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Comparison of Rifampicin Drug Susceptibility Determined by Conventional Culture Method and Pcr-Heteroduplex Analysis in *Mycobacterium Tuberculosis* Strains

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Resistance of Mycobacterium tuberculosis (MTB) to antituberculous drugs has been threatening the public-health grossly. Recently, because of high-level of resistance to rifampicin in MTB, treatment is becoming impossible. In this study, 127 rifampicin resistant and 33 rifampicin susceptible strains were randomly chosen from tuberculosis culture-positive isolates, isolated in the laboratory of the Ataturk Chest Diseases and Chest Surgery Center in Ankara, Turkey, over a 2-year period (1997 to 1998). Clinical samples collected from patients with suspected tuberculosis were studied for acid-fast bacilli stain (AFBS) and cultured for detecting MTB. PCR was used to amplify genetic loci associated with rifampicin resistance. The amplified 305-bp fragment of the rpoB gene was applied to Heteroduplex assay for rapid detection of rifampicin resistance phenotype in MTB. The 127 rifampicin-resistant and 33 rifampicin-susceptible isolates, selected according to conventional culture method, were tested again by PCR-Heteroduplex analysis. According to PCR-Heteroduplex analysis, 105 isolates of 160 M. tuberculosis were rifampicin resistant, 55 strains were rifampicin susceptible. Of 127 rifampicin-resistant isolates detected by conventional culture method, 101 had concordance with Heteroduplex analysis, whereas 26 isolates did not. While 4 strains were detected as rifampicin susceptible by conventional culture technique, they were rifampicin resistant by Heteroduplex analysis. It is thought that there might be a mutation out of fragment amplified, in 26 strains which were not observed as rifampicin resistant by Heteroduplex analysis; or a mistake in the result of resistance defined by classic culture method. In conclusion, however, PCR-Heteroduplex analysis is a rapid and reliable method to detect rifampicin resistance, although it is a relatively expensive technique.

Key Words: M. tuberculosis, Rifampicin Resistance, PCR-Heteroduplex Analysis, Proportional Method.

Mycobacterium tuberculosis Suşlarında Klasik Kültür Metodu Ve Pcr-Heteroduplex Analizi İle Saptanan Rifampisin İlaç Duyarlılığının Karşılaştırılması

Mycobacterium tuberculosis'in (MTB) antitüberküloz ilaçlara karşı direnç geliştirmesi büyük ölçüde halk sağlığını tehdit etmektedir. Son zamanlarda, MTB'deki rifampisin direncinin yüksek seviyede olması nedeniyle, tedavisi zorlaşmaktadır. Bu çalışmada, yaklaşık 2 yıl boyunca (1997-1998) Ankara, Atatürk Göğüs Hastalıkları ve Göğüs Cerrahisi Merkezi laboratuvarından izole edilen, kültürü pozitif izolatlardan 127 rifampisin dirençli ve 33 rifampisin duyarlı suşlar rasgele seçilmiştir. Tüberkülozdan şüphelenilen hastalardan toplanan klinik örneklerden asido resistan basil (ARB)çalışılmıştır ve MTB saptamak için kültüre alınmıştır. Rifampisin direnciyle ilgili gen bölgesini çoğaltmak için PCR kullanılmıştır. rpoB geninin çoğaltılan 305 baz çiftlik parçası için MTB'deki rifampisin direnç fenotipini hızlı saptamak amacıyla Heterodupleks analizi uygulanmıştır. Klasik kültür yöntemine göre seçilmiş 127 rifampisin dirençli ve 33 rifampisin duyarlı izolatlar tekrar PCR-Heterodupleks analizi ile test edilmişlerdir. PCR-Heterodupleks analizine göre 160 M. tuberculosis suşlarından 105'i rifampisin dirençli ve 55 suş ise rifampisin duyarlı bulunmuştur. Klasik kültür metoduyla saptanan 127 rifampisin dirençli izolattan 101'i Heterodupleks analizi ile uyumluydu, fakat 26 izolat uyum göstermemiştir. Dört suş klasik kültür yöntemiyle rifampisine duyarlı olarak saptandığı halde, heterodupleks analizi ile rifampisine dirençli bulunmuşlardır. Heterodupleks analizi ile rifampisin dirençli olarak gözlenmeyen 26 suşta çoğaltılan parçanın dışında bir mutasyon, yada klasik kültür metodu ile saptanan direnç sonuçlarında bir hata olabileceği düşünülmüştür. Sonuç olarak, yine de PCR-Heterodupleks analizi nispeten pahalı bir teknik olmasına rağmen, rifampisin direncini saptamak için hızlı ve güvenli bir metottur.

Anahtar Kelimeler: M. tuberculosis, Rifampisin Direnci, PCR-Heteroduplex Analizi, Klasik Kültür Metodu

Globally, tuberculosis (TB) leads to about 3 million deaths annually.[1] This disease is also Turkey's foremost health problem, and causes considerable economic losses. The treatment of TB is quite important to decrease mortality and to interrupt the chain of transmission. Infection source in TB is especially bacilli discharging patients. Therefore, infected person must be quickly identified, and cured to prevent economic losses, before patient with TB infects another person.
Chemotherapy for TB lasts from 6 months to one year, and drug treatment and hospitalization cost pretty much. Short-course standard therapy designed for TB is that therapy with isoniazid (INH), rifampin (RIF), ethambutol (EB) and pyrazinamide for 2 months, and following that, with INH and RIF for 4 months.[2, 3]

Rifampicin is one of the most important antituberculosis-drugs, because of its preventive impact on the development of TB bacilli.[4] There are various literatures about impact of rifampicin resistance on prognosis.[5,6,7] In Turkey, it is known that approximately 40% of patients with TB are resistant to at least one of the drugs used in therapy.[8, 9] Especially, in the past 10 years, new methods were developed for detection of antibiotic susceptibility.[10,11,12,13,14] It is a fact that, these methods based on molecular biology have led to considerable progression in diagnosis. Rapid tests for drug susceptibility have been developed by detecting the genetic basis of antimicrobial resistance.[15, 16]Heteroduplex analysis, developed in past years is a conformational technique used to detect mutations in DNA fragments amplified by PCR. It is a rapid method, showing if there is any mutation in a gene by comparing with a control DNA.

The subject of our study was selected depending on completely those ideas. In the study, it was objected to detect rifampicin resistant TB cases in a shorter time with PCR-heteroduplex analysis by using sputum samples of patients with TB or bacilli growth in the culture.

MATERIALS AND METHODS

Mycobacteria isolation: MTB strains were isolated from clinical samples, such as sputum, bronchoalveolar lavage, urine and gastric aspirate collected from patients with suspected tuberculosis in the laboratory of the Ataturk Chest Diseases and Chest Surgery Center in Ankara, Turkey, over a 2-year period (1997 to 1998) (Table I). Those samples collected in the laboratory were studied for acid-fast bacilli stain (AFBS) and cultured for detecting MTB. After the susceptibility testing of isolated strains to the 4 major drugs was made, 127 RIF-resistant and 33 RIF-susceptible strains were randomly chosen from tuberculosis culture-positive isolates, and they were stored at 4 °C.

Table I. Dispersions of 160 clinical samples in this study; and the number of *M. tuberculosis* strains isolated from these samples, and their acid-fast bacilli stain (AFBS) results

TYPE OF SAMPLE	NUMBER OF STRAINS ISOLATED	AFBS	
		POSITIVE	NEGATIVE
Sputum	154	123	31
Urine	2	-	2
Gastric aspirate	2	2	-
Bronchoalveolar lavage	2	-	2
TOTAL	160	125	35

Sputum samples were treated with the standard protocol of n-acetylcysteine-NaOH and concentrated by centrifugation.[17,18,19] From the sediments smears were made, stained by Ziehl-Neelsen, examined for AFB. After microscopic examination, cultures were done on Löwenstein-jensen (LJ) medium. 2-4 weeks later, MTB growth on LJ at 37 °C was identified by applying biochemical tests, including niacin production, nitrate reductase activity, catalase - peroxidase activity, and by observing its colony morphology, pigment production, and growing speed.[20,21,22]

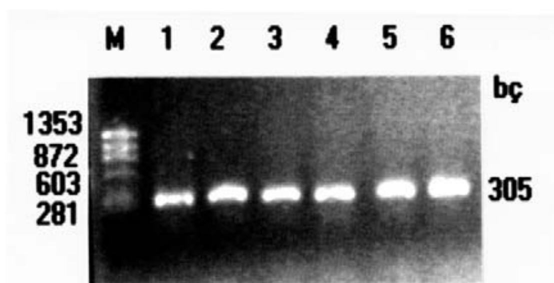
Drug susceptibility testing: Drug susceptibility was determined on LJ by the proportion method of Canetti et al.[23] The susceptibility of mycobacteria analyzed for 4 major antituberculosis agents including INH, RIF, streptomycin (STM), and EB by conventional culture technique.[24] In order to detect antibiotic resistance, suspensions and dilutions of isolates were prepared before the inoculation on LJ. Bacilli were incubated at 37 °C for about 28 days, and were noted one by one if there was any growing for each drug.

***Sample preparation for PCR*:** PCR was used to amplify genetic loci associated with rifampicin resistance. The amplified 305-bp fragment of the *rpoB* gene was applied to Heteroduplex assay for rapid detection of rifampicin resistance phenotype in MTB.

A suspension of a clinical isolate of *M. tuberculosis* having ~10^5 microorganisms per ml was prepared in TE buffer (10 mM Tris [pH 8.0], 1 mM EDTA), by adjusting its turbidity with McFarland standards. Clinical isolate was resuspended in TE buffer 3 times. The last suspension was placed on boiling water for 20 min. After boiling the tube was centrifuged and 100 µL from the top was pipetted into fresh tube to use for PCR amplification.

PCR amplification of mycobacterial strains: In order to detect Rifampicin resistance with Heteroduplex analysis in both TB patient strains and control strains, a 305-bp fragment, in which mutations were frequently observed in the *rpoB* gene associated with Rifampicin resistance, was amplified by PCR. The sequences of the primers were (5' to 3') CAG ACG TTG ATC AAC ATC CG and TAC GGC GTT TCG ATG AAC.[25] A reaction mixture of 50 µL containing the primers (50 pM each), Taq DNA polymerase buffer(1.25U), $MgCl_2$ (1.25 mM), 10 x PCR enhancer, deoxynucleoside triphosphates mix (20 mM), 10 x PCR buffer, and the sample was prepared. DNA was denatured for 3 min at 94ºC; 40 amplification cycles were performed with an automated thermal cycler (Thermal cycler; PTC-100; MJ Research, USA).Each cycle consisted of denaturation at 94ºC for 1 min, annealing of primers at 60ºC for 1 min, and primer extension at 72ºC for 1 min. These amplified products were separated on 1.5 % agarose gel electrophoresis. Gel stained by ethidium bromide was illuminated by UV source. It was detected 305 base pairs DNA bands, target DNA regions. Figure 1 shows gel electrophoresis images of the susceptible control bacterium and the strains isolated from patients amplified by PCR in this study.

Figure I. The gel electrophoresis images of a susceptible control bacterium and the strains isolated from patients amplified by PCR. M: Marker (ϕx174 HaeIII); 1:Positive control(a patient isolate at first known to be positive); 2:Control bacterium (*M. tuberculosis* H37Ra); 3, 4, 5 and 6: Patient isolates; bç: base pair.



Heteroduplex Analysis: Rifampicin resistance was also investigated by Heteroduplex analysis as well as conventional culture techniques. Rifampicin-sensitive *M. tuberculosis* H37Ra obtained from the American Type Culture Collection was used as a control strain. DNA of control strain and DNA of patient strain were mixed at equal amount in a fresh tube. One µL 0.1 M EDTA was added to tube for Taq polymerase inhibition. Tubes were firstly heated until 95ºC in thermal cycler for DNA denaturation. Then, tubes were cooled gradually until 25ºC within 30 minutes so that DNA chain can again annealed with the other chain which is proper. Heteroduplex was produced by annealing again 2 apart chairs. These products were separated on mutation detection gel, Bio Rad Protean II xi electrophoresis containing 15% urea. Gel was stained with ethidium bromide and illuminated on transilluminator.

RESULTS

After the microscopic examinations and cultivations of clinical samples over a 2-year period (1997 to 1998), culture-positive 160 MTB strains were randomly selected for the study.

The 125 (78.1) of 160 MTB strains selected were smear-positive, 35 (21.9) strains were smear-negative (Table II). Culture and drug sensitivity test were made on Loewenstein-Jensen medium. According to susceptibility testing done by conventional culture method, 127 (79.4) of 160 MTB strains were Rifampicin resistant and 33 (20.6) were Rifampicin sensitive. As a result of susceptibility testing, resistance to other antibiotics (INH, EB, STM) alongside rifampicin was also observed in some *M. tuberculosis* isolates. Nineteen strains were resistant to INH-RIF, 10 strains were resistant to STM-RIF and only 1 strain was resistant to EB-RIF. INH-STM-RIF resistance was detected in 35 strains. Five strains were resistant to INH-EB-RIF, whereas 1 strain was resistant to STM-EB-RIF. Resistance to 4 major antituberculosis drugs (INH-STM-EB-RIF) was determined in 19 strains.

Table II. The result of acid-fast bacilli stain (AFBS) of *M. tuberculosis* strains isolated various samples collected from patients with suspected and their susceptibility testing to rifampicin

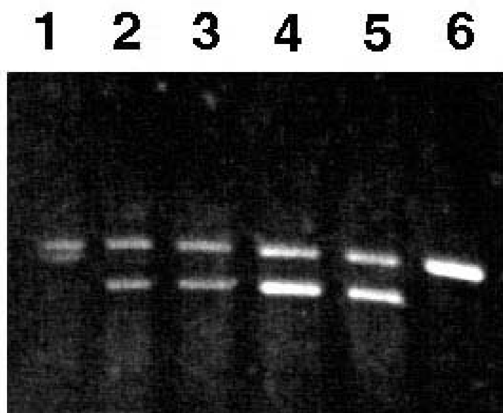
The number of *M. tuberculosis*	ARFS		RIFAMPICIN RESISTANCE	
160	POSITIVE (%)	NEGATIVE (%)	POSITIVE (%)	NEGATIVE (%)
	125 (78,1)	35 (21,9)	127 (79,4)	33 (20,6)

When Heteroduplex analysis was applied, it was found that 105 strains were RIF resistant and 55 strains were RIF susceptible (Table III). The 105 (71.7%) of 127 RIF resistant strains detected by conventional culture method were in parallel with Heteroduplex analysis, while 26 (28.3%) resistant strains were observed to be sensitive to RIF with Heteroduplex analysis. Figure 2 illustrates rifampicin susceptibility of some *M. tuberculosis* strains by Heteroduplex analysis. RIF resistance mutations revealed double bands when the 305-bp PCR products from RIF resistant *M. tuberculosis* strains were analyzed by PCR-Heteroduplex analysis. In contrast, single bands representing the homoduplex at 305-bp were detected when RIF susceptible strains were analyzed.

Table III. The ARFS dispersions of strains detected rifampicin drug susceptibility by conventional method and heteroduplex analysis

ARFS	Conventional technique		Heteroduplex analysis	
	Positive	Negative	Positive	Negative
Positive	103	22	88	37
Negative	24	11	17	18
Total	127	33	105	55

Figure II. Detection of rifampicin-resistant *M. tuberculosis* strains isolated from different patients on mutation detection gel by Heteroduplex analysis. 1, 2, 3, 4, 5: Five different rifampicin-resistant strains isolated from patients. 6:Control bacterium (a patient strain at first known to be rifampicin-susceptible).

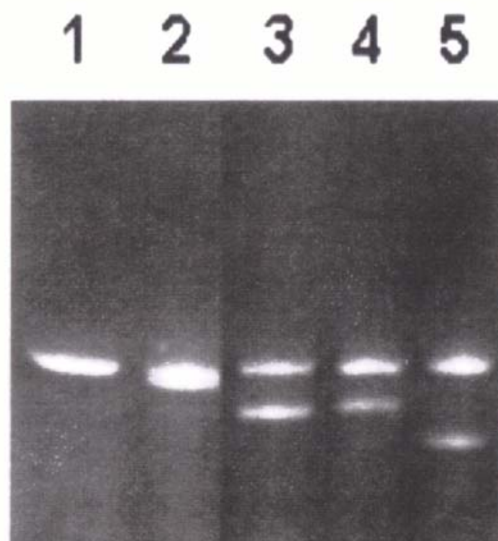


When the bands produced by 105 RIF susceptible strains on the mutation detection gel by heteroduplex analysis were examined, 4 different pair-band were observed. These different appearing band figures have been thought to occur due to mutations in different zones. Table IV shows the frequency of 4 different band-pair shapes in rifampicin-resistant strains detected by heteroduplex analysis. Figure III also shows Heteroduplex figures of various *rpoB* gene mutations in RIF-resistant mycobacteria isolated from patients.

Table IV. The frequency of different band-pair shapes in rifampicin-resistant strains detected by heteroduplex analysis

The kinds of heteroduplex band	The number of resistant strains
Shape 1	12
Shape 2	49
Shape 3	27
Shape 4	17
Total	105

Figure III. Heteroduplex figures of various *rpoB* gene mutations in rifampin-resistant mycobacteria isolated from patients.1:Control bacterium (*M. tuberculosis* 37Ra); 2, 3 ,4, and 5: 4 different band shapes



DISCUSSION

Rifampicin is an important drug for treatment of TB. The resistance developing to that drug causes considerable problems in developing countries. To reduce the morbidity and mortality rates of the patients infected with RIF-resistant MTB strains, it is necessary to identify the RIF-resistant MTB strains accurately in a shorter time.

In the present study, the conventional susceptibility testing by culture was compared with PCR-Heteroduplex analysis for a rapid detection of RIF resistance in *M. tuberculosis* strains.

Rifampicin susceptibilities of total 160 strains that were known to be RIF-resistant and RIF-susceptible as a result of conventional culture method were tested in much shorter time as 2 days by PCR-Heteroduplex analysis which is a rapid molecular method. The 55 (34.4%) of 160 isolates were RIF-susceptible, 105 (65.6%) were RIF-resistant. The 101(79.5%) of 127 RIF-resistant isolates detected by conventional culture method were in concordance with heteroduplex analysis, while 26 (20.5%) strains were sensitive to RIF. The 29 isolates were detected as susceptible according to both methods, 26 isolates were susceptible according to only heteroduplex analysis. The reason for this difference is that mutations can be out of the region amplified by PCR. In our study, 4 different pair-bands were observed by Heteroduplex analysis. It is thought that 4 different pair-bands due to different mutations in various amino acids. The Heteroduplex profiles are specific for each mutation that confers RIF-resistant. DNA sequencing analysis is necessary to identify the region that these mutations lead to RIF resistance. Besides, in our study, RIF resistance of 4 strains was not observed with conventional technique, whereas these 4 strains were detected to be resistant to RIF with heteroduplex analysis. As a result, concordance with conventional culture method and PCR-heteroduplex method were 83%.

Previous studies on the usefulness of PCR-Heteroduplex in assessing RIF resistance have shown good results when compared with other methods.[26,27,28,29,30] Thus, for drug susceptibility tests in slow growing mycobacteria are enough to use only a small amount sample.

Williams et. al. detected mutations in *rpo*B gene encoding RNA polymerase enzyme in *M. tuberculosis* RIF-resistant isolates by using Heteroduplex analysis.[27] They made DNA sequencing analysis of total 120 MTB strains collected from different geographical regions.(110 RIF-resistant and 10 RIF-susceptible).They observed 16 mutations affecting 13 amino acids in RIF-resistant strains. At the result of their study, it was decided that PCR-heteroduplex analysis gave easily interpretable results and did not require radioactively labeled compounds to complete the test and therefore provides an assay which may show promise for application in the clinical laboratory. In our study, we could not detect mutation region because of impossibility in DNA sequencing analysis. Drobniewski and Wilson emphasized that Heteroduplex analysis is the best technique to detect rapidly resistance to RIF and isoniasid in *M. tuberculosis*.[26] Again, Williams et. al. tested sputum specimens of totally 665 patients suspected of TB by using PCR-Heteroduplex analysis.[29] They, firstly, investigated ARFS from sputum samples in microscopic examinations. In susceptibility testing, only 6 of 44 isolates were RIF-resistant. When they repeated susceptibility testing by Heteroduplex analysis, they found 5 of 44 isolates were RIF-resistant. In order to detect RIF resistance in culture-positive samples, Heteroduplex analysis was observed to be susceptible in the rate of 83 %. This rate is in concordance with that of our study. Kim et. al. analyzed RIF-resistant MTB strains by PCR-SSCP (Single- Stranded Conformational Polymorphism) and DNA sequencing analysis.[31] They realized that PCR-SSCP technique would cause incorrect positive results. Therefore, PCR-Heteroduplex analysis used in our study gives more reliable result than PCR-SSCP technique.

Recently, since it has been a recognized target for most of the antituberculosis drugs, Heteroduplex analysis can be also used to detect the resistance produced against other antituberculosis drugs in order to yield accurate analysis in a shorter time. PCR for diagnosis of drug resistance is not suitable in developing countries since it is relatively expensive. However, because the PCR-Heteroduplex method would shorten the duration of secondary susceptibility testing, it can be suggested as an alternative diagnosis of MTB in clinical laboratories.

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