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Evaluation of the genotype MTBDRplus assay for the diagnosis of tuberculosis and rapid detection of rifampin and isoniazid resistance in clinical specimens*

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Evaluation of the genotype MTBDRplus assay for the diagnosis of tuberculosis and rapid detection of rifampin and isoniazid resistance in clinical specimens*

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Aim: To determine the performance of the Genotype MTBDRplus assay for diagnosis of tuberculosis and rapid detection of rifampin (RIF) and isoniazid (INH) resistance in clinical specimens.

Materials and methods: A total of 90 clinical specimens of 57 patients (69 sputum samples, 11 bronchoscopic aspirates, 5 bronchoalveolar lavage, 4 deep tracheal aspirate, 1 lymph aspirate) sent to the Ege University Medical Faculty, Department of Medical Microbiology, Mycobacteriology Laboratory between December 2007 and 2009 during the clinical routine were included in the study.

Results: Overall 80 valid results were obtained for 90 clinical specimens (88.9%) with MTBDRplus. While 74 of 82 (90.2%) smear positive specimens gave interpretable results by MTDRplus, 2 of 8 smear negative specimens gave invalid results. The overall rates of concordance between the results of the MTBDRplus assay and those of the drug susceptibility testing for the assessment of RIF and INH resistance were 97.5% (78/80) and 98.8% (79/80), respectively.

Conclusion: Although the MTBDRplus assay could be a useful tool for rapid identification of RIF- and INH-resistant *Mycobacterium tuberculosis* in both clinical samples and strains, the test results must always be confirmed by culture and drug susceptibility testing.

Key words: *Mycobacterium tuberculosis*, rifampin resistance, isoniazid resistance, Genotype MTBDRplus, clinical specimen

Klinik örneklerden tüberküloz tanısı ve hızlı rifampin ve izoniyazid direnci saptanması için geno type MTBDRPlus testinin değerlendirilmesi

Amaç: Bu çalışmada Genotype MTBDRplus testinin klinik örneklerden tüberküloz tanısı ve hızlı rifampin (RIF) ve izoniyazid (INH) saptanmasıdaki performansının belirlenmesi amaçlanmıştır.

Yöntem ve gereç: Aralık 2007 ve 2009 tarihleri arasında Ege Üniversitesi Tıp Fakültesi Tıbbi Mikrobiyoloji Anabilim Dalı Mikobakteriyoloji Laboratuvarı'na rutin inceleme için gönderilen 57 hastaya ait toplam 90 klinik örnek (69 balgam örneği, 11 bronkoskopik aspirat, 5 bronkoalveoler lavaj, 4 derin trakeal aspirat, 1 lenf aspiratı) çalışmaya alındı. **Bulgular:** Toplam 90 klinik örneğin 80'inde (% 88,9) MTDRplus testiyle geçerli sonuç elde edildi. Direkt bakısı pozitif 82 örneğin 74'ünde (% 90,2) MTDRplus ile geçerli sonuç alınırken, direkt bakısı negatif sekiz örneğin ikisinde geçersiz sonuç alındı. MTBDRplus testiyle ilaç duyarlılık testi arasındaki genel uyumluluk oranı RİF direnci için % 97,5 (78/80) ve INH direncini için % 98,8 (79/80) olarak sapatandı.

Sonuç: MTBDRplus klinik örneklerde ve kökenlerde RIF ve INH'a dirençli *M. tuberculosis*'in tanısında kullanılabilecek yararlı bir araçtır. Buna karşın test sonuçları mutlaka kültür ve ilaç duyarlılık testleriyle doğrulanmalıdır.

Anahtar sözcükler: *Mycobacterium tuberculosis*, rifampin direnci, izoniyazid direnci, Genotype MTBDRplus, klinik örnek

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Introduction

Mycobacterium tuberculosis remains one of the most significant causes of death from an infectious agent. The incidence of pulmonary tuberculosis in Turkey is nearly 27.9 per 100,000 population (1). The proportions of multidrug resistant tuberculosis (MDR-TB) among new cases, previously treated cases and all TB cases are 2.9%, 15.5%, and 4.9%, respectively (1).

Collectively, DNA sequencing studies demonstrate that more than 95% of RIF-resistant *M. tuberculosis* strains have a mutation within the 81-bp hotspot region of the *rpoB* gene (2-4). In contrast, the mutations causing INH resistance are located in several regions (5). Approximately, 34.6% to 94.3% of INH-resistant strains have been found to contain mutations in codon 315 of the *katG* gene (6-10), 2.9% to 21.5% contain mutations in the *inhA* promoter region (6,7,9,10), and an additional 2% to 11.5% have mutations in the *ahpC-oxyR* intergenic region (6,7,9).

Several molecular methods have been developed in recent years for the diagnosis of tuberculosis and rapid detection of drug resistance in clinical specimes, including line probe assays (GenoType MTBDRplus; Hain Lifescience GmbH, Nehren, Germany, INNO LİPA Rif.TB; Innogenetics, Ghent, Belgium) and real-time PCR (Xpert; Cepheid, Sunnyvale, CA, USA). Molecular assays have been established to allow the prediction of drug resistance in clinical specimens within 1 working day and potentially are the most rapid methods for the detection of drug resistance (2,6,11-13). The Genotype MTBDR plus assay (Hain Lifescience GmbH, Nehren, Germany) is a novel kit-based method for the detection of the most common mutations in the *M. tuberculosis rpoB*, *katG,* and *inhA* genes (6,12).

The aim of the present study was to determine the performance of the Genotype MTBDRplus assay for diagnosis of tuberculosis and rapid detection of rifampin and isoniazid resistance in smear-positive clinical specimens. The results obtained by the Genotype MTBDRplus assay were then compared with the results obtained by culture and phenotypic susceptibility testing.

Materials and methods

Clinical specimens

A total of 90 clinical specimens of 57 patients (69 sputum samples, 11 bronchoscopic aspirates, 5 bronchoalveolar lavage, 4 deep tracheal aspirate, 1 lymph aspirate) sent to the Ege University Medical Faculty, Department of Medical Microbiology, Mycobacteriology Laboratory between December 2007 and 2009 during the clinical routine were included in the study. Clinical specimens were processed by the conventional *N*-acetyl-Lcysteine–NaOH method (14). After decontamination, smears were prepared by the auramine-rhodamine acid-fast staining method (14). Decontaminated specimens were inoculated to Löwenstein-Jensen solid medium and MB/BacT liquid medium (BioMérieux, Marcy l'Etoile, France) for growth detection, the leftover sediment of the decontaminated specimen was stored at -20 °C. Smear positive specimens were studied in 2 weeks; smear negative specimens were studied after growth of culture immediately.

Identification of *Mycobacterium tuberculosis* complex (MTBC) strains from cultures and drug susceptibility testing (DST)

After growth of the cultures, species identification and, in cases in which MTBC strains were identified, DST were performed. The identification of *M. tuberculosis* from the grown cultures was confirmed by the GenoType MTBDRplus assay.

The proportional method with 7H10 medium had been used to test resistance to the INH and RIF. Tests were performed with the standard critical concentrations of INH (0.2 µg/mL) and RIF (1 µg/mL).

Genotype MTBDRplus assay

The Genotype MTBDRplus assay was used according to the instructions of the manufacturer. Briefly, for DNA preparation, 1 mL of clinical specimen was used. For PCR amplification, 35 µL of primer-nucleotide mix (provided with the kit), amplification buffer containing 2.5 mM $MgCl_2$, 1.25 U of FastStart *Taq* polymerase (Roche Molecular Diagnostics, Mannheim, Germany), and 5 µL of supernatant in a final volume of 50 µL were used. The amplification protocol consisted of 15 min of denaturation at 95

°C, followed by 10 cycles comprising 30 s at 95 °C and 120 s at 58 °C; an additional 35 cycles comprising 25 s at 95 °C, 40 s at 53 °C, and 40 s at 70 °C; and a final extension at 70 °C for 8 min.

The MTBDRplus strip contains 27 probes, including hybridization (CC) and amplification (AC) controls. *M. tuberculosis* was detected in a sample by the use of the *M. tuberculosis* complex-specific (TUB) probe. The *rpoB*, *katG*, and *inhA*-specific regions were detected by *rpoB*, *katG*, and *inhA* control probes, respectively. The reactivities of an amplified fragment with the 8 *rpoB* wild-type probes (rpoB WT1 through rpoB WT8), 2 *inhA* wild-type probes (*inhA* WT1 and *inhA* WT2), and 1 *katG* wild-type probe were used to detect the mutations that lead to RIF and INH resistance in *M. tuberculosis*. Furthermore, 10 probes (mutant probes) were specifically designed to hybridize the sequences of the 4 most frequently observed *rpoB,* 4 *inhA*, and 2 *katG* mutations: *rpoB* MUT1 (D516V), *rpoB* MUT2A (H526Y), *rpoB* MUT2B (H526D), and *rpoB* MUT3 (S531L), *inhA* MUT1 (-15C/T), *inhA* MUT2 (-16A/G), *inhA* MUT3 (-8T/C), and *inhA* MUT4(-8T/A), *katG* MUT1 (S315T1), and *katG* MUT2 (S315T2).

In conclusion, when all of the wild-type probes gave a positive signal and all of the mutant probes reacted negatively, the *M. tuberculosis* isolate was considered susceptible to RIF and INH. When at least one negative signal was obtained with the *rpoB* wild-type probes, the isolate was considered resistant to RIF, and same was true for the *katG* and inhA wild-type probes. When the resistance to RIF or INH was due to one of the 10 most frequently observed mutations described above, a positive reaction was obtained with at least one of the 10 mutant probes and was always accompanied by a negative reaction with the corresponding wild-type probe.

Results

A total of 90 clinical specimens, consisting of 82 smear-positive and 8 smear negative samples, were included in the study. All clinical specimens tested were culture positive for *M. tuberculosis.* Overall, 80 interpretable results were obtained for 90 specimens (88.9%) by MTDRplus. There was no statistically significant difference in readability rates between clinical samples graded as 4+, 3+, and 2+ compared to 1+ by logistic regression analaysis ($P > 0.05$). While 74 of 82 (90.2%) smear positive specimens gave interpretable results by MTDRplus, 2 of 8 smear negative specimens gave invalid results (Table 1). However the *M. tuberculosis* complex-specific (TUB) control band was detected in 7 of 10 invalid results. Interestingly 3 specimens, in which TUB control band was negative, were smear-positive. As a result, TUB control band was detected in 87 (96.7%) of 90 clinical specimens.

Among the 80 interpretable clinical specimens, obtained from 47 patients, 71 clinical specimens obtained from 43 patients were pansusceptible, 5 clinical samples obtained from 2 patients had INH^r

Table 1. MTBDRplus assay results according to the auramine-rhodamine microscopy staining for the culture positive 90 clinical samples.

MTBDR*plus*	No. (%) of specimens					
test result*	Smear 4+	Smear 3+	Smear 2+	Smear 1+	Smear negative	Total
MTBDR*plus* +	18 (85.7)	19 (100)	22 (88)	12 (70.6)	6 (75)	77 (85.6)
MTBDR*plus* ±	0 (0)	0 (0)	1(4)	2 (11.8)	0 (0)	3 (3.3)
MTBDR*plus* -	3 (14.3)	-	2(8)	3(17.6)	2 (25)	10 (11.1)
Total	21	19	25	17	8	90

* MTBDR*plus* + interpretable and concordant test results with DST, MTBDR*plus* ± interpretable and discordant test results with culture and DST, MTBDR*plus* – invalid test results

and RIF^s pattern, 2 clinical samples obtained from 1 patient had INH^r and RIF^r pattern, and 2 clinical samples obtained from 1 patient had INH^s and RIF^r pattern (Table 2).

The overall rate of concordance between the results of the MTBDRplus assay and those of the DST for the assessment of RIF resistance was 97.5% (78/80; K = 0.655 ± 0.226 P < 0.001). The discordant 2 samples were RIF susceptible with DST, but MTBDRplus assays of these samples gave positive signals both with *rpoB* MUT2B probe and WT *rpoB* probes. Both samples were smear positive and obtained from the same patient and their MTBDRplus assays made from culture gave WT patterns (Table 3). The overall rate of concordance between the 2 tests for assessment of INH susceptibility was 98.8% (79/80; K = 0.926 ± 0.073 P < 0.001). The discordant sample was INH resistant according to the DST but INH susceptible by MTBDRplus assay. The MTBDRplus assay of this sample made from culture also gave katMUT1 pattern. The rate of concordance was also calculated by taking into account only one sample per patient (n = 47). The overall rate of concordance betwen MTBDRplus and DST was 95.7 (45/47). The levels of agreement was 97.9% (46/47; K = 0.657 ± 0.319 P < 0.001) for RIF and (46/47; K = 0.846 ± 0.151 P < 0.001) for INH.

Discussion

The MTBDRplus assay has been widely used in clinical routine for identificatin of *M. tuberculosis* complex and deteciton of RIF and INH resistance from clinical strains. The MTBDRplus assay can also be applied directly to clinical specimens. In several studies performed recently, the rates of valid test results for smear positive and smear negative clinical specimes have been reported to be 91.7%-100% and 46.1%-76%, respectively (15-19). In concordance with the result of Nikolayevsky et al. (15) but not with that of Lacoma et al. (16), Hilleman et al. (17) and Miotto et al. (18), our result with clinical samples

Table 2. Analysis of 9 RIF and/or INH resistant clinical samples obtained from 4 patients according to MTBDRplus assay.

		Molecular DST				Phenotypical DST		
		Sputum		Culture				
		MTBDRplus patterns						
ID	Smear results	RIF	INH	RIF	INH	RIF	INH 0.2	INH 1.0
J2368±	3+	MUT3	WT	MUT3	WT	R	S	S
J2433±	3+	MUT3	WT	MUT3	WT	R	S	S
K170*	1+	WT +MUT2B	katG MUT1	WT	katG MUT1	S	R	R
K171*	1+	WT +MUT2B	katG MUT1	WT	katG MUT1	S	R	R
K822	2+	WT	katG MUT1	WT	katG MUT1	S	R	R
K971¶	2+	WT	inhA MUT1	WT	inhA MUT1	S	R	S
K1019¶	2+	WT	inhA MUT1	WT	inhA MUT1	S	R	S
K1032¶	2+	WT	inhA MUT1	WT	inhA MUT1	S	R	S
K1059¶	1+	WT	inhA MUT1	WT	inhA MUT1	S	R	S

± J2368 and J2433 were obtained from same patients. *K170 and K171 were obtained from same patients.
¶ K971, K1019, K1032, and K1059 were obtained from same patients.

Table 3. Analysis of disagreements between molecular (clinical specimens and cultures) and phenotypical DST results for the 80 interpretable clinical specimens.

		Molecular DST				Phenotypical DST		
		Clinical specimes		Culture				
		MTBDRplus patterns						
ID	Smear results	RIF	INH	RIF	INH	RIF	INH 0.2	INH 1.0
K1435	2+	WT	WT	WT	katG MUT1	S	R	R
K170*	1+	WT +MUT2B	katG MUT1	WT	katG MUT1	S	R	R
K171*	1+	WT +MUT2B	katG MUT1	WT	katG MUT1	S	R	R

*K170 and K171 were obtained from same patients

revealed 90.2% (74/82) valid test results for smear-positive specimens and 75% (6/8) for smear-negative specimens. In contrast with Nikolayevskyy et al. (15) we found that no statistically significant differences in readability rates in clincal samples graded as 4+, 3+, and 2+ compared to 1+.

We found 3 kinds of invalid results in 10 of the 90 clinical specimens. For one smear positive sample graded as 4+, all gene-specific hybridization bands were absent, despite the presence of amplification and the hybridization control bands (Figure, Strip 1). When this sample was retested the same result was obtained. Two postulates can be offered to explain this situation. (i) The inhibitors in the specimen can lead to inhibition of amplification of *M. tuberculosis* genome in the specimen without any effect on the amplification control in the primer-nucleotide mix. (ii) Probably due to the nonhomogeneous distrubition of bacilli in the specimen, there were no bacilli in the PCR mix.

For 2 smear positive samples, there was an absence of TUB band, despite the presence of some gene-specific hybridization bands along the strip (Figure, Strips 2 and 3). In the third kind of invalid result, which was present in 7 cases (2 smear negative, 5 smear positive), the pattern of resistance was incomplete because of the absence of some wild-type probes or presence of mutation bands discordant with wild-type probes (Figure, Strips 4 and 5).

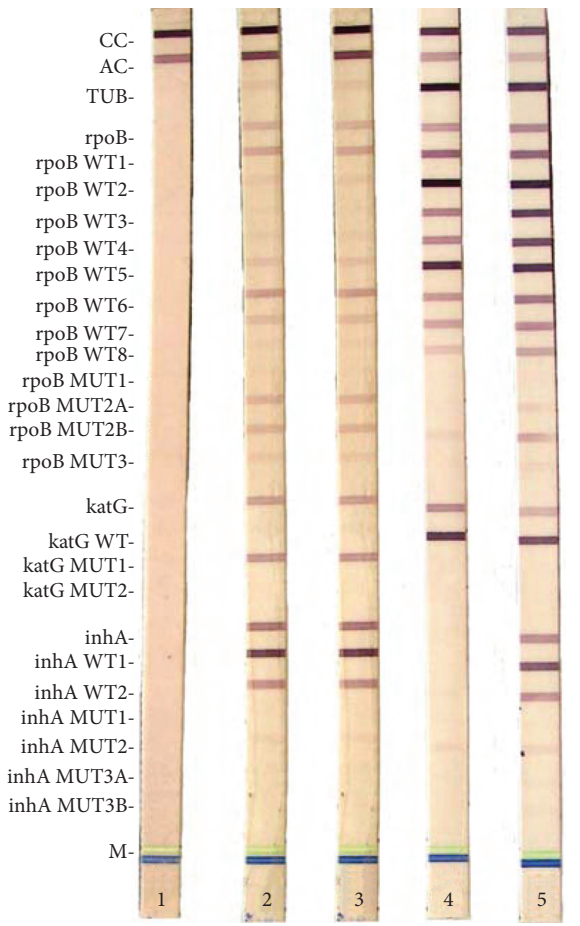

Figure. Examples of invalid results obtained by MTBDR*plus* assay. The positions of the oligonucleotides and the control probes are given on the left.

Of the 80 valid GenoType MTBDRplus assays, 3 clinical specimens obtained from 2 patients gave discordant results with DST. However, the GenoType MTBDRplus assay gave concordant test results with DST in 90 clinical strains, in our study. One smear positive clinical specimen graded as 2+ gave susceptible pattern with GenoType MTBDRplus assay, but GenoType MTBDRplus assay performed from clinical strains of this sample had katG MUT1 pattern and INH resistance was detected by DST (Table 3). Two specimens obtained from one patient, GenoType MTBDRplus assay gave signal with both rpoBWT probes and MUT2B probe despite the presence of WT pattern with GenoType MTBDRplus assay made from clinical strains and RIF resistance was not detected by DST. It is well known that the specimens tested can contain heteroresistant strains. In case of heteroresistance, a mutated as well as wild-type sequence can be detected in the respective sample.

In conclusion, the test has a short turnaround time and simultaneously provides the RIF and INH susceptibility pattern in a working day. Although the MTBDRplus assay could be a useful tool for rapid identification of RIF- and INH-resistant *M. tuberculosis* in both clinical samples and strains, the test results must always be confirmed by culture and DST.

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