RESEARCH ARTICLE

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Investigation of Isoniazid, Rifampicin and Second Generation Antibiotic Resistance Genes in Rifampicin-Resistant *Mycobacterium tuberculosis* Complex Strains Isolated at Düzce University Between 2004-2021

ABSTRACT

Objective: To determine the gene patterns causing antibiotic resistance in *M. tuberculosis* complex (MTBC) strains using molecular methods.

Methods: Nineteen rifampicin-resistant MTBC strains isolated between 2004 and 2021 were included. The species of these strains with MTBC genotype and the gene pattern causing rifampicin resistance with MTBDR plus genotype were analysed.

Results: Nineteen of the isolates were identified as *M. tuberculosis*/*canetti* by the MTBC genotype method. Seven of these isolates were genotypically resistant to rifampicin. One of the resistant isolates had deletion in WT8 and WT6 bands, one had deletion in WT8 band, one had deletion in WT7 band and rpoBMUT2A mutation, and four had deletion in WT8 band and rpoBMUT3 mutation. Seven of the resistant isolates were genotypically INH resistant. Five of them had katGMUT1 mutation with deletion in katGWT band and two of them had only INH AMUT3B mutation. Of the 10 multidrug-resistant MTBC isolates, nine were genotypically resistant to none of the second-generation drugs using the GenoType MTBDR sl ver 2.0 method. However, one isolate could not be evaluated with this assay.

Conclusions: The presence of MDR-TB and RR-TB is an important challenge especially in TB control, which increases the need for molecular methods. Although it still has not replaced culture, there is a need for the use and development of new molecular methods that will benefit us in TB treatment and control.

Keywords: Drug Resistance, *Mycobacterium tuberculosis* Complex, Multi-Drug Resistance Tuberculosis.

Düzce Üniversitesi'nde 2004-2021 Yılları Arasında İzole Edilen Rifampisin Dirençli *Mycobacterium tuberculosis* Kompleks Suşlarında İzoniazid, Rifampisin ve İkinci Nesil Antibiyotik Direnç Genlerinin Araştırılması

ÖZET

Amaç: *M. tuberculosis* complex (MTBC) suşlarında antibiyotik direncine neden olan gen paternlerini moleküler yöntemlerle belirlemek.

Yöntem: 2004-2021 yılları arasında izole edilen rifampisine dirençli on dokuz MTBC suşu çalışmaya dahil edilmiştir. Bu suşların MTBC genotipine sahip türleri ve MTBDRplus genotipi ile rifampisin direncine neden olan gen paterni analiz edildi.

Bulgular: İzolatların on dokuzu MTBC genotip yöntemi ile M. tuberculosis/canetti olarak tanımlanmıştır. Bunlardan yedisi genotipik olarak rifampisine dirençliydi. Dirençli izolatlardan birinde WT8 ve WT6 bantlarında delesyon, birinde WT8 bandında delesyon, birinde WT7 bandında delesyon ve rpoBMUT2A mutasyonu ve dördünde WT8 bandında delesyon ve rpoBMUT3 mutasyonu vardı. Dirençli izolatların yedisi genotipik olarak INH dirençliydi. Bunların beşinde katGWT bandında delesyon ile katGMUT1 mutasyonu ve ikisinde sadece inh AMUT3B mutasyonu. Çok ilaca dirençli 10 MTBC izolatından dokuzu GenoType MTBDR sl ver 2.0 yöntemi kullanılarak ikinci nesil ilaçların hiçbirine genotipik olarak dirençli bulunmamıştır. Bir izolat bu test ile değerlendirilememiştir.

Sonuç: ÇİD-TB ve RR-TB varlığı özellikle TB kontrolünde önemli bir zorluktur ve bu durum moleküler yöntemlere olan ihtiyacı artırmaktadır. Halen kültürün yerini almamış olsa da, TB tedavisi ve kontrolünde bize fayda sağlayacak yeni moleküler yöntemlerin kullanılmasına ve geliştirilmesine ihtiyaç vardır.

Anahtar Kelimeler: İlaç Direnci, *Mycobacterium tuberculosis* Kompleksi, Çoklu İlaca Dirençli Tüberküloz

INTRODUCTION

Tuberculosis (TB), caused by *Mycobacterium tuberculosis* complex (MTBC), is the most common infectious disease causing death with an annual mortality rate of 1.5 million deaths worldwide. Although the global incidence of TB has been on a downward trend since 2000, the emergence and spread of drug-resistant TB strains has significantly impacted efforts to control and eradicate the disease (1). The World Health Organisation has reported an estimated half a million cases of MDR-TB in recent years, of which 8.5% were MDR-TB (2). Currently, the advent of multidrug resistance (MDR) is a major burden on the global tuberculosis control program. The spread of MTB-MDR is rising throughout the world in both fresh tuberculosis cases and patients cured of MTB with the commonly used drugs (RIF and INH). RIF-resistance (≥90% cases) in MTB-infected patients have been classified as a main biomarker for drug resistance detection (3).

Early detection of MTBC isolates and accurate drug susceptibility testing (DST) are vital to prevent transmission of MDR-TB strains. Due to the long duration of DST with culture-based methods, various probe-based and sequence analysis-based molecular methods have been developed today to provide results in a short time and with high accuracy and to detect the mutation associated with resistance. Probe-based methods include GeneXpert MTB/RIF test (Cepheid, USA) using molecular probes and MTBDRplus and MTBDR sl (Hain LifeScience GmbH, Germany) using line probes (1). Sequence analysis based methods include Sanger sequencing, pyrosequencing and next generation sequencing. Sequence analysis-based methods are used to obtain the sequences of wild-type isolates or mutants, while probe-based methods are used to detect the presence of mutations (4).

The GenoType MTBDRplus test, based on PCR-based reverse hybridisation, is one of the most widely used commercial molecular tests (5). While this test detects MTBC species and RIF and INH resistance, the GenoType MTBDR sl test can detect resistance to secondary drugs as well as MTBC.

The *Mycobacterium tuberculosis* complex is a group of Mycobacteria that comprises of *M. tuberculosis, M. africanum, M. bovis, M. caprae, M. canettii, M. africanum, M. microti, M. pinnipedii* and *M. caprae*. Additionally, two novel species (*M. oris* and *M. mungi*) are also referred to as MTBC (6). GenoType MTBC test (Hain LifeScience GmbH, Germany) is a molecular method based on reverse hybridisation for typing MTBC members. It is the oldest and probably the most widely used molecular test to differentiate the causative agents of tuberculosis (7).

MATERIAL AND METHODS

This study was carried out with the approval of Düzce University Faculty of Medicine, Non-Interventional Health Research Ethics Committee with the decision dated 18.10.2021.

In our study, resistant strains selected among 854 MTBC isolates isolated from various clinics between 2004 and 2021 at the Tuberculosis Unit of the Medical Microbiology Laboratory of Düzce University Health Application and Research Centre were included.

Culture and species identification of the isolates were performed according to standard mycobacteriological procedures. Anti-TB drug susceptibilities of the isolates were performed on a BACTEC MGIT 320 (Becton Dickinson, USA) according to the standard procedure recommended by the manufacturer. Drug concentrations include streptomycin (SM) 1.0 μg/mL, INH 0.1 μg/mL, RIF 1.0 μg/mL and 5.0 μg/mL for EMB. All strains were sub-cultured in skimmed milk stock medium and stored at -20 °C until molecular studies were performed. Positive MGIT tubes with simultaneous growth were stored at +4 ºC (1,4-5).

In order to revitalise the strains before the commencement of the study, LJ medium was inoculated from the stored positive MGIT bottles and stock media and incubated at 37 ºC. Equivalent MGIT liquid medium was inoculated simultaneously and placed in the BACTEC MGIT 320 device. Weekly growth controls were performed. DNA isolation for PCR was performed for the isolates in which growth was observed (1,4).

MTBC subspecies determination was performed by GenoType MTBC (Hain LifeScience GmbH, Germany) test and rpoB gene mutation for RIF resistance, katG gene for high level INH resistance, promoter region of inhA gene for low level INH resistance were analysed by GenoType MTBDRplus (Hain LifeScience GmbH, Germany) test. In addition, the molecular resistance pattern against second-line fluoroquinolones (FLQ) (gyrA and gyrB genes) and second-line injectable drugs (SLID) (rrs and eis genes) was investigated in 10 MDR-TB isolates by GenoType MTBDR sl VER 2.0 (Hain LifeScience GmbH, Germany) (7).

Statistical Analyses: IBM SPSS 22.0 package programme was used for statistical analysis of the data. The relationships between categorical variables were analysed using Chi-square and Fisher's Exact tests. $p<0.05$ was considered statistically significant.

RESULTS

Of the 854 MTBC strains phenotypically tested for antibiotic susceptibility, 24 (2.8%) were found to be RIF resistant. Of these strains, 14 (1.6%) were determined to be MDR-TB with combined RIF+INH resistance. Three of the 24 rifampicin-resistant MTBC strains could not be resuscitated by passages and 21 of them were included in the study.

Of the 21 patients with phenotypic rifampicin resistance, 7 (33.3%) were female and

14 (66.7%) were male with a mean age of 45.0±17.1 years (Table 1).

Table 1. Demographic characteristics of patients from whom rifampicin-resistant MTBC strains were isolated

Cinsiyet	n	%
Female	7	33.3
Male	14	66,7
Total	**21**	**100**

When the distribution of the isolates included in the study was analysed according to the years, MDR-TB was not detected in eight of the eighteen years. Of the MDR-TB isolates, 2 (9.5%) each were isolated in 2007, 2010, 2011, 2012, 2014, 2021; 4 (19%) in 2009 and 5 (23.8%) in 2016. Although there was no statistical difference between the years, the highest number of resistant strains was found in 2016 (p=0.066) (Figure 1).

Twelve (57.1%) of the rifampicin-resistant MTBC isolates were positive by EZN staining, while nine (42.9%) were negative by EZN staining (Figure 2).

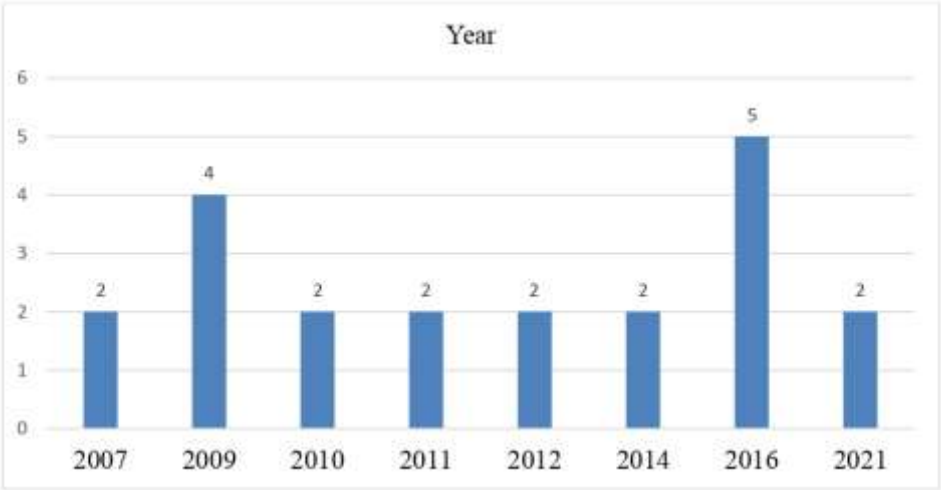

Figure 1. Distribution of rifampicin-resistant MTBC strains according to years.

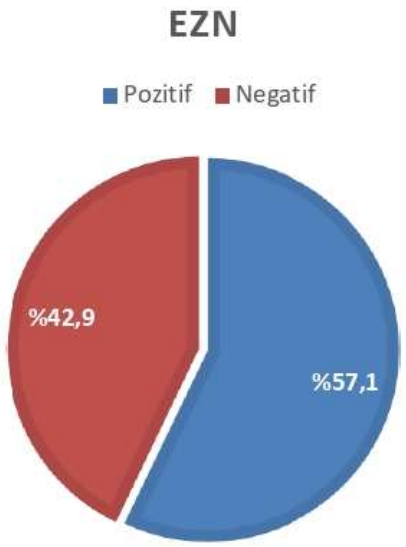

Figure 2. EZN positivity of rifampicin-resistant MTBC strains.

f the 21 rifampicin-resistant MTBC isolates, nine (42.9%) were resistant to streptomycin (SM) and seven (33.3%) were resistant to ethambutol (EMB). While nine (42.9%) of the isolates were resistant to RIF alone (RR-TB), 12 (57.1%) were MDR-TB with RIF+INH co-resistance. There was no significant difference between RIF and INH co-resistance and the others (p=0,482). (Table 2).

Table 2. Drug susceptibility results of rifampicin resistant MTBC strains by MGIT SIRE method.

Anti-TB Drug (n=21)	Resistance		p
	n	%	
RIF+SM *	9	42.9	p=0.482
RIF+EMB**	7	33.3	
RIF only	9	42.9	
RIF+INH	12	57.1	

*RIF+SM: Rifampicin +Sreptomycin,
**RIF+EMB: Rifampicin+ Ethambutol

Genotypic drug susceptibilities of phenotypically RIF 21 resistant MTBC isolates were investigated by GenoType MTBDRplus method. However, two isolates were excluded from the study because they did not meet the evaluation criteria of this test (Figure 3).

When the genotypic resistance status of 19 isolates detected with the Genotype MTBDRplus kit was evaluated, genotypic RIF resistance was detected in seven of the isolates (36.8%). In four of these resistant isolates (57.1%), deletion in the WT8 region and rpoBMUT3 mutation were found together. In the other isolates, one (14.3%) had deletion in WT8 and WT6 bands and one (14.3%) had deletion in WT8 band. In one (14.3%) isolate, deletion in WT7 band and rpoB MUT2A mutation were observed together.

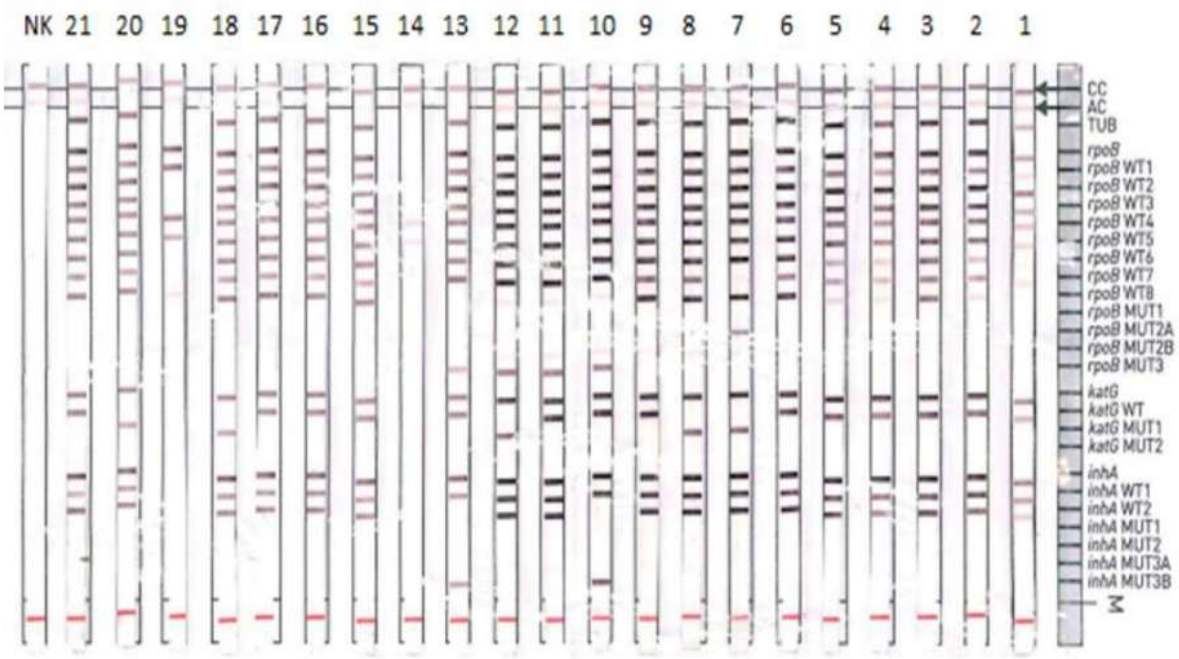

Figure 3. GenoType MTBDR plus test results

Seven (36.8%) of the 19 isolates were genotypically resistant to INH by Genotype MTBDRplus kit, while 12 (63.2%) were susceptible. Five (71.4%) of the resistant patients had katG WT band deletion and katGMUT1 mutation together and two (28.6%) had inhA MUT3B mutation (Table 3).

Table 3. Genotype MTBDRplus test results

MTBDR drug name/Resistance gene-mutation site		**Number**	**%**
Genotype MTBDRplus-RIF	Resistant	7	36.8
	Sensitive	12	63.2
Pozitive WT band	WT8/WT6	1	14.3
	WT8	1	14.3
	WT7/ rpoBMUT2A	1	14.3
	WT8/ rpoBMUT3	4	57.1
Genotype MTBDRplus-INH	Resistant	7	36.8
	Sensitive	12	63.2
Pozitive WT band	katGWT/ katGMUT1	5	71.4
	ınhAMUT3B	2	28.6

Eight of the 19 isolates detected with the Genotype MTBDRplus kit were identified as RR-TB and 11 as MDR-TB. Genotypic RIF resistance was detected in 25% (2/8) of RR-TB isolates with Genotype MTBDRplus kit. Moreover, RIF resistance was genotypically detected in 45.5% (5/11) of MDR-TB isolates by Genotype MTBDRplus kit.

While 12.5% (1/8) of RR-TB isolates were phenotypically INH resistant, genotypically INH resistance was detected with GenoType MTBDRplus kit. Genotypic INH resistance was observed in 54.5% (6/11) of MDR-TB isolates. No significant difference was observed between the presence of RIF and INH resistance in terms of test results ($p>0.05$) (Table 4).

Nine of the 10 MDR-TB isolates evaluated were genotypically resistant to none of the second generation drugs by Genotype MTBDR sl ver 2.0 method. One of these isolates could not be interpreted because it did not meet the evaluation criteria of this test (Figure 4).

Table 4. Comparison of genotypic RIF and INH resistance in RR-TB and MDR-TB isolates

Genotypic Susceptibility Profiles		RR-TB		MDR-TB		p*
		n	%	n	%	
Genotype MTBDRplus-RIF	Resistant	2	25	5	45.5	0.633
	Sensitive	6	75	6	54.5	
Pozitive WT band	WT8/WT6	-	-	1	20	0.714
	WT8	1	50.0	0	0	
	WT7/rpoBMUT2A	0	-	1	20	
	WT8/ rpoBMUT3	1	50	3	60	
Genotype MTBDRplus-INH	Resistant	1	12.5	6	54.5	0.147
	Sensitive	7	87.5	5	45.5	
Pozitive WT band	katGWT/katGMUT1	1	100	4	66.7	0.495
	ınhAMUT3B	-	-	2	33.3	

*Chi-square analysis was performed

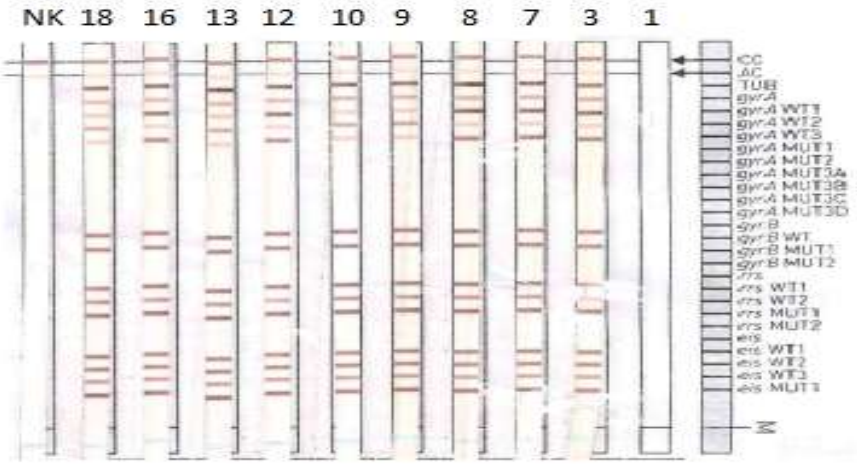

Figure 4. Genotype MTBDR sl ver 2.0 test results.

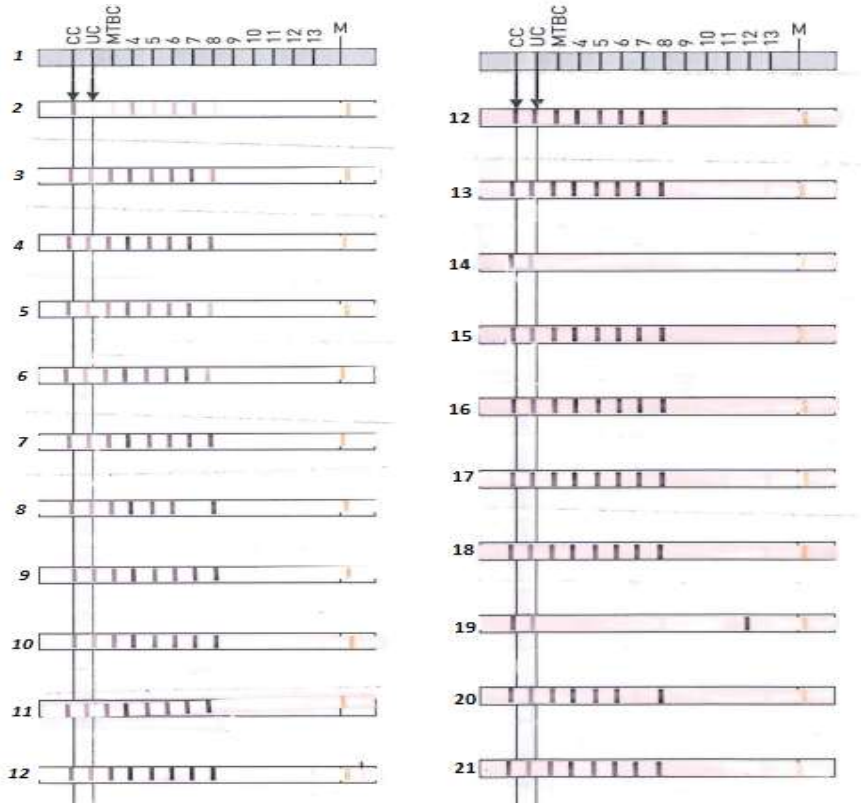

Figure 5. GenoType MTBC test results

When 21 rifampicin-resistant isolates were evaluated by Genotype MTBC method, 19 (90.4%) were found to be *M. tuberculosis*/*canetti*. However, two of them could not be evaluated because they did not meet the interpretation criteria of this test (Figure 5).

DISCUSSION

According to the WHO data, it is estimated that approximately 440,000 RR-TB/MDR-TB cases and 25,000 MDR-TB cases occur annually and 150,000 MDR-TB cases die each year (8). Similarly, the Turkish Tuberculosis Control Report stated that the rate of MDR-TB was 2.6% in new cases and 9.9% in previously treated cases. In the same report, eight (4.5%) of 176 MDR-TB cases in 2018 were identified as MDR-TB cases (9). In a study conducted by Kumar et al. (10) including 164 MTBC isolates, 13.4% of the strains were found to have MDR-TB. Similarly, Yazıcı et al.(11) at Akdeniz University reported that 68 (6.9%) of 974 MTBC isolates were MDR-TB. In a study conducted by Yılmaz et al.(12) in Erzurum, where 419 MTBC isolates were evaluated, the rate of MDR-TB was found to be 3.6% and their results were within the average of Turkey. In our study, 14 (1.6%) of 854 MTBC isolates were found to have MDR-TB. It was observed that the rate of MDR-TB was lower in our region compared to the data in the world and in our country.

Tuberculosis is generally more common in males (8). Liu et al.(13) analysed 139 MDR-TB cases in China and found that 99 (71.4%) were male, 40 (28.6%) were female and the mean age was 51 years. Soeroto et al.(14) analysed 492 cases in a study conducted in Indonesia, where the prevalence of MDR-TB is high, and found that MDR-TB was more common in patients aged <45 years. In a study conducted by Apoorva et al.(15), which included 452 MTBC specimens, 283 of the patients were male (62.3%) and 169 were female (37.2%) and 42.1% were in the 40-59 age group. Of the 12 MDR-TB strains detected in our study, four (33.3%) were female and eight (66.7%) were male patients and the mean age was 43 years. When the data we determined and the literature were evaluated together, it was determined that the age of MDR-TB incidence in our study was similar to the literature and it was more common in males.

Rifampicin is an important first-line anti-TB drug. RIF resistance is an important factor in determining the treatment regimen and prognosis of TB. Therefore, more attention has been paid to the mechanisms of rifampicin resistance (16). Mutations related to RIF resistance are mostly located in the rifampicin resistance determining region (RRDR) of the rpoB gene. This makes RIF more advantageous than other drugs in the application of genotypic-based drug susceptibility testing. Mutations related to RIF resistance are mostly mutations in codons 516, 526 and 531 of the rpoB gene. In a multicentre study conducted by Campbell et al.(17) with 314 clinical samples, mutations in the rpoB gene region were detected in 97% of 174 strains known to be phenotypically RIF resistant. The detected mutations were found between codons 507-533 defined as the RRDR region (17). In a study conducted by Javed et al.(18) on 53 MDR-TB strains in Pakistan using Genotype MTBDRplus test, 42 (79.2%) isolates were genotypically RIF resistant. Among these, the most common mutation was found to be S531L pattern with rpoMUT3 mutation in 34 (64.1%) isolates. Of these 34 isolates, 32 (60.3%) had deletion in the WT8 band, one (1.8%) had deletion in the WT3/WT4 bands and one (1.8%) had deletion in all WT bands. Additionally, five (9.4%) of the other isolates showed rpoMUT1 mutation with WT3/WT4 band deletion. WT7 band deletion was found in two (3.7%) isolates. One (1.8%) isolate showed different mutation patterns with deletion in WT5/WT6 band. In the study of Kumar et al. (19) involving 442 RR-TB isolates, MTBDRplus rifampicin resistance was found to be highest in the combination of both WT8 and MUT3 with 60.6%, and this was shown to include the 530-533 codon. Sağlam et al.(20) at Uludağ University reported 11 (84.6%) of 13 phenotypically RIF resistant strains had mutations in the rpoB gene. They further highlighted that three of these isolates had WT5 band deletion in the rpoB gene region and two of these three isolates had S531L gene pattern with both WT5 band deletion and rpoB MUT3 mutation, while the other three isolates had rpoB WT2 band deletion.

In our study, seven (36.8%) of 19 phenotypically RIF resistant isolates were genotypically RIF resistant by Genotype MTBDRplus test. Deletion in the WT8 band region was detected in six (85.7%) of these isolates. Four of these isolates (57.1%) had a mutation in the rpoB MUT3 gene region and were found to have the S531L gene pattern at codon 530-533. In one (14.2%) isolate, deletion of the WT6 band was observed together with WT8. In one (14.2%) of the seven isolates in which RIF resistance was genotypically determined, a mutation was observed in the rpoB MUT2A gene region with deletion in the WT7 band, and this isolate had the H526Y gene pattern at codon 526-529. When compared with various studies conducted in our country and in the world, the RIF resistance found in our study was genotypically lower. The fact that the resistance we found was most frequently detected in the S531L region was considered to be compatible with the literature.

Additionally, genotypic RIF resistance was observed in five (50%) of 10 MDR-TB isolates in this study. Genotypic resistance was observed in two (22.2%) of the nine isolates with phenotypic RIF resistance alone. This suggests that genotypic RIF resistance in MDR-TB isolates may be more

likely to develop due to gene mutation compared to isolates with RIF resistance alone.

The proportion of isoniazid-resistant TB cases is increasing globally. Mutations in the katG gene play a very prominent role in mediating INH resistance (21). The most common S315T resistance pattern has been reported to be associated with moderate or high levels of resistance to INH (22). A strong correlation between this mutation and the transmission dynamics of MDR-TB and MDR-TB has been previously reported (23). In a recent systemic review on INH resistance, it was shown that 64% and 19% of all INH resistance was associated with katG 315 and inhA-15 mutations, respectively (24). In a study conducted by Javed et al.(18) on 53 MDR-TB isolates phenotypically using Genotype MTBDRplus method in Pakistan, 38 (71.7%) isolates were genotypically INH resistant. In all but one of these isolates (37/53; 69.8%), S315T gene pattern was detected in the katG gene, and in 21 (39.6%) of these, WT band deletion was additionally reported. In the same study, four (7.5%) isolates were found to have mutations in the inhA promoter, and two different resistance patterns were detected: C-15T (MUT1-WT) in three (5.6%) and C-15T and T-8C (MUT1-MUT3A) in one (1.9%). In the study of Kumar et al.(25) involving 442 RR-TB isolates, 11.7% had inh A resistance pattern while 90% had fold G resistance pattern and the most common mutation for fold G was shown as Mut1 mutation and WT deletion in which point mutations occurred at codon 315. In our study, INH resistance patterns were evaluated in 19 phenotypically resistant RR-TB isolates and 10 MDR-TB isolates by Genotype MTBDRplus method. Six of 10 MDR-TB isolates (60%) were genotypically INH resistant. Four of them (40%) had S315T1 gene pattern showing deletion in katG WT band and katG MUT1 gene mutation. Two of the isolates (20%) had T8-A gene pattern with INHA-MUT3B gene mutation. INH resistance was detected genotypically in the katG gene region in a strain that was not phenotypically INH resistant. In our study, the rates of both phenotypic and genotypic INH resistance in MDR-TB strains and the fact that genotypic resistance was mostly seen as S315T1 pattern were evaluated in accordance with the literature. In our study, although there was no phenotypic resistance in one strain, genotypic resistance pattern was observed, which is important in terms of showing the incompatibility between the two tests.

Conventional DST for extensively drug-resistant MTBC strains is performed sequentially. This is a long and laborious two-step procedure starting with culture and first-line drug testing, with the need for further drug testing in case of multidrug resistance. A systematic review to evaluate Genotype MTBDR sl, which is considered to be the only commercially available molecular test for second-line anti-TB drug resistance, showed that it has good accuracy in detecting resistance to FQs, amikacin and capreomycin. However, it is not a suitable choice for kanamycin and ethambutol due to poor sensitivity (26). In our study, nine of 10 phenotypically resistant MDR-TB isolates were susceptible to second generation anti-TB drugs by Genotype MTBDR sl test. One isolate could not be evaluated because it did not meet the interpretation criteria of this test. Considering that the rate of MDR-TB is low in our country, our results were considered to be compatible with the literature. However, since phenotypic susceptibility testing could not be performed on the isolates, the inability to comment on genotypic resistance concordance was considered a limitation in our study.

MTBC consists of a genetically homogenous group. In this group, *M. tuberculosis subsp. tuberculosis* is the most common species causing tuberculosis in humans, while *M. bovis subsp. bovis, M. bovis subsp. caprae, M. canettii, M. africanum* and *M. microti* are the second most common species (6). The distribution and frequency of MTBC strains and sub-strains causing tuberculosis vary in different parts of the World (27). The origins of 19 MTBC isolates included in our study were analysed by Genotype MTBC test and all of them were identified as *M. tuberculosis*.

In conclusion,diagnosis of MTBC and drug susceptibility tests usually takes a long time with traditional methods. Newly developed molecular methods are very advantageous in terms of providing accurate and rapid results in both diagnosis and determination of drug susceptibility. The presence of MDR-TB and RR-TB is an important challenge especially in TB control, which increases the need for molecular methods. Although it has not still replaced culture, there is a need for the use and development of new molecular methods that will benefit us in TB treatment and control. Moreover, the disadvantage of molecular methods is the incompatibility with phenotypic resistance in cases where resistance develops by other mechanisms. We think that our study will contribute to the existing literature in this sense.

Conflict of interest: The authors declare no conflict of interest.

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