



ARAŞTIRMA / RESEARCH

Molecular epidemiological typing of *M. tuberculosis* isolates isolated from Turkey's Eastern Anatolia with in house PCR method

Türkiye'nin Doğu Anadolu bölgesinden izole edilen *M. tuberculosis* izolatlarının inhouse PCR yöntemi ile moleküler epidemiyolojik tiplendirilmesi

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Abstract

Purpose: The aim of this study as to compare microscopy and culture results of samples, determine drug resistance rates of the isolates, evaluate epidemiological relationship between the strains with ERIC-PCR, RAPD-PCR, OUT-PCR based on in house PCR technique.

Materials and Methods: Direct microscopy and culture results of 2010 samples were analyzed. Drug sensitivity results were obtained from TULSA. The typing of isolates based on in house PCR was carried out in the microbiology laboratory of a faculty of medicine in a state university.

Results: Of positive samples, 2.68% had Acid-resistant bacilli (ARB) positive + culture positivity, 2.93% had ARB positive and culture negative in smear, and 1.34% had ARB negative and culture positivity in smear. Resistance to primary antituberculous (anti-TB) drugs wasn't observed in 33 culture positive isolates, whereas resistance to one or more primary anti-TB drugs was observed in 9.09%. Single drug resistance was 3.03%, resistance to Isoniazid (INH) and INH critical drugs was 6.06%. Isolates were divided 3 groups by ERIC-PCR, 5 groups for OUT-PCR and 6 by RAPD-PCR.

Conclusion: Positivity rates were low due to low rate of studied samples, negative samples taken during treatment process. Molecular techniques like ERIC-PCR, RAPD-PCR and OUT-PCR are easy, fast and inexpensive methods for the epidemiological typing of *Mycobacterium tuberculosis* (MTB) in evaluating distinctions, similarities between origins.

Keywords: *Mycobacterium tuberculosis*, EZN, ERIC-PCR, RAPD-PCR, OUT-PCR

Öz

Amaç: Örneklerin mikroskopi ve kültür sonuçlarının karşılaştırılması, izolatların ilaç direnç oranlarının belirlenmesi, suşlar arasındaki epidemiyolojik ilişkilerin ERIC-PCR, RAPD-PCR, OUT-PCR ile ev içi PCR tekniğine dayalı olarak değerlendirilmesi amaçlandı.

Gereç ve Yöntem: 2010 numunenin direkt mikroskopi ve kültür sonuçları analiz edildi. İlaç duyarlılık sonuçları TULSA'dan elde edildi. İzolatların tiplendirilmesi, bir devlet üniversitesinin tıp fakültesinin mikrobiyoloji laboratuvarında, in house PCR yöntemi ile gerçekleştirilmiştir.

Bulgular: Pozitif örneklerin %2.68'inde Aside dirençli basil (ARB) pozitif + kültür pozitifliği, yaymada %2.93'ünde ARB pozitif ve kültür negatif, yaymada %1.34'ünde ARB negatif ve kültür pozitifliği vardı. Kültür pozitif olan 33 izolatta primer antitüberküloz (anti-TB) ilaçlara direnç görülmezken, bir veya daha fazla primer anti-TB ilaca direnç %9.09'da gözlemlendi. Tek ilaç direnci %3.03, Isoniazid (INH) ve INH kritik ilaçlara direnç %6.06 idi. İzolatlar ERIC-PCR ile 3 gruba, OUT-PCR için 5 grup ve RAPD-PCR ile 6 gruba ayrıldı.

Sonuç: Çalışılan numune oranının düşük olması, tedavi sürecinde alınan numunelerin negatif olması nedeniyle pozitiflik oranları düşüktü. ERIC-PCR, RAPD-PCR ve OUT-PCR gibi moleküler teknikler, kökenler arasındaki farklılıkları, benzerlikleri değerlendirmede *Mycobacterium tuberculosis*'in (MTB) epidemiyolojik tiplendirilmesi için kolay, hızlı ve ucuz yöntemlerdir.

Anahtar kelimeler: *Mycobacterium tuberculosis*, EZN, ERIC-PCR, RAPD-PCR, OUT-PCR

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INTRODUCTION

Tuberculosis (TB) is an infectious disease caused by *Mycobacterium tuberculosis* (MTB) bacillus, one of the top 10 causes of death worldwide, and it is an important public health problem. In 2019, approximately 10 million new cases were TB, including HIV-positive (0.2 million) individuals, and 1.4 million people died from this disease ¹.

As a global problem, TB continues to be a major public health problem in Turkey ². According to the 2019 Tuberculosis in Turkey report, published by the TR Ministry of Health, General Directorate of Public Health, The Head Department of Tuberculosis, 12,046 detected tuberculosis cases were found in 2017. It was seen that 11,101 (92%) of them were new cases. Turkey's 2017 estimated incidence rate was 17 per 100 thousand, and the estimated mortality rate was given as 0.53 per 100 thousand ³.

The development of rapid and reliable diagnostic tests is extremely important in providing the correct treatment early and reducing TB cases ⁴. Direct microscopy of Ziehl-Neelsen stained sputum smears used for TB diagnosis is a relatively fast, inexpensive and highly specific method. However, direct Ziehl-Neelsen microscopy's sensitivity is low (50-60%), and it is less sensitive in HIV co-infected patients, children, and patients with extra-pulmonary TB ⁵. Ziehl-Neelsen-stained sputum smears made by decontamination and centrifugation using chemicals such as NaOH and sodium hypochlorite (Isolab) slightly increase this sensitivity ⁶. Today, the method accepted as the gold standard in diagnosing TB disease is the culture method and has an incubation period of 6-8 weeks. The advantage of culture methods is detecting live mycobacteria, isolating bacilli, and the ability to study antimicrobial susceptibility tests ⁷. Correct isolation with culture is the most important step in the definitive diagnosis of tuberculosis. False-positive cultures are caused by contaminated clinical equipment, errors in the preanalytical and analytical process, or cross-contamination of samples. Rapid diagnosis of *M. tuberculosis* complex (MTBC) can be made directly from the clinical sample at the species level or culture by molecular methods. Besides, molecular methods are used for drug resistance detection and TB epidemiology studies ⁸. WHO has approved the use of recently produced commercial tests designed in different formats based on real-time PCR for TB diagnosis and resistance determination⁹⁻¹¹.

False-positive results for TB have been a cause of concern when the clinical, treatment success and social effects of the misdiagnosis of TB are considered ¹²⁻¹³. Simultaneously, these false-positive cultures lead to an overestimation of the incidence and prevalence of tuberculosis in humans. In studies conducted, the contamination frequency of MTBC has been reported between 0.1% and 3%. Contamination indications include culture results that do not match the patient's clinical course, undesirable and unexpected drug resistance, single culture-positive samples, and cultures with low colony numbers ¹⁴⁻¹⁶. Another concern is TB treatment and control problems due to the widespread resistant isolates ¹⁷. Drug resistance surveillance in TB is an important component of the TB control program ¹⁸. Turkey is not among the 30 countries with the highest disease burden in multidrug-resistant tuberculosis (MDR-TB). In the 2018 Global TB report, it was estimated that in Turkey Rifampicin-resistant/multidrug-resistant-tuberculosis (RR / MDR-TB) ratio was reported as 3.3% in new TB cases and 14% in previously treated cases ².

Molecular genotyping methods are widely used in epidemiological typing of MTB isolates in the laboratory. It is important to investigate the relationship between bacterial origins, identify the infection source, and take necessary control measures. Molecular methods used for epidemiological typing purposes are IS6110 RFLP, spoligotyping, MIRU-VNTR and MST ¹⁹⁻²⁰. Among these, the accepted method as the gold standard is the IS6110 RFLP method. These methods cannot be implemented everywhere due to the need for infrastructure, cost and experienced personnel. For this purpose, epidemiological typing methods based on in-house PCR (ERIC-PCR, OUT-PCR, RAPD-PCR) are used for small-scale epidemiological origin identification and identification in-laboratory cross-contamination ²¹. In the future, different molecular typing tools such as ERIC-PCR, RAPD-PCR and OUT-PCR can be used to determine the original relationship between MTB isolates.

For tuberculosis, classical laboratory methods are used and it takes a long time to determine the culture results. It is necessary to wait for a long time for the diagnosis of the disease. In this study, it was aimed to compare the epidemiological relationship between the isolates diagnosed with tuberculosis and tested for drug susceptibility tests, with three typing methods (ERIC-PCR, RAPD-PCR, OUT-PCR)

based on the domestic PCR technique, instead of the classical methods in the literature.

MATERIALS AND METHODS

Study design

This study was conducted with the approval of the Ministry of Health Van Training and Research Hospital Clinical Research Ethics Committee (Decision No: 2020/17). In our study, the results of 2010 various clinical samples pre-diagnosed with TB sent to our Tuberculosis Department of Public Health Laboratory between January 2019 and December 2019 were examined, and positive results were included in the study.

As a result of the examination, direct microscopy and culture-positive results of 2010 samples were recorded. These consist of 130 sputa, 1 urine, 4 fasted human gastric fluid (HGF), 4 broncho-alveolar lavages (BAL), 1 cerebrospinal fluid (CSF) sample. In the study, TB laboratory sample logbook and Tuberculosis Laboratory Surveillance Network (Tulsa) were used. Epidemiological typing tests were carried out in Adiyaman University Faculty of Medicine, Department of Medical Microbiology. The age and gender of positive cases were recorded. After documentation of the obtained data, the following parameters were evaluated:

1. Smear positive, culture positive
2. Smear positive, culture-negative
3. Smear negative, culture positive
4. Contamination
5. Sensitivity results of positive samples to anti-tuberculosis drugs.
6. Epidemiological typing

Microscopy and culture methods

Homogenization and decontamination of non-sterile clinical samples such as sputum, Fasted HGF, BAL was performed using commercial kits based on N-acetyl-L-cysteine and 4% sodium hydroxide method. Decontamination was not applied to sterile clinical samples. Direct and processed smears prepared from clinical samples were stained with the Ehrlich-Ziehl-Neelsen (EZN) method and examined under a light microscope for the presence of Acid Resistant Bacilli (ARB). The solid medium was inoculated into

Lowenstein-Jensen (LJ) medium and left for incubation from the prepared samples. Growth in LJ medium was checked once a week for eight weeks. At the end of this period, non-reproductive samples were evaluated as negative. Growing media were recorded.

Drug sensitivity tests

Isolates identified as MTBC in our study were sent to the Ministry of Health National Tuberculosis Reference Laboratory for verification and Drug Sensitivity Tests (DST). Resistance rates to first-choice anti-TB drugs such as INH, rifampicin (RIF), ethambutol (ETM), streptomycin (SM) and sensitivity of the samples with resistance to other drugs (Pyrazinamide (PZA) and INH critical) were obtained from Tulsa.

Epidemiological methods

In our study, three typing methods (ERIC-PCR, RAPD-PCR, OUT-PCR) based on in-house PCR technique were performed on culture-positive isolates. 13 of 44 MTBC isolates could not be evaluated because their DNA amount was low. A total of 31 isolates, 2 AMS, 2 BAL and 27 sputum samples, were studied for ERIC-PCR, RAPD-PCR and OUT-PCR tests. All studies on clinical material were performed in a class II Biosafety cabinet, and clinical material processing, microscopy and culture procedures were performed following the standards defined by the World Health Organization (WHO)^{11,22}. DNA extraction from isolates and control strains was done according to the boiling method -. DNA extract of each sample was stored at -20 ° C. For OUT-PCR, CGG GGG TTC CCGGAC III (I: Inosine) (İontek, Bursa) A single primer was used to amplify IS6110 in the outer region. The PCR reaction was subjected to 40 cycles of amplification. (3 min at 94 ° C, 1 minute at 94 ° C, 1 minute at 62 ° C), At 72 ° C, this was followed by a minimum extension of 1 minute.

For ERIC-PCR, ERIC1R (Tb-2) 5'ATG TAA GCT CCT GGG GATTCA C and ERIC2 (Tb-3) AAG TAA GTG ACT GGG GTG AGC G (İontek-Bursa) primer pairs were used. The composition of the PCR mix (50µl) was 5µl 10x Taq PCR buffer (500mM KCl, 100mM Tris-HCl, pH: 8.3), Master Amp™, 5µl 10x PCR Enhancer (Center, Technologies), 3µl MgCl₂ (25mM), 5µl dNTP (mix2mM), 0.75µl ERIC1R (87 pmol / µl) primer, 0.9µl ERIC2 (73 pmol / µl) primer and Taq polymerase 1U (0.2µl) and 10µl template

DNA. The PCR reaction was performed for 35 cycles of amplification (2 minutes at 94 ° C, 45 seconds at 94 ° C, 1 minute at 52 ° C) and a final extension at 72 ° C for the last 20 minutes. The amplified product's presence was confirmed with 1.4% NuiSieve agarose gel (Sigma, StLouis, MO, USA). For DNA band analysis, UVI soft, UVI tape, Windows Application V99.06 (BioRad, UK) program was used.

Statistical analysis

Statistical analyses were performed using SPSS software version 23.00. Descriptive statistics were used for demographic data sample types, culture and ARB results. Descriptive analyses were presented using the frequencies.

RESULTS

Of the positive patients, 9.2% (13) were under the age of 15, 15% (2) of the patients under the age of 15 were male and 85% (11) were female. Of the patients over 15 years old, 64.6 % (82) were male and 35.4 % (45) were female. While the mean age of positive samples was 43 for female patients, it was 40 years for male patients. When the data is examined, 92.86% (130) of the positive samples were sputum, 2.86% (4) was BAL, 2.86% (4) was fasted HGF, 0.71% (1) was a urine sample, and 0.71% (1) was a sample of CSF. ARB and culture positivity in smear were most frequently detected in sputum samples. ARB and culture distributions of the positive samples are included in Table 1.

Table 1. ARB and culture distributions of Positive Samples. N (%).

	Culture Positive	Culture Negative	Total
ARB Negative	27 (1.34%)		
ARB Positive	54 (2.68%)	59 (2,93%)	113 (5.67%)
Total	81(4.02%)		

ARB: Acid resistant bacilli

Table 2. Distribution of anti-tuberculosis drug susceptibility test results

Anti-TB medication	Anti-TB drug sensitivity studied.		Resistant Isolate n (%)
	Resistant Isolate n	Total Isolate n	
Streptomycin (SM)	1	34	2.94
Isoniazid (INH)	2	34	5.88
Rifampicin (RIF)	0	34	-
Ethambutol (ETM)	0	34	-
Pyrazinamide (PZA)	0	34	-
Isoniazid (INH) critical	2	34	5.88
Single drug resistance			
Streptomycin (SM)	1	34	2.94
Isoniazid (INH)	0	34	-
Rifampicin (RIF)	0	34	-
Ethambutol (ETM)	0	34	-
Two drug resistance			
INH/RIF	0	34	-
INH/ETM	0	34	-
INH/SM	0	34	-
INH/INH CRITICAL	2	34	5.88
RIF/ETM	0	34	-
RIF/SM	0	34	-
ETM/SM	0	34	-

SM; Streptomycin, INH; Isoniazid, RIF; Rifampicin, ETM; Ethambutol, PZA; Pyrazinamide

As shown in Table 1, the rate of ARB positivity in smear was 5.67% (113 /2010), and the rate of culture positivity was 4.02% (81/2010). In 2.68% (54/2010) of the samples, both ARB positive and culture positivity in the smear, ARB positive and culture-negative in 2.93% (59/2010) smear, ARB negative and culture positive in 1.34% (27/2010) of the samples were detected. 0.04% (1/2010) of the samples were evaluated as contamination. Distribution rates of anti-tuberculosis drug susceptibility test results of culture-positive samples within the scope of the study are given in Table 2.

In 34 isolates found to be culture-positive in the study, no resistance was observed against first-choice anti-TB drugs, whereas resistance was observed against one or more first-choice anti-TB drugs in 8.82% (3/34). Single drug resistance was detected in 2.94% (1/34) of the isolates, and resistance to INH and INH critical drugs was found simultaneously in 5.88% (2/34) of the isolates. In the comparisons made to determine the effect of gender on anti-TB drug resistances, single resistance rates to at least one anti-TB agent or drugs (INH, RIF, ETM, and SM) were seen only in our female patients, and it was found that all of them were over 15 years old.

In our study, three typing methods (ERIC-PCR, RAPD-PCR, OUT-PZR) based on in-house PCR technique were performed on culture-positive isolates. 13 of 44 MTBC isolates could not be evaluated because their DNA amount was low.

A total of 31 isolates, 2 AMS, 2 BAL and 27 sputum samples, were studied for ERIC-PCR, RAPD-PCR and OUT-PCR tests.

In the OUT-PCR method, 5 main groups were determined 100% compatible with each other. These samples, which were compatible with each other, were collected in the same group genotypically (Figure 1).

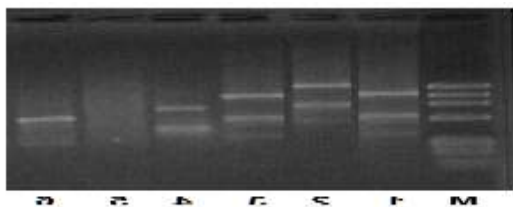


Figure 1. OUT PCR Results

Nusieve Agarose isolates with ERIC-PZR were collected in 3 groups according to two

electrophoresis results. However, these groups were not sufficient for the degree of separation between isolates (Figure 2).

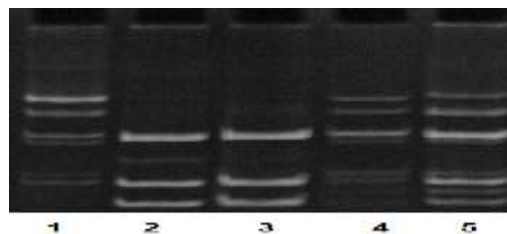


Figure 2. ERIC PCR Results

In the study we conducted with the RAPD-PCR method, isolates were divided into 6 groups (Figure 3).

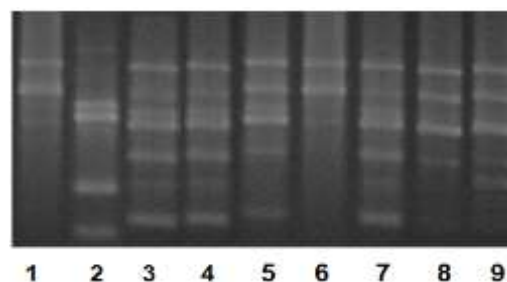


Figure 3. RAPD-PCR Results

When each PCR test is evaluated within itself; It has been observed that it can make precise identification within the group in determining the origin relationships among the strains examined. When the same samples were evaluated comparatively for each PCR test, 20% agreement was observed between OUT-PCR and ERIC-PCR, 8% between OUT-PCR and RAPED-PCR, and 17% between ERIC-PCR-RAPED PCR in the same strain.

DISCUSSION

The emergence of approximately ten million new TB cases with HIV epidemics every year, the increase in MDR-TB and XDR-TB cases worldwide in recent years, the death of approximately one and a half million people from TB disease caused the necessity to examine TB disease¹. Although direct microscopy examination, which is widely used in TB diagnosis, is fast and inexpensive, it has low sensitivity. The culture method, which is the gold standard in diagnosis, is time-consuming and requires trained personnel with additional biosecurity measures²³⁻²⁴.

Increasing resistance to TB drugs and MDR-TB development has been one of the most important problems encountered in the world in providing TB control. The treatment of patients with MDR-TB is less effective, more difficult, low in success rate, high toxicity and requires expensive drugs, and the mortality rate is higher than the drug treatment used in first-line anti-TB treatment^{11,25,26}.

Molecular typing methods are an important indicator for determining the source in cross-contamination cases in tuberculosis laboratories¹⁴⁻¹⁵. This method is important to determine the epidemiological relationship by determining some similar genotypes that cause a false increase in the group^{19,20,27}.

In our study, the mean age of positive samples was 43 for female patients and 40 for male patients. 9.2% of the patients were under the age of 15, 15% of the patients under the age of 15 were male, and 85% were female. While 64.6% of the patients over the age of 15 were male, 35.4% were determined to be female.

Other study observed that the male/female ratio was 2.9, and 98.5% of the patients were in the adult age group, while 1.5% was in the pediatric age group²⁸. In other studies, the mean age of the cases was found to be 27.9, and tuberculosis was reported to be more common in young adults²⁹. Also, in Turkey, in different studies, it was observed that the men/female ratio of 1.5-2.5, while the proportion of adult patients has been shown to vary from 67 to 82.5%³⁰⁻³³.

ARB positivity rate was 5.67%, and culture positivity rate was 4.02%. In the study of Özen et al., these rates were 4%, 3.4%, respectively, and 3% and 4.6% in the study of Alışkan et al.^{28,34}. In different studies, it was observed that the rate of ARB positivity ranged from 1.6-6.5%, and culture positivity ranged from 1.7-7.4%^{30,31,32,35}. In 2.68% of the samples, both ARB positive and culture positivity were detected in both smears.

In our laboratory, 92.9% of MTBC isolates were isolated from respiratory system samples. Özen et al. found this rate as 98% in their study²⁸. In different studies carried out in Turkey this ratio is between 56.2-91%^{34,30,31}.

The contamination rates in culture were found to be 0.04% in our study. Özen et al., Kurtoğlu et al. reported the contamination rates as 4.3% and 4%, respectively^{28,30}. In different studies, the frequency of contamination has been reported between 0.1% and

3%¹⁴⁻¹⁶. Contamination rates are recommended to be between 3-5%³¹.

Resistance to anti-TB drugs creates an important problem in our country, as in the whole world^{3,33}. Our study determined that the highest rate of resistance developed against INH (5.88%), the rate of at least one drug-resistant isolate was 8.82%, and the MDR-TB rate was 0%. When the literature was examined, it was seen that the resistance against INH was highest³²⁻³⁴. Özen et al. determined that the resistance against INH was 12.8%, the rate of isolate resistance to at least one drug was 22%, and the MDR-TB rate was 1%²⁸.

In our study, the NTM growth rate was found to be 0.19%. In different studies, NTM rates were reported to range from 1.1-2.5%³⁶⁻³⁷. The sample type in which NTM was observed in our study was compatible with the literature, 100% of its growth was isolated from respiratory tract samples, and the prevalence of non-respiratory NTM infections is low, as in our study³⁸⁻³⁹.

The strains obtained from the clinical isolates examined in our study were divided into 3 groups with ERIC-PZR, 5 groups with OUT-PZR and 6 groups of RAPD-PZR. Tarhan et al. reported that they divided them into 3 groups by ERIC-PCR, 5 groups by OUT-PCR and 6 by RAPD-PCR⁴⁰.

The positivity rates in the samples taken from the regions we examined were lower than in many other regions of our country. We think that this low positivity may be due to the low sample rate at the time of diagnosis, the study of patient samples with reduced bacillus load, and the negative repetitive samples taken during the treatment follow-up of smear/culture-positive pulmonary tuberculosis patients, and the patients being diagnosed outside the province.

As a result, this study was carried out on 140 samples with suspected tuberculosis sent to the public health laboratory within a one-year period. The data obtained covers a single center. Our data is a cross-sectional data and is planned as point surveillance. One of the limitations of this study is the necessity of conducting a comprehensive study in the whole region and multicenter.

Molecular techniques such as ERIC-PCR, RAPD-PCR and OUT-PCR have not been encountered in molecular epidemiological typing studies in MTBC isolates with in-house PCR methods. In our

experience, each test alone gave reliable results in establishing the basic distinction between strains and in determining the relationship between strains. These tests can also be used to understand cross-contaminations seen in routine work in laboratories⁴⁰. ERIC-PCR, RAPD-PCR and OUT-PCR methods that we used for molecular epidemiological typing of MTB in this study are easy to apply, simple, fast and inexpensive methods for identifying the origins of strains and their relationships with each other. Another limitation of this study is the need for a multicenter with more samples to use these methods in routine practice.

Yazar Katkıları: Çalışma konsepti/Tasarımı: EA, GT, FŞ, SE ; Veri toplama:EA ; Veri analizi ve yorumlama:EA,GT ; Yazı taslağı: EA, GT, FŞ, SE ; İçeriğin eleştirel incelenmesi:GT ; Son onay ve sorumluluk: EA, GT, FŞ, SE ; Teknik ve malzeme desteği: EA, GT;Süpervizyon:GT ; Fon sağlama (mevcut ise): yok.

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