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Diagnosis of Tuberculosis by Conventional and Molecular Methods in Our Laboratory: A 4-Year Assessment

Laboratuvarımızda Konvansiyonel ve Moleküler Yöntemlerle Tüberküloz Tanısı: 4 Yıllık Bir Değerlendirme

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ABSTRACT

Objective: *The aim of this study was to compare direct microscopy, culture and Polymerase Chain Reaction (PCR) methods and to present the antibiotic resistance profile of the last 4 years comparatively by conventional and molecular methods.*

Material and Method: *Bacterial culture, EZN and PCR methods were applied to all samples. Direct rapid resistance test was performed for EZN positive samples.*

Results: *968 patients were included in the study. Culture was positive in 81 (8%), PCR in 78 (8%) and EZN in 39 (46%) patients. PCR performed on the same day in both respiratory and other samples showed very good agreement with culture, while EZN staining showed moderate agreement. It was observed that the rapid resistance test detected rifampicin resistance which was not detected in culture, and in the case of INH, culture antibiogram and rapid resistance test were fully compatible. Application of the rapid resistance test to every patient with positive EZN staining resulted in very early detection of resistance.*

Conclusion: It was concluded that PCR tests are useful in the rapid diagnosis of tuberculosis and resistance in suspicious clinical samples.

ÖZET

Amaç: *Bu çalışmanın amacı, tüberküloz tanısında direkt mikroskopi, kültür ve Polimeraz Zincir Reaksiyonu (PCR) yöntemlerini karşılaştırmak ve son 4 yılın antibiyotik direnç profilini konvansiyonel ve moleküler yöntemlerle karşılaştırmalı olarak sunmaktır.*

Gereç ve Yöntemler: *Tüm örneklere Ehrlich-Ziehl-Neelsen (EZN) boyama, Mycobacterium kültürü ve PCR testleri yapıldı. EZN boyama ile aside dirençli basil (ARB) saptanan örneklere direkt hızlı direnç testi yapıldı.*

Bulgular: *Çalışmaya 968 örnek dahil edildi. Bunların 81'inde (%8) kültür, 78'inde (%8) PCR ve 39'unda (%46) EZN pozitif bulundu. Hem solunum hem de diğer örneklerde aynı gün yapılan PCR kültür ile çok iyi uyum gösterirken, EZN boyaması orta düzeyde uyum gösterdi. Hızlı direnç testinin kültürde saptanmayan rifampisin direncini saptadığı, INH durumunda ise kültür antibiyogramı ile hızlı direnç testinin tam uyumlu olduğu görülmüştür. Hızlı direnç testinin EZN boyaması pozitif olan her hastaya uygulanması, direncin çok erken tespit edilmesini sağlamıştır.*

Sonuç: *Şüpheli klinik örneklerde tüberküloz ve direncin hızlı tanısında PCR testlerinin yararlı olduğu sonucuna varılmıştır.*

Keywords:
Mycobacterium tuberculosis
Polymerase chain reaction
Rapid diagnosis
Rapid resistance test

Anahtar Kelimeler:
Mycobacterium tuberculosis
Polimeraz zincir reaksiyon
Hızlı teşhis
Hızlı direnç testi

INTRODUCTION

Tuberculosis has been an important health problem and cause of death for centuries. Its control is difficult since it is transmitted by droplet infection. In order to reduce transmission, patients should be identified as soon as possible (1,2). For definitive diagnosis of tuberculosis, Mycobacterium tuberculosis should be isolated from clinical specimens. The fastest and cheapest method for this is direct microscopic examination of clinical specimens stained with the EZN method. However, it has low sensitivity (35-80%) (3). When the number of mycobacteria in the clinical sample is less than 104 bacillus/ml, it cannot be detected, and also the differentiation between tuberculosis and non-tuberculous mycobacteria cannot be made.

The gold standard in diagnosis is culture. The detection limit of *M. tuberculosis* in cultures is 100 bacillus/ml. It is necessary to wait for a long period of 4-8 weeks for reproduction. Although this period is reduced to 10-12 days with automatic controlled liquid media, even this

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period can be long for many patients. For this reason, real-time Polymerase Chain Reaction (PCR) test that can be performed directly on the samples, which is a fast, specific and sensitive method have been developed for diagnosis of M.tubeculosis (4-7). PCR is a diagnostic method, where unlike culture method the number and viability of the microorganism in the sample to be examined is unimportant, and a small number of genetic materials can be reproduced (8-15). However, factors such as clinical sample type, method, contamination, evaluation as well as personal factors affect the performance of the tests.

Although we have a gold standard diagnostic method like the reason for investigating another method is the need for rapid diagnosis in clinical cases caused by this slow-growing bacterium. With the PCR method, diagnosis can be made with a sensitivity of 15-30 bacilli/ml, and antibiotic susceptibility tests can also be studied from the sample at the same time.

The aim of our study is to compare the direct microscopy, culture and PCR methods studied in the samples that were sent to our laboratory for the diagnosis of tuberculosis and to reveal the antibiotic resistance profile of the last 4 years. The study was approved by the Non-Interventional Health Research Ethics Committee of Düzce University Faculty of Medicine with the date 07.11.2023 and number E-050.99-360085.

MATERIALS AND METHODS

In our study, microscopic examination, culture and PCR test results of sputum, bronchoalveolar lavage (BAL), deep tracheal aspirate (DTA), biopsy, urine, sterile body fluid samples examined between January 2017 and December 2020 in the Tuberculosis Laboratory of Düzce University were examined. Only one of the multiple samples from the same patient, which was examined by all three methods, was included in the study. Patients were divided into two age groups as above and below 65 years of age. The samples were compared by dividing them into two groups as respiratory tract samples and other. EZN staining (Merck, Turkey), culture [Löwenstein-Jensen(LJ) (RTA Laboratories, Turkey) and BACTEC MGIT 960 (Becton, Dickinson and Company Sparks, USA)] and FluoroType® MTB (Hain Lifescience, Germany) methods were applied to the samples. Growth times in culture were recorded. The culture was accepted as the gold standard and compared with PCR, EZN and clinical findings. Steptomycin, INH, rifampicin and ethambutol susceptibilities were determined by the BACTEC MGIT 960 (Becton, Dickinson and Company Sparks, USA) method of those in whom *M. tuberculosis* growth was detected in the culture. In addition, rapid resistance test [GenoType® MTBDRplus (Hain Lifescience, Germany)] was performed directly from the samples that were positive only in the EZN dye. The presence of wild type probes and mutation probes in the katG and inhA gene regions for INH resistance and in the rpoB gene regions for rifampicin resistance were investigated by rapid resistance test.

Statistical analysis:

SPSS 17 (SPSS Inc, Chicago, IL, USA) was used for statical evaluation. Categorical data were summarized as frequency and percentage. The compatibility of the diagnostic methods used was determined by McNemar and Kappa methods. Pearson Chi-square and Fisher Exact tests were performed for the relationships between categorical variables. $p<0.05$ was considered statistically significant.

RESULTS

A total of 968 patients, 645 (67%) male and 323 (33%) female, were included in the study. The mean age of the patients was 55.5 ± 19.8 (min:2-max:95).

Of patients with growth in culture, which is accepted as the gold standard method, 56 (69%) were male and 25 (31%) were female, with a mean age of 55.6 ± 19.5 (min: 9-max: 94). Of the patients, 54 (67%) were under the age of 65 and 27 (33%) were over the age of 65. There was no difference between these two groups in terms of *M. tuberculosis* culture positivity (p=0.817).

When the samples that were sent to our laboratory was examined, the distribution was as follows: 18% (179) BAL, 70% (686) sputum, 4.9% (48) sterile body fluid, 1% (11) urine, 1% (18) other samples (gastric fasting fluid, wound, tissue). The evaluation of *M. tuberculosis* growth according to age groups, sample types and sex is shown in Table 1.

Bacterial culture, EZN and PCR methods were applied to

Table 1: Association of age group, gender and sample type with M. tuberculosis positivity

		Culture positive sample n(%)	Culture negative sample n(%)	p value
Age	>65 Old Year	27 (%8)	307(%92)	0.817
	<65 Old Year	54 (%9)	580(%91)	
Gender	Women	25(%8)	298(%92)	0.618
	Men	56(%9)	589(%91)	
Sample type	Sputum	42(%6)	644(%94)	0.000
	Bronchoalveolar lavage	34(%19)	145(%81)	
	Biopsy	4(%15)	22(%85)	
	Urine	0(%0)	11(%100)	
	Sterile Body Fluid	0(%0)	48(%100)	
	Other*	1(%6)	17(%94)	

**: Gastric lavage, wound*

Table 2: Diagnostic values of EZN and PCR results according to the culture results of the samples

	EZN	PZR
Respiratory system samples	Total: 865	
Sensitivity (%)	46	82
Specificity (%)	99	98
PPV*(%)	97	84
NPV(%)**	95	98
Harmony with culture	Middle	Too big
Non-respiratory system samples	Total: 103	
Sensitivity (%)	40	80
Specificity (%)	100	100
PPV*(%)	100	100
NPV(%)**	97	99
Harmony with culture	Middle	Very good

**PPV: positive predictive value ** NPV: negative predictive value*

Table 3: Antibiotic resistance status in M. tuberculosis specimens with growth

Antibiotic	Sensitivity n%	Resistant n %	p value
Streptomycin	68 (%84)	13(%16)	0.000
INH	70 (%86)	11(%14)	
Rifampin	81(%100)	0(%0)	
Ethambutol	72 (%89)	9(%11)	

Table 4: Evaluation of INH rapid susceptibility tests

		INH rapid susceptibilty test (GenoType® MTBDRplus)	
		Sensitive(n)	Resistant(n)
INH rapid susceptibilty test (Bactec 960 TB Culture)	Sensitive (n)	39	0
	Resistant (n)	0	8

Table 5: Evaluation of Rifampin rapid sensitivity tests

		Rifampin susceptibilty test (GenoType® MTBDRplus)	
		Sensitive(n)	Resistant(n)
Rifampin rapid susceptibilty test (Bactec 960 TB Culture)	Sensitive (n)	45	2
	Resistant (n)	-	-

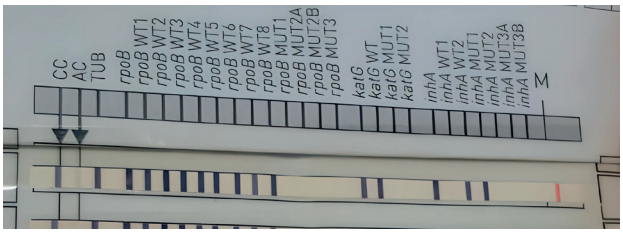

Figure 1: INH resistance detected in the inhA locus (loss of InhA wild type 1)

all samples. Culture was positive in 8% (81), PCR in 8% (78), EZN in 46% (39) of patients.

The mean growth period of *M. tuberculosis* in culture was 12.56±8.24 days (min:3-max:36). With the PCR method, the result was obtained on the day of sample arrived.

Out of 81 patient samples with *M. tuberculosis* growth 37 (46%) were detected by EZN staining and 66 (81%) by PCR method. Sensitivity, specificity, positive predictive value and negative predictive values of EZN staining were determined as 42%, 85%, 95% and 16%, respectively. For the PCR test, these values were determined as 80%, 98%, 84% and 98%, respectively. The gold standard culture method was found to be moderately compatible with EZN staining and very well compatible with PCR method. The diagnostic values of EZN and PCR results according to the culture results of respiratory and non-respiratory samples are shown in Table 2.

PCR method was positive in 12 patients (1%) while culture and EZN methods were negative. 5 of these 12 patients were clinically and radiologically compatible with tuberculosis and cured with treatment. Four of them had malignancy and died. The PCR positivity in the remaining 3 patients was not clinically compatible. EZN method was positive in one patient while culture and PCR tests were negative. This patient had no clinical and radiological findings and was considered as contamination.

Antibiogram of 81 specimens with *M. tuberculosis* growth showed that all specimens were susceptible to rifampicin, while 13 (17%) were resistant to streptomycin, 11 (14%) to isoniazid (INH), and 9 (11%) to ethambutol. Rifampin was found to be statistically more sensitive than other antibiotics (p=0.000). Antibiotic sensitivities are shown in Table 3.

In our study, rapid resistance test was performed on 47 (58%) of 81 samples with culture growth. While there was 100% correlation between rapid resistance test and antibiotic susceptibility tests for INH; Incompatibility was detected in two samples for rifampin (Tables 4 and 5).

In the INH resistance study with the rapid resistance test, it was observed that “low level INH resistance detected in the inhA locus” was also detected in the antibiotic susceptibility test (Figure 1)

While the mutations detected in the rpoB locus are detected both by rapid resistance test and antibiotic resistance tests; It was observed that wild type probe deletions could not be detected. This showed that rifampin antibiotic resistance could be detected earlier with rapid resistance tests (Figure 2,3).

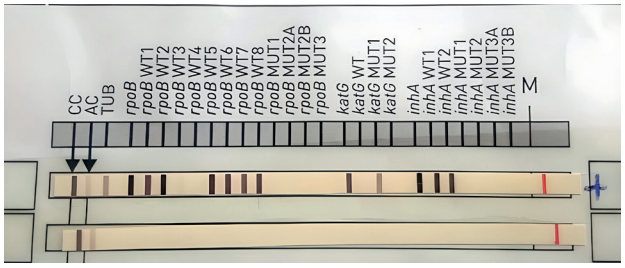

Figure 2: Rifampin resistance by mutations in the rpoB locus (loss of rpoB wild type2 and 3)

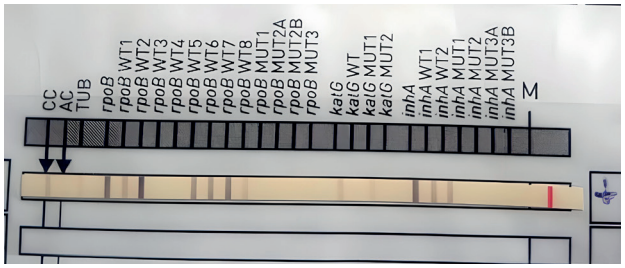

Figure 3: Rifampin resistance by mutations in the rpoB locus (loss of rpoB wild type2 and 3) and INH resistance detected in the katG locus (loss of katG wild type 1) and detection of kat G MUT 1)

DISCUSSION

Tuberculosis is a life-threatening disease for centuries. Diagnosis of this disease and determination of antibiotic susceptibility will provide treatment with the right drug regimens and reduce the infectiousness (16). There are many methods used for diagnosis. Although staining with EZN is the fastest method, its sensitivity is low (3). With this method, it is not possible to distinguish between species and live/dead bacteria. In order to detect positivity, it is necessary to have at least 103-104 bacteria / ml in the sample (16,17).

In a study by Abdulmajed et al(15), the sensitivity and specificity of the EZN staining method was 32% and 66% respectvely. And they found a low level of agreement between the culture and the EZN method. Sensitivity rates were similar to our study, but moderate agreement was found between culture and EZN in our study. Although by EZN staining method results can be obtained within twenty-four hours and it is a cheap test, it has low sensitivity rates that vary depending on the quality of the microscope used, the type of specimen, the thickness of the smear, the decolorization time during staining, the speed of the centrifugation process and the experience of the person evaluating the smear preparation, and the prevalence of tuberculosis in the population studied (16)

Tuberculosis culture method is the accepted gold standard method (18,19). For maximum efficiency, liquid and solid media should be used together. For culture growth an average of 2 weeks required (7-30 days) and the detection limit is approximately 100 bacteria/ml (16). Detection and the planning the treatment strategy for tuberculosis patients as soon as possible is of great importance (16). For this reason, nucleic acid methods have been developed in recent years (20-25). With these methods, the presence of *M. tuberculosis* and antibiotic susceptibility can be studied directly from the patient sample by PCR method (26-30). With the PCR method, detection can be made with a sensitivity of 15-30 bacillus/ml. In our study, 5 of 12 patients (41%) with culture negative and PCR positive were clinically and radiologically compatible with tuberculosis and healed with cure. Detection of positivity by PCR in all of these patients is valuable in terms of not to miss the patients who expelled a small number of bacilli or could not be detected due to being under treatment. PCR test is an important diagnostic tool in cases where there is no growth in culture (7,8,9,10). If there is clinical suspicion in a patient with a positive PCR test, beginning treatment and taking precautions without waiting for culture results will provide early infection control (27). It is also emphasized that it will be very useful in the differential diagnosis of tuberculosis and non-tuberculosis and also in the diagnosis of patients receiving inadequate tuberculosis treatment (12,13).

In this study, culture, PCR and microscopic examination methods performed on samples in our tuberculosis laboratory were compared. In our study, similar to the studies in the literature, the PCR methods of the samples received both from respiratory and non-respiratory systems performed on the same day had high agreement with the culture and has moderate agreement with the EZN staining method. (9,10, 21, 25, 28, 29)., The sample type and the amount of bacillus in the sample besides the PCR method used also play an important role in obtaining different sensitivity results. In theory, even a few bacilli in the sample is enough for PCR positivity. However, in practice, many studies have shown that the sensitivity of PCR for tuberculosis is not that high. Inability to reveal the *M. tuberculosis* DNA, loss of the bacilli during procedure and the presence of inhibitory substances in the sample may be shown as reason (20,21).

In the antibiogram of the samples with *M. tuberculosis* growth, 59 of 81 (72.8%) samples were found to be sensitive to all drugs and all samples were found to be sensitive to rifampin, while 13 (17%) were resistant to streptomycin, 11 (14%) to INH, 9 (11%) to rifampin. Rifampin was found to be statistically more sensitive than other antibiotics. In a study conducted in our laboratory in 2005, the rates of streptomycin, INH, rifampin, ethambutol resistance were reported as 11.3%, 8%, 4.8%, and 0%, respectively. Accordingly, streptomycin, INH, ethambutol resistance rates increased; It was observed that the rate of rifampin resistance decreased (11). In a study by Abdulmajed et al. (15), the survival rates for antituberculosis drugs were 12%, 4%, 13.2% and 4% for streptomycin, INH, rifampin, ethambutol, respectively; In the study of Saygan et al. (26), resistance rates were found to be 9.1%, 13.2%, 4% and 3.3% for streptomycin, INH, rifampin, ethambutol, respectively, to antituberculosis drugs. It has been observed that there may be regional differences in antibiotic resistance rates.

Studies show that both automated and manual systems are good in detecting INH and rifampin sensitivity, but are not so as for ethambutol and streptomycin (28,29).

In the rapid resistance test, incompatibility with the

antibiogram was detected in 2 patients (4%). While mutation probes detected in the rpoB locus with rapid resistance test are also detected with antibiotic resistance tests; wild type probe deletions could not be detected. This showed that rifampicin antibiotic resistance could be detected in the early period with rapid resistance tests. In the INH resistance study, it was observed that "low level INH resistance detected in the inhA locus" was also detected in the antibiotic susceptibility test. Early detection of mutations is important for these two drugs, that are very important in tuberculosis treatment. Acharya et al. (25) in their review; found the sensitivity of the rapid resistance test as 98% in rifampicin resistance and 84% in INH resistance. In a study by Barnand et al.(14), the sensitivity of Genotype MTBDR plus test in detecting rifampin and INH resistant strains was 99% and 94%, respectively, in 536 EZN positive sputum samples; the specificity is 99% and 100%; On the other hand, Ling et al.(27) determined that the specificity and sensitivity were 98% and 99%, respectively; Dorman et al.(30) determined the sensitivity of the test to determine rifampin resistance 86%, specificity 97%; They found INH resistance to be 62% and 98%, respectively. The findings support the recommendations that the GenoType MTBDR plus assay should not be used in sputum specimens where microscopy is negative or bacilli are rare. The use of rapid resistance tests is beneficial not only can be performed on the same day, but also for detecting the mutations that not have yet been reflected in antibiotic susceptibility tests. This guides the clinician during the treatment.

In conclusion; it should be considered that PCR tests are useful in the rapid diagnosis of tuberculosis in suspicious clinical samples in routine practice and that these tests definitely should not be used for screening purposes, but they are thought to be valuable in supporting the clinic together with conventional tests. In our study, it was observed that it is very important using staining and nucleic acid tests together, as well as culture methods.

Conflict of Interest: No conflict of interest was declared by the authors.
Ethics: The study was approved by the Non-Interventional Health Research Ethics Committee of Düzce University Faculty of Medicine with the date 07.11.2023 and number E-050.99-360085.
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Approval of final manuscript: All authors.

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