

ORIGINAL ARTICLE

Performance of genotype-MTBDR test directly on clinical specimens

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ABSTRACT

Objectives: Most important point for the control and effective treatment of multidrug resistant tuberculosis (MDR-TB) is early diagnosis and rapid determination of the resistance. The aim of this study is to assess the performance of the Genotype-MTBDR assay applied directly on sputum samples and compare the results with those obtained by DNA sequencing and phenotypic susceptibility testing.

Materials and methods: Between November 2005 and February 2006, 93 smear and culture positive sputum samples were included in the study. Drug susceptibility results for rifampin (RIF) and isoniazid (INH), obtained by proportion method on L-J medium, Genotype-MTBDR and DNA sequencing were compared.

Results: The rate of concordance between the results of the Genotype-MTBDR and DNA sequencing was 93.5% and 96.7% for RIF and INH, respectively. Moreover, Genotype-MTBDR detected all the RIF (24) and INH (18) resistant strains obtained by sequencing (100%). Compared to the DNA sequencing method; the sensitivity, specificity, positive predictive and negative predictive value for RIF and INH were 100%, 91.3%, 80%, 100% and 100%, 96%, 85.7%, 100% respectively.

Conclusion: Genotype-MTBDR, one of molecular assays, distinctly shortens the time for diagnosis and detection of resistance to INH and RIF, essential for management of MDR-TB. The test appears to have good sensitivity and specificity when also used directly on sputum specimens. *J Microbiol Infect Dis* 2012; 2(4): 135-141

Key words: Mycobacterium tuberculosis; drug resistance; genotype-MTBDR.

Genotype-MTBDR testi performansının doğrudan balgam örneklerine uygulanarak değerlendirilmesi

ÖZET

Amaç: Çoklu ilaç dirençli tüberkülozun (ÇİD-TB) kontrol ve etkin tedavisinde erken tanı ve ilaç direncinin hızlı bir şekilde saptanması yaşamsal bir rol oynamaktadır. Bu çalışmanın amacı; Genotype-MTBDR testinin doğrudan balgam örneklerine uygulandığında performansını değerlendirmek ve fenotipik duyarlılık testleri ve DNA dizi incelemesi ile elde edilen sonuçları karşılaştırmaktır.

Gereç ve yöntem: Kasım 2005 ve Şubat 2006 tarihleri arasında yayma ve kültür pozitif toplam 93 balgam örneği çalışmaya dahil edildi. Lowenstein-Jensen agarda agar proporsiyon, Genotype-MTBDR testi ve DNA dizi inceleme yöntemi ile belirlenen rifampin (RIF) ve isoniazid (INH) duyarlılık sonuçları birbiri ile karşılaştırıldı.

Bulgular: Genotype-MTBDR testi ve DNA dizi inceleme yöntemi ile elde edilen ilaç duyarlılık sonuçları arasında; rifampin (RIF) açısından %93,5, isoniazid (INH) açısından %96,7 oranında uyum saptandı. Aynı zamanda bu moleküler test; DNA dizi inceleme yöntemi ile RIF (24) dirençli ve INH (18) dirençli olarak belirlenen tüm suşları saptamıştır (%100). DNA dizi inceleme yöntemi ile karşılaştırıldığında; Genotype-MTBDR testinin RIF ve INH için duyarlılık, özgüllük, pozitif prediktif ve negatif prediktif değerleri sırasıyla; %100, %91,3, %80, %100 ve %100, %96, %85,7, %100 olarak saptandı.

Sonuç: Moleküler yöntemlerden biri olan Genotype-MTBDR; ÇİD-TB tanısı ve direnç belirlenme süresini belirgin olarak kısaltmaktadır. Direkt olarak balgam örneklerine de uygulandığında da testin duyarlılık ve özgüllüğü yüksektir.

Anahtar kelimeler: Mycobacterium tuberculosis; ilaç direnci; genotype-MTBDR.

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INTRODUCTION

Mycobacterium tuberculosis is still one of the most significant causes of death from an infectious agent. One third of the world population is currently infected with the tuberculosis bacillus, nearly 1% of this population is newly infected and it is killing nearly two million people every year. Poor control programs, irregular and inappropriate drug usage are the reasons for the development and increasing rate of multidrug-resistant tuberculosis (MDR-TB), which is defined as *Mycobacterium tuberculosis* resistant at least to rifampin (RIF) and isoniazid (INH).^{1,2}

Since fatality rates for MDR-TB are much higher, the most important point for the control and effective treatment of MDR-TB is early diagnosis and rapid determination of the resistance. However, drug susceptibility testing by conventional methods takes several weeks.^{3,4} Recently, there has been considerable progress in understanding of the mechanisms of resistance to the antituberculosis drugs, especially INH and RIF.^{5,6} Drug resistance in tuberculosis is the result of random genetic mutations in particular genes conferring resistance. The vast majority of RIF resistance (95%) is caused by mutations located in the 81-bp region of the *rpoB* gene. In contrast, the mutations causing INH resistance are located in several genes and regions. Approximately 50-95% of INH resistant strains contain mutations in codon 315 of the *katG* gene, 20-35% contains mutations in the *inhA* regulatory region and 10-15% have mutations in the *ahpC*-*oxyR* intergenic region.^{5,8}

Based on all these knowledge to shorten the duration of drug susceptibility tests; several molecular methods such as DNA sequencing, line probe assay (LIPA) and DNA microarray, have been developed.⁸ Two commercial DNA strip assays; the INNO-LIPA Rif TB (Innogenetics, Ghent, Belgium) and the Genotype-MTBDR (Hain Lifescience, Nehren, Germany), both based on multiplex PCR and reverse hybridization have been introduced to detect the most important gene mutations conferring the resistance. The Genotype-MTBDR is one of DNA strip assays and distinct to the INNO-LIPA Rif TB, it has an advantage of being able to detect the presence of mutations in both INH and RIF simultaneously. It is based on the hybridization between *rpoB* and *katG* amplicons to membrane-bound probes. The DNA

strip covers five *rpoB* wild-type probes, four *rpoB* mutant probes (D516V, H526Y, H526D, S531L mutations), one *katG* wild-type probe, and two *katG* mutant probes (S315T1 and S315T2 mutations).^{9,10} In 2007, after we completed the study, Genotype-MTBDR test was replaced by Genotype-MTBDRplus test (HAIN Lifescience GmbH, Germany) which detects additional mutations in *inhA* gene that confer resistance to INH.¹¹⁻¹³

The aim of this study is to assess the performance of the Genotype-MTBDR assay applied directly on sputum samples and compare the results with those obtained by DNA sequencing and phenotypic susceptibility testing.

MATERIALS AND METHODS

Atatürk Chest Diseases and Chest Surgery Research and Educational Hospital is a 600-bed tertiary care hospital and it is the reference hospital for tuberculosis in Ankara, in the capital city of Turkey. The sputum samples obtained from patients (1 specimen per patient) admitted to this hospital between November 2005 and February 2006 was collected.

Strains and phenotypic susceptibility testing

The sputum samples were decontaminated and homogenized by NALC-TSC-NaOH (3%NaOH, 1.47% Trisodium Citrate) method and neutralized with phosphate buffer (0.067 M, pH 6.8). After centrifugation, half of the sediment (500 µl) was stored in falcon tubes at -70°C for the Genotype-MTBDR Assay and the other half was used for EZN staining and cultured on Löwenstein-Jensen (L-J) medium.

The cultures were incubated at 37°C for 6 weeks. The solid media were evaluated every day in the first 5 days and weekly thereafter. Drug susceptibility testing (DST) for RIF and INH was performed by proportion method on L-J medium, using concentrations 20 and 40 µg/ml for RIF and 0.2 and 1 µg/ml for INH.¹⁴

DNA Extraction

A total of 93 smear and culture positive sputum samples were preserved in the falcon tubes at -70°C and then they were transported to molecular laboratory of 19 Mayıs University, Faculty of Medicine, Department of Clinical Microbiology and Infectious Diseases for the molecular steps.

DNA was extracted from mycobacteria by RTP Spin Mycobacteria DNA Kit (Invitex, Germany). According to kit information; NAC Buffer, Resuspension Buffer R, Proteinase K, Binding solution, Wash Buffer 1 and 2 and Elution Buffer D were used respectively. The extracted DNA was stored at -20°C in order to be used at the further steps.

Genotype-MTBDR

The Genotype-MTBDR assay (Hain Life-science, Nehren, Germany) was carried out according to the manufacturer's instruction with the reagents provided in the kits. The assay consists of PCR amplification, hybridization of the PCR products to the probe-containing strips, and detection and interpretation of the results. The amplification protocol consisted of 5 min of denaturation at 95°C; 10 cycles of 30 sec at 95°C and 2 min at 58°C; 30 cycles of 25 sec at 95°C, 40 sec at 53°C, and 40 sec at 70°C; and a final extension at 70°C for 8 min. PCR products were analyzed in 1.5% agarose gel for the control of incomplete or marginal amplification. Each strip contains 17 probes, including amplification and hybridization. The *katG* and *rpoB* specific regions were detected by *katG* and *rpoB* control probes, respectively. These strips have five wild-type (WT) *rpoB* probes that cover the whole 81-bp resistance-determining region of the gene and one *katG* WT probe. Furthermore, the strips contain six mutation probes which were designed to hybridize to the sequences of the four most frequently observed *rpoB* and two *katG* mutations: *rpoB* D516V, *rpoB* H526Y, *rpoB* H526D, *rpoB* S531L, and *katG* S315T1, *katG* S315T2.

DNA Sequencing

Genomic bacterial DNA, used for sequencing, was extracted from bacteria grown on L-J medium. Firstly a part of the culture was suspended in 1 ml of water and killed at 95°C for 20 min. Then DNA was extracted according to the procedure in Invisorb Spin Bacteria DNA Mini Kit (Invitex, Germany). The extracted DNA was stored at -20°C. Amplification was performed in a GeneAmp thermal cycler (GeneAmp® PCR System 9700, Applied Biosystems) and the protocol consisted of an initial denaturation step of 10 min at 95°C, followed by 35 cycles of 1 min at 95°C, 1 min at 55°C, and 2 min at 72°C, ending with an extension step of 8 min at 72°C. PCR products

were analyzed in 1.5% agarose gel. Purifications before and after sequencing were performed by DyeEx 2.0 Spin Kit. The gene *katG* was amplified and sequenced by using the primers (Tb86: 5'-GAAACAGCGGCGCTGATCGT and Tb87: 5'-GTTGTCCCATTTCGTCGGGG), and *rpoB* was amplified and sequenced by using the primers (*rpo95*: 5'-CCACCCAGGACGTGGAGGCGATCACAC and *rpo397*: 5'-CGTTTCGATGAACCCGAACGGGTTGAC).^{15,16} The gene targets were sequenced using Big Dye Terminator cycle sequencing kit version 3.1 (Applied Biosystems, Foster City, CA) in an ABI PRISM 310 DNA sequencer (Applied Biosystems).

RESULTS

Ankara Atatürk Chest Diseases and Chest Surgery Research and Educational Hospital is one of the National Reference Centers for the patients with tuberculosis in Turkey. Between November 2005 and February 2006, a total of 126 sputum samples from 126 patients were collected during the study period. A total of 93 smear and culture positive sputum samples were included in the study, smear negative-culture positive or smear positive-culture negative samples were excluded. INH-RIF susceptibility test results obtained by the Genotype-MTBDR test were compared with those obtained by proportion method on L-J medium and sequencing.

In our study, DST with DNA sequencing revealed that, 63 (67.7%) *M. tuberculosis* isolates were susceptible to both antibiotics, 18 (19.4%) of those were resistant to INH, 24 (25.8%) were resistant to RIF. The rate of MDR-TB was 12.9% (12/93).

The rate of concordance between the results of the Genotype-MTBDR and conventional DST was 82.7% for RIF and 84.9% for INH. However when the Genotype-MTBDR results were compared with the sequence results; the concordance rates were 93.5% and 96.7% for RIF and INH, respectively. Compared to the sequencing method; the sensitivity, specificity, positive predictive and negative predictive value for RIF and INH were 100%, 91.3%, 80%, 100% and 100%, 96%, 85.7%, 100% respectively.

The Genotype-MTBDR assay detected 23 of 32 (71.8%) RIF resistant strains obtained by proportion method. The other 9 strains were sus-

ceptible to RIF due to Genotype-MTBDR assay. When these 9 strains were also sequenced, all were susceptible to RIF. Of the 27 INH resistant strains, 17 (62.9%) were correctly identified by Genotype-MTBDR. The other 10 strains were susceptible to INH due to both Genotype-MTBDR assay and DNA sequencing.

Genotype-MTBDR detected all the RIF (24) and INH (18) resistant strains obtained by sequencing, with at least one negative signal on WT probes (100%). Eight different types of mutations were detected in 24 RIF-resistant *M. tuberculosis* isolates. Twelve (50%) isolates carried the most common mutation, Ser-531-Leu. Four

(16.6%) isolates showed His-526-Tyr mutation, 3 (12.5%) isolates showed Asp-516-Val mutation, one (4.1%) isolate had Asp-516-Tyr and Leu-511-Arg mutations, one (4.1%) had Ser-512-Arg, one (4.1%) had Glut-513-Leu, one (4.1%) had Asp-516-Tyr and one isolate (4.1%) carried Leu-533-Pro mutation (Table 2). The *katG* gene in 18 INH resistant isolates was examined by sequencing, and mutations in codon 315 were detected in all isolates. Sixteen (88.8%) of these mutations in codon 315 were in the form of Ser-315-Thr mutation, one (5.5%) in the form of Ser-315-Gly and one (5.5%) of the isolates showed Ser-315-Ala mutation (Table 3).

Methods	RIF		INH	
	Sensitive n (%)	Resistant n (%)	Sensitive n (%)	Resistant n (%)
Agar Proportion (n:93)	61 (65.6)	32 (34.4)	66 (71)	27 (29.0)
Genotype-MTBDR (n:93)	63 (67.7)	30 (32.3)	72 (77.4)	21 (22.6)
DNA Sequencing (n:93)	69 (74.2)	24 (25.8)	75 (80.6)	18 (19.4)

Table 1. The Susceptibility Results with Agar Proportion, Genotype-MTBDR and DNA Sequencing

Table 2. Frequency of mutations concerned with RIF resistance in *rpo B* gene region detected by DNA Sequencing

Location of mutations	Nucleotide changes	Amino acid changes	No (%) of strains n:24
Codon 531	TCG TTG	Ser Leu	12 (50.0)
Codon 526	CAC TAC	His Tyr	4 (16.6)
Codon 516	GAC GTC	Asp Val	3 (12.4)
Codon 511 & 516	CTG CGG and GAC TAC	Leu Arg and Asp Tyr	1 (4.2)
Codon 512	AGC CGC	Ser Arg	1 (4.2)
Codon 513	CAA CTA	Glut Leu	1 (4.2)
Codon 516	GAC TAC	Asp Tyr	1 (4.2)
Codon 533	CTG CCG	Leu Pro	1 (4.2)

Table 3. Frequency of mutations concerned with INH resistance in *kat G* gene region detected by DNA Sequencing

Location of mutations	Nucleotide changes	Amino acid changes	No (%) of strains, n:18
Codon 315	AGC ACC	Ser Thr	16 (89.0)
Codon 315	AGC CGC	Ser Gly	1 (5.5)
Codon 315	AGC GCC	Ser Ala	1 (5.5)

DISCUSSION

The World Health Organization (WHO) estimates the rates of MDR-TB at 3.6% (95% confidence

interval (CI): 3.0-4.4) among all incident TB cases globally. Almost 50% of MDR-TB cases worldwide are estimated to occur in China and India. Treatment success in MDR-TB patients is low

because of high frequency of death, default and treatment failure.¹⁷

Prompt identification of MDR and initiation of MDR treatment is the most important point for the control and effective treatment of MDR-TB. However, using conventional DST methods yields results within weeks (for liquid media) or months (for solid media). Early diagnosis and initiation of MDR-TB treatment gives a better chance of cure and prevents the development and spread of further resistance and reduces the mortality. Specimens for culture should be obtained especially from all previously treated patients and DST should be performed for at least INH and RIF at the beginning of the therapy.³

LJ medium is inexpensive, widely used since decades and is still being preferred in many countries. However, it is not recommended for susceptibility testing by WHO since it is solidified by heating (that may destroy antibiotics) and includes abundant amount of proteins (that may bind antibiotics), which may lead to false identification of higher antibiotic resistance rates, if preparation methods are not standardized. Since 2008; WHO has endorsed the use of liquid culture and rapid species identification as preferable to solid culture-based methods alone. Besides lowering the duration of time required for obtaining results (about 10 days), liquid systems are also more sensitive for detecting mycobacteria and may increase the case yield by 10% compared to solid media.^{3,18} However; L-J medium was the preferred medium at one of reference hospitals (Ankara Atatürk Chest Diseases and Chest Surgery Research and Educational Hospital) in Ankara during the study period. In our study the resistance rates by proportion method on L-J medium were higher than the others. They were; 29% and 34.4% by proportion method on LJ while resistance rates by sequencing were 19.3% and 25.8% for INH and RIF respectively. In order to have a better idea about the accuracy of Genotype-MTBDR, we compared the results obtained by this test with the ones obtained by DNA sequencing.

Although these resistance rates show similarities with the meta-analysis results of Yolsal et al. (1984-1989; INH 27.8%- RIF 22.3% and 1990-1995; INH 23.8%-RIF 22.1%), those were higher than the rates reported by Kısa et al. from Ankara (MDR 1.7%) and Öztürk et al. from North-

West Region of Turkey (INH 8%, RIF 4.8%, MDR 4.8%).¹⁹⁻²¹ As we did not request any data from the laboratory for the history of patients in this study, it is not clear whether the rates are primary or secondary resistance rates. In contrast with all other studies; rate of resistance to RIF was higher than for INH.

As reported by several studies, the concordance rates of Genotype-MTBDR test with susceptibility results obtained by culture methods, are high when isolates are tested after grown in culture media.^{8-10,22,23} But the rates are lower when the assay was directly applied on clinical samples.^{1,16} Since 2008; WHO recommended the use of line probe assays, for rapid screening of patients at risk of MDR-TB.¹⁸ We used the test directly on sputum samples and the rate of concordance between the results of the Genotype-MTBDR and sequencing was 93.5% and 96.7% for RIF and INH, respectively. Moreover Genotype-MTBDR detected all the RIF (24) and INH (18) resistant strains obtained by sequencing (100%). Its sensitivity and negative predictive value were 100% for both INH and RIF.

There were discordant results between the tests. 6 samples were resistant by Genotype-MTBDR but sensitive by DNA sequencing (Table 4), 3 samples were resistant by Genotype-MTBDR but sensitive by DNA sequencing (Table 5). Furthermore 4 isolates for RIF and 3 isolates for INH were resistant by Genotype-MTBDR and DNA sequencing but susceptible by phenotypic susceptibility testing. Discordance between the results of different tests may be explained by heteroresistance, which is the presence of resistant and sensitive bacilli in the same sputum samples. While mutant bacilli were detected by molecular assays, sensitive bacilli might have grown on other medias. The other reason for the differences between the tests may be the rare mutations in Turkey.^{12,13,24}

There were no newly reported mutations at different codons of INH or RIF resistant strains in our study.^{7,21,25-27} The codons most frequently involved in mutations with RIF-resistant isolates were codon 531 (50%), codon 516 (20.86%) and codon 526 (16.6%). Mutations at codon 531 have been reported in the majority of RIF resistant strains from Greece, Russia and Middle Eastern countries while most patients from South Asia contained mutations at codon 526.²⁸⁻³² The rest

of the mutations (%13) were at codons 511, 512, 513 and 533 (Table 2). Mutations associated with INH resistance were all in katG codon 315 (Table

3), comparable to previously reported rates from Russia and South Asian countries but higher than described in Kuwait.^{12, 33}

DNA Sequencing	Genotype-MTBDR, n (%)		Agar Proportion, n (%)	
	Sensitive	Resistant	Sensitive	Resistant
RIF sensitive, (n:69)	63 (91.3)	6 (8.7)	57 (82.6)	12 (17.4)
RIF resistant, (n:24)	0 (0.0)	24 (100.0)	4 (16.7)	20 (83.3)

Table 4. The Susceptibility Results of Rifampin

DNA Sequencing	Genotype-MTBDR n (%)		Agar Proportion n (%)	
	Sensitive	Resistant	Sensitive	Resistant
INH sensitive, (n:75)	72 (96.0)	3 (4.0)	63 (84.0)	12 (16.0)
INH resistant, (n:18)	0 (0.0)	18 (100.0)	3 (16.7)	15 (83.3)

Table 5. The Susceptibility Results of Isoniazid

Our study has some limitations. First of all; socio-demographic data and the HIV status of the patients were not considered. Secondly, since performing the molecular part of the study in a different city from where the samples were collected, these sputum samples had to be stored at deep freeze (-70°C) for a long time. Keeping frozen at low temperature may damage the mycobacteria or decrease the ability of the test to detect the resistant strains. Besides this, we could not reanalyze the samples with the new version of the test, Genotype-MTBDRplus, since we could not provide enough budgets at that time. As well as the refrigerator was broken 3 months after the study and the majority of the samples were melted. Additionally, we were unable to compare mutations with MIC values so we don't know the level of resistance to antituberculosis drugs. Finally; the results of Genotype-MTBDR were compared with those obtained by sequencing due to the disadvantages of L-J medium mentioned before.

In conclusion; rapid diagnosis and identification of resistant strains are essential for early and efficient treatment and control of the MDR-TB. So the primary aim of using these expensive molecular tests is to shorten the time needed for diagnosis and detection of drug susceptibility among populations at high risk for MDR-TB in routine daily practice. The test appears to have good sensitivity and specificity when also used directly on sputum specimens. With the new version of the test, Genotype-MTBDRplus assay (HAIN Lifescience GmbH, Germany), its ability to detect

the resistance directly from clinical samples has also been improved. New molecular assays do not eliminate the need for conventional culture and DST since direct use of line probe assays on smear negative clinical specimens is not recommended. Besides this, these methods shorten distinctly the time for diagnosis and detection of resistance which essential for management of MDR-TB.

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