Susceptibility Rates of Pulmonary and Extrapulmonary 229 *Mycobacterium tuberculosis* Isolates to Major Antituberculous Drugs and Comparison of E test and Standard Proportion Methods

Akciğer ve Akciğer Dışı Kaynaklı 229 *Mycobacterium tuberculosis* İzolatının Majör Antitüberküloz İlaçlara Duyarlılık Oranları ve E Test ile Standart Proporsiyon Yöntemlerinin Karşılaştırılması

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ABSTRACT Objective: Following the antimycobacterial resistance that has been emerging, and struggling the against resistance has become as important as effective treatment in tuberculosis control. The drug susceptibility test (DST) results of 229 *M.tuberculosis* (MTB) which had been isolated on Lowenstein-Jensen (L-J) medium from various clinical samples were evaluated. **Material and Methods:** All isolates are originated from112 pulmonary samples and 117 extrapulmonary samples. After decontamination and L-J culture procedures, all isolates were identified as MTB complex by using conventional biochemical tests including niacin, nitrate, catalase and catalase tests implemented at 68°C. DST was performed by E test (AB Biodisk, Sweeden) method for isoniazid (INH), rifampicin (RIF), ethambuthol (EMB) and streptomycin (SM) according to manufacturer advices. L-J proportion method was performed for 59 MTB isolates in addition to E test. **Results:** The overall resistance rates of INH and RIF were 4% and resistance to SM was 2% and of EMB was 1%. The resistant isolates were found generally in the isolates originated from pulmonary samples. The resistance rates to INH and RIF were 7%, to SM 3% and to EMB 2% for pulmonary; and to INH, RIF and SM were 1% and to EMB was 0% for extrapulmonary isolates, respectively. INH and RIF resistance was significantly higher in pulmonary isolates ($p< 0.05$). In 59 MTB isolates, agreement between E test and L-J proportion was 95% for INH and EMB, 86% for RIF, and 85% for SM. **Conclusion:** According to DST results, antimycobacterial resistance rates in extrapulmonary MTB isolates were significantly lower than pulmonary isolates. E test method which is known as a reasonably fast test in comparison with conventional susceptibiliy tests may be used as a rapid diagnostic test method for DST. However, there is a need for further E test studies comparing standard methods in more MTB isolates.

Key Words: *Mycobacterium tuberculosis*; tuberculosis, multidrug-resistant; tuberculosis; microbial sensitivity tests

ÖZET Amaç: Ortaya çıkmakta olan antimikrobiyal direncin ardından dirence karşı mücadele tüberküloz kontrolünde etkili tedavi kadar önemli hale gelmiştir. Çeşitli klinik örneklerden alınan, Lowenstein-Jensen (L-J) besiyerinde izole edilen 229 *M. tuberculosis* (MTB) 'un duyarlılık test sonuçları değerlendirildi. **Gereç ve Yöntemler:** Tüm izolatların 112'si akciğer örneklerinden ve 117'si akciğer dışı örneklerden meydana gelmiştir. Kontaminasyon ve L-J kültür işlemlerinden sonra, tüm izolatlar niasin, nitrat, katalaz ve 68°C'de yapılan katalaz testlerini içeren klasik biyokimyasal testler kullanılarak MTB kompleksi olarak tanımlandı. Antimikrobiyal duyarlılık testleri üreticisinin önerilerine göre izoniazid (INH), rifampisin (RIF), etambutol (EMB) ve streptomisin (SM) için E test (AB Biodisk, İsveç) yöntemiyle yapıldı. Elli dokuz MTB suşu için E test ile birlikte L-J proporsiyon metodu uygulandı. **Bulgular:** Genel direnç oranları INH ve RIF'e karşı %4, SM'e karşı %2 ve EMB 'e karşı%1 idi. Dirençli izolatlar genellikle akciğer örneklerinden alınan izolatlardı. Direnç oranları akciğer izolatları için INH ve RIF'de %7, SM'de %3 ve EMB'de %0 ve akciğer dışı izolatlar için INH, RIF ve SM'de %1 ve EMB'de %0'dı. INH ve RIF direnci akciğer izolatlarında belirgin olarak yüksekti ($p< 0.05$). L-J proporsiyon ve E test metodu arasında 59 suş için saptanan uyumluluk; INH ve EMB için %95, RIF için %86 ve SM için %85 idi. **Sonuç:** Çalışmamızda elde edilen ilaç duyarlılık sonuçlarına göre, ektrapulmoner kökenli MTB izolatlarında direnç, pulmoner izolatlara kıyasla anlamlı olarak düşüktü. Konvansiyonel duyarlılık testlerine kıyasla daha kısa sürede sonuç verdiği bilinen E test yöntemi, antimikobakteriyel duyarlılıkla ilgili hızlı tanı testi olarak kullanılabilir. Ancak, agar proporsiyon yöntemi ile karşılaştırıldığı çok daha fazla sayıda suşla yapılmış karşılaştırmalı çalışmalara ihtiyaç vardır.

Anahtar Kelimeler: *Mycobacterium tuberculosis*; tüberküloz, çok ilaç direnci; tüberküloz; mikrobiyal duyarlılık testleri

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Tuberculosis (TB), which is one of the oldest diseases in history, still is a threat for the public health. The incidence of the disease decreased until 1980 as a result of chemotherapy and tuberculosis control strategies. In the next 20 years, not only HIV infection but also the nontreatable resistant cases will continue to infect community and relevant diseases will rise again.[1] Clinical forms of extrapulmonary TB that are the most frequent cause of fever of unknown origin increased related to the HIV cases.[2]

Nowadays the basis of the control strategy is early detection of cases and completion of treatment. Rapid detection of microorganisms and DST are important in treatment of cases and infection control of TB, especially in extrapulmonary forms which are difficult to diagnose.

Although the disease has been known for a long time, effective chemotherapeutic agents were only found in the last century. Despite most cases can be treatable with current drugs, antimycobacterial resistance could not be prevented. Today we face with multidrug resistant TB and we need new effective therapeutic agents. Thus far application of directly observed treatment strategy (DOTS) will prevent emergence of secondary resistance. In addition, clinical microbiology laboratories have an important role in TB control for early and prompt diagnosis and follow up for drug resistance.[3]

The aim of this study is to (1): detect antimycobacterial susceptibilities of 229 *Mycobacterium tuberculosis* (MTB) isolates by E test, (2): evaluate significant resistance difference between pulmonary and extrapulmonary originated isolates.

MATERIAL AND METHODS

SETTING AND PATIENT POPULATION

Our hospital, in which mostly extrapulmonary TB cases are followed up, is a 550-bed tertiary referral center hospital in Istanbul, Turkey. In this study, some pulmonary isolates (59 samples) obtained during treatment of naive TB dispencery patients were evaluated besides pulmonary and extrapulmonary isolates obtained from in/outpatients of our hospital. Two hundred twenty nine MTB isolates isolated within the last 10 years were included in this study. Most of the isolates were obtained from the patients with pulmonary and extrapulmonary TB (especially tuberculous meningitis, tuberculous lymphadenitis, Pott disease, and etc.) from in/outpatients of our hospital.

CULTURE AND IDENTIFICATION

Samples were cultured in L-J medium following the standard digestion-decontamination procedure developed by Kent and Kubica for 45 days and growth control was done weekly.[4] Species identification was performed by using niacin, nitrate, catalase and catalase tests implemented at 68°C.[5]

ANTIMYCOBACTERIAL SENSITIVITY

The minimal inhibitory concentration (MIC) values of isolates identified as MTB were investigated for primary antituberculosis agents [isoniazid (INH), rifampicin (RIF), ethambutol (EMB) andstreptomycin (SM)] using E test. Bacterial suspensions prepared in liquid Middlebrook 7H9 medium from L-J medium equal to a McFarland 3 density were spread on solid Middlebrook 7H11 medium, then E test strips containing different concentrations of INH, RIF, EMB and SM were placed. Control medium plates prepared with H37Rv (ATCC 27294) isolate and all plates were incubated at 37°C for 5-7 days and then were evaluated according to the manufacturer advices. MIC values and MIC_{50}/MIC_{90} values were determined. All prosedures were performed in a class 2A biosafety cabin (Jouan™ MSC9, France).

Breakpoint values used for determination of antimycobacterial susceptibilities for MTB by E test are as follow.[6]

INH	0.2 μg/ml
SM	2 μg/ml
RIF	1 μg/ml
EMB	5 μg/ml

The drug susceptibility test (DST) results of 59 pulmonary isolates obtained from TB dispencery patients were also investigated by means of conventional L-J proportion method.

STATISTICAL ANALYSIS

All data input and statistical analysis were made using SPSS 17.0 Windows program (SPSS, Chicago, IL, USA) licensed to our institution. We researched the difference between pulmonary and extrapulmonary MTB isolates in terms of resistance to four drugs by Chi-Square test (Fisher's exact test) and a p value < 0.05 was accepted as a significant difference.

RESULTS

DST results of 112 pulmonary and 117 extrapulmonary MTB isolates were evaluated after 5-10 days from inoculation. The material distribution is shown in Table 1.

The resistance rates of 229 isolates were found considerably low. In Figure 1, MIC values of MTB isolates by E test are shown. In Table 2, MIC values of 229 MTB isolates for four major antituberculous drugs were summarized.

Most MIC values were detected in low concentrations. Resistance rates of extrapulmonary isolates were lower than pulmonary ones. Detected MIC_{50} and MIC_{90} values were close to each other and all were susceptible.

According to DST results of 229 MTB isolates, resistance rate was 4% for INH and RIF, 2% for SM and 1% for EMB. Resistance rates in pulmonary and extrapulmonary isolates are shown in Table 3.

TABLE 1: Material distribution of 229 *M.tuberculosis* strains.

Samples	n	%
Pulmonary Samples		
Sputum	102	44.5
Stomach fluid	10	4.4
Extrapulmonary Samples		
Abscess	47	20.5
Cerebrospinal fluid	30	13.1
Urine	10	4.4
Lymphadenopathy biopsy	9	3.9
Pleural fluid	7	3.1
Sinovial fluid	6	2.6
Ascites fluid	5	2.2
Pott biopsy	3	1.3
Total	229	100

There was a significant difference in DST results between pulmonary and extrapulmonary isolates ($p< 0.005$). Pulmonary isolates were more resistant to INH and RIF ($p< 0.004$, $p< 0.002$). Resistant isolates were mostly isolated from sputum, cerebrospinal fluid (CSF), stomach fluid (FSF) and ascites fluids, in rank order (Table 4).

Six multidrug resistant isolates were isolated from sputum (5) and CSF (1). One of 30 CSF-originated MTB isolates was multi-drug resistant (MDR). However, this strain was isolated from a patient with pulmonary and meningeal MDR TB and he had transferred to our hospital as a secondary MDR TB case from a Chest Diseases Hospital.

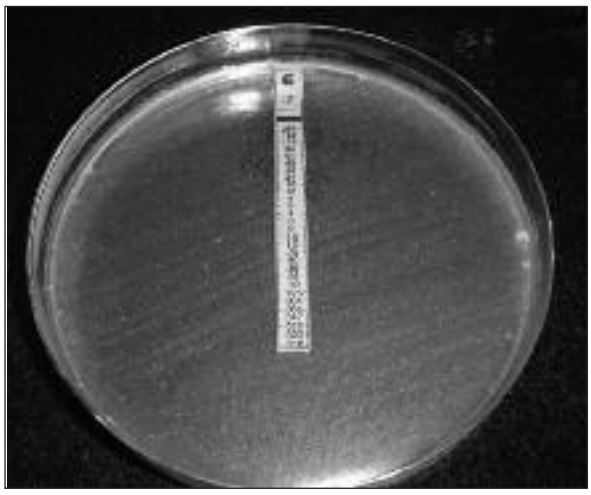

FIGURE 1: MIC value of a MTB isolate by E test.

TABLE 2: MIC values of 229 *M.tuberculosis* strains.

	EMB	INH	RIF	SM
	5 µg/ml	0.2 µg/ml	1 µg/ml	2 µg/ml
MIC_{50} values	0.02	0.02	0.02	0.06
MIC_{90} values	0.09	0.02	0.06	0.25
Break point value	**EMB**	**INH**	**RIF**	**SM**
0.002			55	
0.003			1	
0.008			3	
0.016	154	206	116	
0.023	9	2	8	
0.032	13	4	10	
0.047	18	3	3	
0.064	10	1	11	178
0.094	6		3	8
0.125	1	2	3	5
0.19	4	1	2	6
0.25	1	1	1	10
0.38	2	1		6
0.5	2			6
0.75	1			2
1	1	1	4	1
1.5	1		1	
2	1	5		
3		1		1
4	1			
6			1	
8			1	
12				
16				2
32			4	
48				1
256	4	2	1	2
1024				1

EMB: Ethambutol, INH: İzoniazid, RIF: Rifampicin, SM: Streptomycin.

TABLE 3: Resistance rates of 229 MTB isolates.

	EMB	INH	RIF	SM
	%	%	%	%
Pulmonary	2	7	7	3
Extrapulmonary	0	1	1	1
Total	1	4	4	2

EMB: Ethambutol, INH: Izoniazid, RIF: Rifampicin, SM: Streptomycin.

DST results of 59 MTB isolates obtained from TB dispencery patients were investigated with both E test and conventional L-J proportion method and then, these results are compared in Table 5. In addition; positive predictive value (PPV), negative predictive value (NPV), sensivity and specifity of E test method for 59 MTB isolates according to L-J proportion test results are summarized in the same table.

DISCUSSION

Although TB has been affecting human since ancient times, antimycobacterial chemotherapeutics were invented in the middle of twentieth century. Although the present chemotherapeutics can cure most of the infections, as a consequence of inappropriate use of antituberculous drugs in susceptible cases, the resistant mutants have been emerged. Today we are in an age of resistant variants and new more effective drugs are necessary.[7] Now, if patients take an appropriate regimen and follow it regularly for a sufficient time, all the patients will be cured. However, indetermined drug resistance, inappropriate drug regimens and mostly incomplete therapy can cause treatment failure.

In this study DST results of 229 MTB were searched, resistance rates for INH and RIF were 4%, for SM 2% and EMB 1%. Only six MDR isolates were detected (3%) and they were isolated from sputum and CSF. There are few reports of drug susceptibility patterns of MTB isolated from cases of tuberculous meningitis.[1,8] Multidrug resistance in tuberculous meningitis is not considered as a serious problem in these reports in keeping with present report. The general resistance rates and MDR rates of this study were close to other studies in which extrapulmonary samples were investigated. In Thwaites et al.'s study, the resistance rates aga-

TABLE 4: Distribution of resistant isolates.

	EMB	INH	RIF	SM
	n	n	n	n
Sputum	5	11	12	7
CSF	1	1	1	1
FSF	1	1		1
Ascites fluid				1

EMB: Ethambutol, INH: Izoniazid, RIF: Rifampicin, SM: Streptomycin.

TABLE 5: DST results of E test and conventional L-J proportion methods of 59 MTB isolates and PPV, NPV, sensivity and specificity of E test method.

n: 59	L-J Proportion method		E Test method		Agreement (%)	PPV (%)	NPV (%)	Sensivity (%)	Specificity (%)
	S (%)	R (%)	S (%)	R (%)					
INH	85	15	86	14	95	88	96	78	98
RIF	88	12	85	15	86	44	94	57	90
SM	78	22	90	10	85	83	85	38	98
EMB	95	5	97	3	95	50	96	33	98

INH: Isoniazide, RIF: Rifampicin, SM: Streptomycin, EMB: Ethambuthol, PPV: Positive predictive value, NPV: Negative predictive value, S: Sensitive, R: Resistant.

inst INH and RIF detected were detected as 6% among 180 isolates isolated from CSF. Resistance was established the most important factor related to death in their study.[1] It is remarkable that MDR isolates had extrapulmonary origin in this study and the results were close to ours. The limited number of MDR isolates were not evaluated statistically in our study. However, five of six isolates had pulmonary origin, only one extrapulmonary isolate was isolated from CSF. This patient also had pulmonary MDR TB, therefore the resistance was evaluated to be pulmonary origin.

Antituberculous drug resistance rates were found high in some researches in patients with pulmonary TB.[9-13] A study from our country, resistance rates in 214 isolates from patients admitted to regional TB dispenceries were found as 29, 27, 21 and 10% for SM, INH, RIF and EMB respectively and MDR was found 11%.[14] A similar study conducted in 2007, DST results of 505 MTB isolates were searched and of strains, 76.2% were found sensitive to all drugs tested. The resistance rates of 505 MTB isolates were detected as 13.3% for INH and RIF, 9.1% for SM and 3.4% for EMB.[15] High resistance rates in these studies may be originated from pulmonary TB patients with high secondary resistance rates. The difference of general resistance rates may be originated from the patient population's characteristics. Resistance rates of pulmonary TB patients followed in regional TB dispenceries may vary related to primary or secondary resistance. The resistance detected in incompliant patients who has been treated many times in different durations is usually secondary and higher than primary resistance rates. In Turkey, the rate of MDR isolates is reported as 3% in new TB cases and 18.3% in previously treated cases. The reported INH, RIF, SM and EMB resistances were 13.8%, 6.6%, 7.5% and 4.3%, respectively.[16] Istanbul Health Directorate implemented a DOTS covering 98% of the patients to get their medicine by a health professional since 2006. After this strategy it is important to follow the resistance rates of dispencery patients in a couple of years.

The resistance rates to INH and RIF were significantly higher in pulmonary isolates ($p< 0.005$) compared to extrapulmonary ones in this study. The isolates resistant to INH and RIF were obtained from treatment of naive dispencery TB patients. Therefore, these resistance rates to INH and RIF were assessed as "primary resistance" in this study. In two studies from Turkey, Altıntop et al.[17] and Korkmaz et al.[18] detected very high same multidrug primary resistance rates in their studies (6%). Similarly, in some studies performed in our country, reserachers found high overall resistance rates for all first-line antituberculous drugs.[19,20] The difference between resistance rates of these reports and present study may be due to differences between patient populations. In studies mentioned above, the clinical data about cases were not noted and they could be included in both primary and secondary resistant cases. Indeed, extrapulmonary isolates having lower resistance rates comprised the half of the study. Therefore, we thought that the lower overall resistance rates in this study were related to the patient population. However our resistance rates in pulmonary isolates were similar to studies on treatment of naive pulmonary TB cases and on primary resistant cases.[2,21,22]

The low resistance rates of extrapulmonary isolates that were included in present study may be related to different factors. The time period among

the patients that realize a complaint and apply to a health institution is much shorter in extrapulmonary TB cases in contrast to pulmonary ones. Since the disease does not hinder daily activities except terminal period, delayed diagnosis and inadaptable treatment about pulmonary TB cases may be seen more frequently than extrapulmonary TB cases. Whereas severe headache or neurological symptoms in TB meningitis, dyspnea in pleuresy, existence of a palpable mass in lymphadenitis are the most prominent and severe symptoms for patients in early admittance to a health institute and strictness to therapy. For this reason patient, who is compatible to treatment of extrapulmonary TB, especially TB meningitis which has a high mortality and morbidity is much better than patients with pulmonary TB.[23] These patients were compatible to their treatment and never changed or quitted the therapy by themselves. In addition, pulmonary TB cases are usually quit their TB therapy when they get well and incompleted therapy may result in secondary drug resistance in these patients. We concluded that these reasons were likely to provide lower resistance rates in extrapulmonary TB cases.

PPV of E test for RIF and SM were low (44% and 50%), but agreement between E-test and L-J proportion methods for INH and EMB were 95%, for RIF was 86% and for SM was 85%. In this study, good correlation between two tests showed that E-test may be considered as an alternative method to standard proportion methods. In addition, E test may provide some advantages for developing countries such as easy application, obtaining results within a week, and not requiring an expertize or special equipment. Several studies performed in our country and other countries showed that this method gives rapid result and the results are similar to the standard proportion method. In these studies, E test method was emphasized as a fast, easy, reliable, and feasible method for routine use in detecting antimycobacterial susceptibility.[11,12,19,21,24] Akcali et al. have also found an excellent agreement for INH, RIF, SM and EMB (100%) with E test and agar proportion results in their study.[19] In another study, Muralidhar et al suggested that correlation between L-J proportion and E test method was 87%.[25] However, Freixo et al. and Hausdorfer et al. noted a significant number of false sensitive and false resistant results with E test in comparison with agar proportion.[13,22] Similarly, some researchers suggested that E test should not be used routinly due to its false positive and false negative results for SM. The specialized equipment required (CO_2 incubator), and its cost, all make this method less useful in developing countries.[19,26]

Various studies have shown that E-test is a promising new method for the susceptibility testing of slowly growing mycobacteria as well.[19,25] However costs may be considered a disadvantage of this method. We believe that E test method has advantages such as to obtain the results in seven days and easy implementation and no need for expertize except its cost compared to conventional methods.

Comparison of E test and standard proportion methods in only 59 strains, may be a probable limitation of this study. We therefore suggest that, comparison of E test with standard method should be performed with more number of MTB isolates in further studies.

CONCLUSION

In this study, we found that antimycobacterial resistance rates of extrapulmonary isolates were significantly lower than pulmonary ones. Therefore; drug resistance in extrapulmonary TB is not yet a serious problem according to our results. Comparing with multidrug resistant pulmonary TB patients, higher treatment success should be expected in extrapulmonary TB patients. Today it is advised to get susceptibility results routine prior to antituberculous treatment especially in countries with high INH resistance rates. L-J proportion method is considered as the gold standard, but it is a time consuming application. Diagnostic delays in TB may increase treatment failure and poor outcomes. It will be possible to get antimycobacterial susceptibility results in a shorter time with developing rapid, quantitative, DSTs. We found a good corre-

lation between L-J proportion and E test methods in 59 MTB strains. Therefore, E test seems to be a rapid, reliable and simple method for determining antimycobacterial resistance, but there is a need for further studies comparing it with standard methods with more number of MTB isolates.

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