 to 29 strains and 5 unique isolates were identified; and it was stated that the largest cluster (25.7%) belonged to the T1 family and that the LAM7TUR family ranked second (5.4%) (33). Having attributed their finding of the prevalence of LAM7TUR above the world averages to the country's location as a neighbor of Turkey, the researchers highlighted the importance of migrations in the spreading of the *M. tuberculosis* genotypes (33).

In our study, it was established that 3 isolates resistant to 4 drugs (SIRE) (3/32, 9.4%) and 2 of the isolates resistant to 3 drugs (2/32, 6.3%) belonged to the T1 family. Nevertheless, no significant difference was found between the resistant strains detected in the *M. tuberculosis* complex isolates and the family types (P = 0.983). However, the Beijing genotype, determined to be associated with multiple drug resistance, was not encountered in our study. The Beijing genotype of *M. tuberculosis* has been observed to be highly prevalent in the northwestern federal region of Russia (34). According to a study conducted by Velayati et al. (35) in Iran, the major *M. tuberculosis* superfamilies were found to be African-Indian (24%), central Asian (20.8%), T clade (20.7%), Haarlem I (4.4%), and Beijing (3.2%).

There is a limited number of studies on the typing of mycobacteria with the method of spoligotyping in Turkey. It is seen in these studies that the common genotype in Turkey is the T1 family (36,37). Bulut et al. (36) obtained 15 different genotype patterns in the spoligotyping of 48 primary drug-resistant isolates they had isolated from the Kelkit Valley and failed to type 4 isolates. In that study, they stated that the most prevalent spoligotype families were TI (37.5%), S (25%), and LAM-7 TUR (8.3%) (36). Moreover, in a study performed in Adana region, it was stated that, depending on the method of spoligotyping, 443 (94.9%) of 467 isolates formed 21 clusters, 24 isolates could not be typed, and the most prevalent family was the T1 family with 239 isolates (51.9%), followed by the LAM7 TUR family with 54 isolates (11.5%) (37).

In conclusion, the PCR-RFLP analysis is a specific, quick, and effective method for the identification of species and can be used in the routine identification of mycobacteria. It is seen that the method has potential for quicker and more accurate identification than the conventional methods of identification based on the morphological and biochemical properties of mycobacterial isolates. The quick diagnosis of the disease will allow the early initiation of treatment by clinicians, and therefore a decrease in morbidity and mortality. Spoligotyping is an ideal method of determining the diversity of genotypes of mycobacteria, typing them, and conducting epidemiological studies. Thus, the obtained information will be beneficial for the accurate planning of treatments for MDR tuberculosis patients in Turkey and for the prevention of their infectivity.

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