RESEARCH / ARAŞTIRMA
DOI: 10.26650/IUITFD.1051730
İst Tıp Fak Derg 2022 / J Ist Faculty Med 2022

GENE MUTATION PATTERNS OF RIFAMPICIN IN MULTIDRUG-RESISTANT *MYCOBACTERIUM TUBERCULOSIS* COMPLEX STRAINS

ÇOĞUL İLACA DİRENÇLİ *MYCOBACTERİUM TUBERCULOSİS* KOMPLEKS SUŞLARINDA RİFAMPİSİNİN GEN MUTASYON PATERNLERİ

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Cite this article as: Yildirim Servi E, Uzun M. Gene mutation patterns of rifampicin in multidrug-resistant *mycobacterium tuberculosis* complex strains. J Ist Faculty Med 2022;85(2):216-22. doi: 10.26650/IUITFD.1051730

ABSTRACT

Objective: The aim of this study was to evaluate the GenoType®MTBDR*plus* test for detection of rifampicin (RIF) resistance in MDR *Mycobacterium tuberculosis* complex strains.

Materials and Methods: Twenty-five multidrug-resistant (MDR) *Mycobacterium tuberculosis* clinical strains were used, gene mutations causing RIF resistance were investigated by GenoType®MTBDR*plus* and the results were compared with the results of the BACTEC 460 TB system. The strain was sequenced if there was no mutation and absence Wild Type (WT). *Mycobacterium tuberculosis* ATCC 35838 was used as a quality control (QC) strain.

Results: There was 96% compliance between the GenoType®MTBDR*plus* and BACTEC 460 TB system for the finding of RIF resistance. The most frequent mutation zone in MDR strains was *rpoB* S531L promotor zone (13 strains, 52%). The other *rpoB* gene mutations were H526Y (three strains, 12%) and H526D (three strains, 12%), while five strains (20%) had Δ2-Δ5 mutations in the wild type probes. There was no mutation in only one strain (4%) by GenoType®MTBDR*plus* but it was found to be as resistant to rifampicin by the BACTEC 460 TB system. This strain was sequenced and detected to have triple mutations. Mutations were found on codons 489, 493, and 503.

Conclusion: GenoType®MTBDR*plus* showed good compatibility with the BACTEC 460 TB system in detecting rifampicin resistance, and it was thought that GenoType®MTBDR*plus* could be an effective and reliable test for RIF susceptibility testing in MDR-TB patients, providing a significant advantage in technical time.

Keywords: Tuberculosis, rifampicin, resistance, GenotypeMTBDR*plus*, *Mycobacterium*, *rpoB*

ÖZET

Amaç: Bu çalışmada, çoğul ilaca dirençli (ÇİD) *Mycobacterium tuberculosis* kompleks suşlarında rifampisin (RIF) direncinin saptanmasında GenoType®MTBDR*plus* testinin değerlendirilmesi amaçlanmıştır.

Gereç ve Yöntem: Yirmi beş ÇİD *Mycobacterium tuberculosis* klinik suşu kullanılmış, RIF direncine neden olan gen mutasyonları GenoType®MTBDR*plus* ile araştırılmış ve sonuçlar BACTEC 460 TB sistemi sonuçları ile karşılaştırılmıştır. Eğer mutasyon veya Vahşi Tip (VT) bandı yoksa suş sekanslanmıştır. Kalite kontrol (QC) suşu olarak *Mycobacterium tuberculosis* ATCC 35838 kullanılmıştır.

Bulgular: Rifampisin direncinin saptanmasında GenoType®MTBDR*plus* ve BACTEC 460 TB sistemi arasında %96 uyum bulunmuştur. ÇİD suşlarda mutasyonun en sık *rpoB* S531L promotor bölgede (13 suş, %52) olduğu görülmüştür. Diğer *rpoB* gen mutasyonları H526Y (üç suş, %12) ve H526D (üç suş, %12) bölgesinde iken, beş suşta (%20) vahşi tip problarında Δ2-Δ5 mutasyonları saptanmıştır. Bir suşta (%4) GenoType®MTBDR*plus* ile mutasyon bulunamamış, ancak BACTEC 460 TB sistemi ile rifampisine dirençli olduğu belirlenmiştir. Bu suş sekanslanmış, 489, 493 ve 503 kodonlarında üçlü mutasyona sahip olduğu tespit edilmiştir.

Sonuç: Rifampisin direncinin saptanmasında GenoType®MTBDR*plus*, BACTEC 460 TB sistemi ile iyi uyum göstermiştir ve ÇİD-TB hastalarında RIF duyarlılık testi için GenoType®MTBDR*plus*'ın etkili ve güvenilir bir test olabileceği ve bunun teknik zaman açısından da önemli bir avantaj sağlayacağı düşünülmüştür.

Anahtar Kelimeler: Tüberküloz, rifampisin, direnç, GenotypeMTBDR*plus*, *Mycobacterium, rpoB*

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Submitted/Başvuru: 31.12.2021 • **Revision Requested/Revizyon Talebi:** 14.02.2022 •
Last Revision Received/Son Revizyon: 22.02.2022 • **Accepted/Kabul:** 23.02.2022 • **Published Online/Online Yayın:** 10.03.2022

INTRODUCTION

Tuberculosis (TB), caused by *Mycobacterium tuberculosis* complex (MTBC), is one of the leading causes of disease in the world. *M. tuberculosis* is responsible for 97.9% of the disease. The incidence of the disease has increased and decreased over the past thousands of years, but this disease has remained a permanent threat to public health (1-3).

According to the 2020 data of the World Health Organization (WHO), 10 million new cases of tuberculosis were determined and approximately 1.4 million people died from tuberculosis in 2019. Global cases were reported 44% from Southeast Asia, 25% from Africa, 18% from the West Pacific, 8.2% from the Eastern Mediterranean, 2.5% from Europe and 2% from the Americas. India, Indonesia, China, Nigeria, Pakistan, Bangladesh and South Africa have been reported as countries with the highest number of cases (4).

According to the TB dispensary in Turkiye 2020 report, 11,786 TB patients were identified in 2018. Of the patients, 6,778 (57.5%) were male and 5,008 (42.5%) were female. The case rate was 16.5/100 000 in men and 12.3/100 000 in women. The total case rate was determined as 14.4/100 000 in 2018. The most common drug resistance in new tuberculosis cases whose drug susceptibility tests were studied was isoniazid with 11.6%. The total resistance rate was 19.2% in the cases in which DST was studied, while the MDR-TB rate was 2.6% in new cases and 9.9% in previously treated cases (5).

In many developed countries, a rapid rise has been detected in tuberculosis with the increase in Human Immunodeficiency Virus (HIV)/Acquired Immunodeficiency Syndrome (AIDS) cases. In addition to HIV/AIDS infections, cases of multidrug-resistant tuberculosis (MDR-TB; least resistant to isoniazid (INH) and rifampicin (RIF) from first-line anti-TB drugs) also played an important role in the re-increase of tuberculosis. In addition to MDR strains, the emergence of extensively drug-resistant TB (XDR-TB; resistant to isoniazid, rifampicin, any quinolone and one of kanamycin, capreomycin or amikacin) strains, non-compliance with treatment, patient management mistakes, unsupervised treatment, limited or intermittent drug supply, use of substandard drugs and bad tuberculosis control programs have also been among the factors causing the increase in tuberculosis (4, 6, 7).

In the last 20 years, new molecular methods have been developed for diagnosis of tuberculosis. These methods allow resistance to various antituberculosis drugs, especially RIF, to be detected in a very short time and to start treatment in a short time. At the same time, epidemiological data are obtained by investigating which gene mutations are dominant in many countries through these methods (8, 9).

In this study, it was aimed to evaluate the GenoType®MTBDR*plus* test for detection of rifampicin resistance in MDR *Mycobacterium tuberculosis* complex strains.

MATERIALS AND METHODS

Bacterial strains

Bacterial strains were isolated from clinical specimens, which came to the Istanbul University, Istanbul Faculty of Medicine, Department of Medical Microbiology, Mycobacteriology Laboratory using the BACTEC 460 TB system. Sensitivity to antituberculosis drugs (INH, RIF, ethambutol (EMB), streptomycin (SM)) was investigated using this system, and was determined to be resistant to rifampicin and isoniazid.

Ethical statement

The approval of the Istanbul University Faculty of Medicine Ethics Committee was obtained for this study (Date: 30.11.2011, No: 2118).

Identification of rifampicin resistance by molecular assay

Gene mutations causing RIF resistance were investigated by GenoType® MTBDR*plus* assay version 2.0 (HainLife Sciences, Nehran, Germany) (10).

DNA isolation

One milliliter of culture was taken from the liquid medium and transferred to a sterile screw-capped Eppendorf tube and incubated for 20 minutes at 95°C in a heat block. DNA was obtained by incubating in an ultrasonic water bath for 15 minutes.

DNA amplification

The total reaction volume for each strain was set to be 50 µl; 35 µl of the primer nucleotide mixture in the kit, 5 µl of 10x PCR buffer, 2 µl of $MgCl_2$, 3 µl of ultra pure water, 0.2 µl of TaqGold polymerase and 5 µl of the sample were added in sterile PCR tube. The PCR mix was subjected to the PCR steps listed in Table 1 (10, 11).

Table 1: PCR conditions

	Pre-PCR	PCR	PCR	Extension
Temperature/time	95°C→15 min	95°C→30 sec 65°C→2 min	95°C→25 sec 50°C→40 sec 70°C→40 sec	70°C→8 min
Cycle number	**1**	**10**	**20**	**1**

Hybridization

Hybridization and detection were performed according to the manufacturer's instructions. Twenty microliters of denaturation solution were added to 20 µl of amplified sample and mixed. The solution was incubated at 21°C for 5 min. One milliliter of hybridization buffer was added and shaken. After, the membrane strips were placed in the hybridization solution and incubated for 30 min at 45 °C. Each strip was washed twice. One milliliter of diluted substrate was added and colorimetric detection of the hybridized amplicons was obtained (12).

The Genotype MTBDR*plus* strip includes 27 probes. It contains hybridization (CC) and amplification (AC) controls to confirm the test protocols. *M. tuberculosis* is identified by the use of the *M.tuberculosis* complex-specific (TUB) probe. The *rpoB*, *katG*, and *inhA* control probes detect *rpoB*, *katG*, and *inhA* regions, respectively. The eight *rpoB* wild-type probes (WT1 to WT8), two *inhA* wild-type probes (WT1 and WT2), and one *katG* wild-type probe are used to determine the mutations that lead to RIF and INH resistance. The ten mutant probes were specifically designed to hybridize sequences of the frequently detected four *rpo*B, four *inh*A and two *kat*G mutations: *rpoB* MUT1 D516V, *rpoB* MUT2A H526Y, *rpoB* MUT2B H526D and *rpoB* MUT3 S531L, *inhA* MUT1 C15T, *inhA* MUT2 A16G, *inhA* MUT3A T8C and *inhA* MUT3B T8A, *katG* MUT S315T1 and *katG* MUT S315T2 (13).

If a band occurs at the *rpo*B mutation site, the strain is resistant to RIF. In addition, the strain is accepted as resistant to RIF if there is no band in the mutation region but there was a faint band in the WT region (10).

DNA sequencing

The strain was sequenced in the absence of mutation or absence of the WT band. A 411bp fragment of the *rpoB* gene was amplified using *rpoB* primers (Forward primer: 5'- TACGGTCGGCGAGCTGATCC-3', Reverse primer: 5'-TACGGCGTTTCGATGA ACC-3') (14). Product size (411 bp) was verified by agarose (2%) gel electrophoresis. PCR product was cleaned up using ExoSAP-IT™ PCR Product Cleanup kit (Thermo Fisher Scientific, USA). Cycle sequencing was performed with BigDye™ Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, USA). After that, sephadex purification was done and the product was loaded in the ABI 3130 XI 16 Capillary Genetic Analyser (Hitachi, Japan). Clustal Omega online tool (https://www.ebi.ac.uk/Tools/msa/clustalo/) was used for DNA sequence comparisons (15).

Quality control

Mycobacterium tuberculosis ATCC 35838 was used as a quality control (QC) strain for all methods. This strain is resistant to rifampicin.

RESULTS

Both male 15 (60%) and female 10 (40%) TB patients' samples were included in the study. Twenty-five strains were isolated from 24 sputum and one abscess samples.

Drug susceptibility testing of 25 strains was determined using the BACTEC 460 TB method. Resistance was determined to be INH+RIF in six strains (24%), INH+RIF+EMB in eight strains (32%), INH+RIF+SM in four strains (16%) and INH+RIF+SM+EMB in seven strains (28%) (Figure 1).

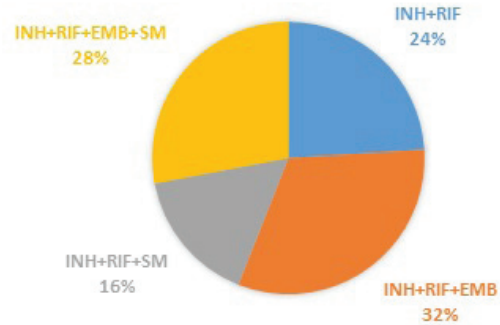

Figure 1: Sensitivity test results by the BACTEC 460 TB method

Gene mutations causing RIF resistance were investigated by GenoType® MTBDR*plus*. The most frequent mutation zone in MDR strains was the *rpoB* S531L promotor zone (13 strains, 52%). The other *rpoB* gene mutations were H526Y (four strains, 16%), H526D (one strain, 4%) and D516V (one strain, 4%) while five strains had Δ2-Δ5 mutations (20%) in the WT probes. There was no mutation in only one strain (4%) by GenoType®MTBDR*plus* but it was found to be as resistant to rifampicin by the BACTEC 460 TB system (Table 2).

In seventeen of the twenty-five isolates, WT1/WT4/WT7 (one; 4%), WT2/WT3/WT4 (one; 4%), WT3/WT4 (two; 8%), WT6/WT7/WT8 (one; 4%), WT8 (12; 48%) faint bands were present. On the other hand, WT6/WT7/WT8 (one; 4%), WT3/WT4 (one; 4%), WT3/WT7 (one; 4%), WT6/WT8 (one; 4%), and WT7 (four; 16%) absence bands were available in eight isolates (Table 2).

The strain that was found susceptible by GenoType® MTBDR*plus* was sequenced and triple mutations were detected. Mutations were found on codons 489, 493 and 503 (Table 3).

DISCUSSION

Molecular methods provide faster diagnosis than traditional methods. However, it is recommended that these tests be applied in conjunction with gold standard culture methods. The sensitivity and specificity of studies in

Table 2: Gene mutation pattern detected by GenoType MTBDR*plus*

Gene	Number of isolates	Wild type band faint	Wild type band absence	Mutation band	Mutation
	1	WT2/3/4	WT6/7/8	-	-
	2	WT3/4	-	-	-
	1	-	WT3/4	*rpo*B MUT1	D516V
	1	-	WT3/7	-	-
	1	WT6/7/8	-	*rpo*B MUT2A	H526Y
rpoB	1	WT1/4/7	WT6/8	-	-
	3	-	WT7	*rpo*B MUT2A	H526Y
	1	-	WT7	*rpo*B MUT2B	H526D
	12	WT8	-	*rpo*B MUT3	S531L
	1	-	-	*rpo*B MUT3	S531L
	1	-	-	-	-

Table 3: Sequence result of the susceptible strain

Codon	Amino acid change	Nucleotide change
489	Gln → Hist	CAG → CAC
493	Asn → Isoleucin	AAC → ATC
503	Lys → Isoleucin	AAG → ATC

which RIF resistance is detected by molecular methods vary between 92-100% and 85-100%, respectively (16-22).

In our study, the performance of the GenoTypeMTBDR*plus* assay for the detection of RIF-resistant strains of *M. tuberculosis* was evaluated on 25 MDR *M. tuberculosis* isolates. Twenty-four (96%) of the 25 isolates, which were determined to be rifampicin-resistant with the BACTEC 460 TB method, were found to be rifampicin-resistant with GenoType MTBDR*plus*. Our result was similar to other reports from India, France, Germany and Ethiopia (16, 23-25). One isolate (4%) was susceptible to rifampicin. It was observed that the GenoType MTBDR*plus* showed good accordance with the BACTEC 460 TB system. The resistance in the mutation undetectable strain is thought to be related to the gene region not detected by the GenoType MTBDR*plus* kit. Our sequence study confirmed this idea. We found triple mutation on codons 489, 493 and 503. These codons are outside the gene region range included in the GenoType MTBDR*plus* kit. Cavusoglu et al. detected rifampin resistance in 22 (53.7%) of 41 isolates with Genotype MTBDR assay. But two RIF-resistant isolates (4.8%) were identified as RIF sensitive. One strain had an Gln-490-His mutation and the other isolate had a CGG insertion between codons 514 and 515. These mutation regions were outside the 81-bp hotspot region and not detected by Genotype MTBDR assay (26). In our study, the result is identical with these study results.

Molecular methods provide information about the mutation regions that occur in the genes that cause resistance, as well as providing faster results. Studies have shown that mutations in different codons of the relevant *rpoB* gene are effective at different rates in the emergence of RIF resistance, and it has been reported that the percentage of mutations in the codon S531L of the *rpoB* gene is higher than the other codons (27, 28).

In our study, RIF-resistance-specific mutations were determined on *rpoB* MUT probes in 19 (76%) of the 25 MDR isolates by the GenoType MTBDR*plus* assay. This frequency rate was found to be similar with other studies (29-31).

In 2018, in the study by Kamiri et al., among the 35 specimens identified as RIF-resistant using the GenoTypeMTBDR*plus* assay, 23 (65%) carried the S531L, eight isolates (22%) showed mutation in codon H526Y and four isolates (11%) in D516V codon (32). In 2013, in the study by Maurya et al., the frequency of rpoB mutation was 28 in S531L (62.3%), eight in D516V (17.7%), six in H526Y (11.1%), one in the H526D (2.2%) region and nine (20%) unknown mutations (absence of one or more wild-type) among a total of 45 MDR-TB strains (23). In 2017, in the study by Abanda et al., among the 48 isolates determined as RIF-resistant using the GenoTypeMTBDRplus assay, 41 (85%) had the S531L mutation and three isolates (6%) showed mutation in codon H526Y. The frequency of codon 531 mutation in this study was 85%, which is higher than generally reported (19). In 2013, in the study by Yadav et al., among all 66 RIF-resistant strains, 51 (72%) had a mutation in *rpoB* S531L. Four strains (6%) had a mutation in *rpoB* H526D (4/66), two in (3%) *rpoB* D516V (2/66) and two (3%) in *rpoB* H526Y (33).

The result of this study showed that of the 19 specific mutations of the *rpoB* gene, 13 mutations (52%) were at

codon S531L. The remaining 16% (4/25) had mutations at codon H526Y, 4% (1/25) at codon H526D and 4% (1/25) at codon D516V. Mutations at codon S531L and H526Y are known to be the most prevalent RIF resistance. Our results displayed a similar profile with the previous study (30, 34, 35). But our frequency rate is lower in codon S531L. This may be due to the variability of strains between countries. Mutation percents in the D516V and H526Y codon were found to be lower in our study compared with percents reported from Europe (36). We think that smaller sample sizes cause the difference. This idea is supported by the work by Farooqi et al. (37). On the other hand, five of our MDR isolates have only WT band absence and faint band but don't have a corresponding MUT band. This result is associated with mutations that cause drug resistance. In addition to that, silent mutation that doesn't result in any amino acid difference may cause this pattern, or the presence of less common mutations in the *rpoB* gene that cannot be detected by the GenoType MTBDR*plus* test may lead to this result (11, 16).

The present study showed that TB is more common among males (60%) and affects all age groups. This finding was in alignment with several reports (38-40). Males generally are known to be more susceptible to TB than females because of their relatively large social network, which increases their risk of infection, in addition to the higher prevalence of smoking, which has a confirmed association with TB (41, 42).

Consistent results were detected in this study, when compared with the results obtained worldwide. However the frequency rate of mutations was found to be low. We think that the use of a small number of isolates led to this result, because when previous studies are reviewed it appears that more isolates are used. The GenoType MTBDR*plus* assay has high sensitivity and specificity. However it identifies limited mutation regions.

CONCLUSION

In conclusion, GenoType® MTBDR*plus* showed good compliance with the BACTEC 460 TB system in the detection of rifampicin resistance and it was thought that GenoType® MTBDR*plus* might be an effective and reliable test for the susceptibility testing of rifampicin in MDR-TB patients. Additionally, this technique also provided a significant advantage in terms of time.

Informed Consent: Written consent was obtained from the participants.

Ethics Committee Approval: This study was approved by the Clinical Research Ethical Committee of the Istanbul University, Istanbul Faculty of Medicine (Date: 30.11.2011, No: 2118).

Peer Review: Externally peer-reviewed.

Author Contributions: Conception/Design of Study- E.Y.S., M.U.; Data Acquisition- E.Y.S., M.U.; Data Analysis/Interpretation- E.Y.S., M.U.; Drafting Manuscript- E.Y.S., M.U.; Critical Revision of Manuscript- E.Y.S., M.U.; Approval and Accountability- E.Y.S., M.U.

Conflict of Interest: Authors declared no conflict of interest

Financial Disclosure: This study was partially funded by Scientific Research Projects Coordination Unit of Istanbul University. Project number: 37697. This study was supported by Istanbul University Scientific Research Projects Unit (Project No: 21970).

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