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**ORIGINAL ARTICLE**

# Comparison of Culture, Direct Microscopy, and Polymerase Chain Reaction Results for Detection of Mycobacterium Tuberculosis Complex in Clinical Specimens

# Klinik Örneklerde Mycobacterium Tuberculosis Kompleksi'nin Saptanması İçin Kültür, Direkt Mikroskopi Ve Polimeraz Zincir Reaksiyon Sonuçlarının Karşılaştırılması

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**ABSTRACT**

**Aim:** Tuberculosis is a chronic, necrotizing disease known since prehistoric times. The most important stage of the tuberculosis control program is the microbiological identification, typing and detection of drug resistance of the Mycobacterium tuberculosis complex for the detection of active cases. The aim of this study is to compare the results of the methods used in the detection of M.tuberculosis complex in clinical samples.
**Materials and Methods:** The results of the samples sent to the laboratory for the purpose of investigating M. tuberculosis complex between January 2016 and January 2022 from patients with pre-diagnosis of tuberculosis were evaluated retrospectively. In microbiological diagnosis, Ehrlich-Ziehl-Neelsen (EZN) staining method, culture (BACTEC MGIT 320 automated system and Löwenstein Jensen (LJ) medium, and polymerase chain reaction (PCR) tests were used.
**Results:** A total of 1706 samples with all three requests (EZN, culture and TB-DNA) were included in the study. The mean age of the patients was 46.49±25.77 years and 1025 (60.1%) were male. Culture in 48 (2.8%) samples, PCR positivity in 40 (2.3%) and EZN positivity in 32 (1.9%) samples were mostly sent from the departments of chest diseases (52.8%) and pediatrics (24.1%).
**Conclusion:** It was determined that the culture method gave more reliable results than PCR and EZN in demonstrating the presence of M. tuberculosis complex. As a result, since the culture method, gives late results, extensive routine use of molecular tests such as PCR is needed for accurate diagnosis of tuberculosis in a short time and to detect drug resistance.

**Keywords:** Mycobacterium tuberculosis, EZN, Löwenstein Jensen medium, TB-DNA

**ÖZ**

**Amaç:** Tüberküloz insanlık tarihi kadar eski bir geçmişe sahip olan kronik, nekrotizan bir hastalıktır. Tüberküloz kontrol programının en önemli aşaması olan aktif olguların tespiti için Mycobacterium tuberculosis kompleksin mikrobiyolojik yöntemlerle tanımlanması, tiplendirilmesi ve ilaç direncinin saptanması gerekmektedir. Bu çalışmanın amacı klinik örneklerde M.tuberculosis kompleksin tespitinde kullanılan yöntemlerin sonuçlarının karşılaştırılmasıdır.
**Gereç ve yöntem:** Tüberküloz ön tanılı hastalardan Ocak 2016 - Ocak 2022 tarihleri arasında M. tuberculosis kompleks araştırılması amacıyla laboratuvara gönderilen örneklerin sonuçları rektospektif olarak değerlendirildi. Mikrobiyolojik tanıda; Ehrlich-Ziehl-Neelsen (EZN) boyama, kültür (BACTEC MGIT 320 otomatize sistemi ve Löwenstein Jensen (LJ) besiyeri ve polimeraz zincir reaksiyonu (PZR) testi kullanıldı.
**Bulgular:** Çalışmaya her üç istemi olan (EZN, kültür ve TB-DNA) toplam 1706 örnek alındı. Hastaların yaş ortalaması 46,49±25,77 olarak saptanmıştır ve 1025'i (%60,1) erkektir. Örneklerin 48'inde (%2,8) kültür, 40'ında (%2,3) PZR ve 32'sinde (%1,9) EZN pozitifliği saptandı. Örnekler en çok göğüs hastalıkları (%52,8) ve pediatri (%24,1) kliniklerinden gönderilmiştir.
**Sonuç:** M. tuberculosis kompleks varlığının gösterilmesinde kültür yönteminin PZR ve EZN'ye göre daha güvenilir sonuç verdiği saptandı. Sonuç olarak kültür yöntemi geç sonuç verdiğinden tüberkülozun kısa sürede doğru tanısı ve ilaç direnci tespiti için PCR gibi moleküler testlerin geniş rutin kullanımına ihtiyaç duyulmaktadır.

**Anahtar Kelimeler:** Mycobacterium tuberculosis, EZN, Löwenstein Jensen besiyeri, TB-DNA

## Introduction

Tuberculosis (TB) is one of the most important fatal diseases caused by a single infectious agent. According to the World Health Organization (WHO) 2019 Global TB Report; approximately 10 million people were infected with TB in 2018 (1). Neglect and rejection of treatment by the patients increases the Multi-Drug Resistant Tuberculosis (MDR-TB) rate. In addition, increasing anti-TB drug resistance has become an important problem due to travel and migration (2,3). Along with the developments in molecular diagnostic methods, around 200 Mycobacterium species have been identified recently, and most of them are disease agents in humans (4). The most isolated mycobacterium species in TB cases is Mycobacterium tuberculosis (5). Since tuberculosis is one of the diseases that cause the highest mortality and morbidity in the world, epidemiological studies should be reported regularly. It is extremely important to identify M. tuberculosis as

soon as possible in order to prevent the spread of the infection and to start specific treatment. Examples of traditional approaches used in the diagnosis of tuberculosis are microscopic examination by staining with acid-resistant staining (ARB) and identification of the agent by culture (6).

The diagnosis of TB is made by detecting the agent in respiratory tract samples such as sputum, bronchoalveolar lavage (BAL) or body fluids such as urine, blood, sterile body fluids (pleura, peritoneum, pericardium, joint, cerebrospinal fluid). Although new molecular diagnostic methods continue to develop in recent years, culture method maintains its importance as the "gold standard" in the diagnosis of Mycobacterium spp. (7).

There are three types of media used in the culture. Hard-boiled eggs [eg Löwenstein Jensen (LJ) ], solid agar (eg. Middlebrook 7H11), liquid broth (eg. Middlebrook 7H12). Incubation in liquid broth requires 1-3 weeks, while solid media requires 3-8 weeks. 5-10% carbon dioxide accelerates reproduction. Among the automated rapid culture methods, commercial systems such as "Mycobacterium growth indicator tube" (MGIT) 960 system, BACTEC 460 TB system, BACTEC 9000 MB system, BACTEC MGIT 320, ACT Myco E SP 2 system can be counted. With these methods, rapid primary isolation of mycobacteria, differentiation of "Mycobacterium tuberculosis complex (MTC)" and non-TB mycobacteria and detection of susceptibility of mycobacteria to anti-tuberculosis drugs are performed (8).

Culture method takes 3-4 weeks for the bacilli to reproduce and form a visible colony. In addition to traditional methods in the diagnosis of tuberculosis, molecular diagnostic methods have been developed with high sensitivity and specificity (6). Molecular methods are useful to identify mycobacteria grown in culture at the species level in a short time, to investigate their susceptibility to tuberculosis drugs, to detect drug-resistant cases, and to detect mutations related to resistance (9).

In this study, it was aimed to investigate and compare the results of culture, direct microscopy and PCR methods in the detection of Mycobacterium tuberculosis complex strains in various clinical samples. In addition, it was aimed to investigate the distribution of tuberculosis positivity according to hospital units.

**Materials and Methods**

In the study, 1706 clinical samples that EZN staining, culture and PCR methods were performed in Selçuk University Medical Faculty Hospital Microbiology Laboratory from various clinics between 01.01.2016-01.01.2022 were analyzed retrospectively. Culture, EZN stain, and TBC DNA results of the patients were evaluated and compared.

Samples such as sputum, bronchoalveolar lavage (BAL), abscess, joint fluid, biopsy, fine needle aspiration were subjected to pre-treatment (homogenization, decontamination, concentration). Homogenization-NALC (N-Acetyl-LCysteine+Sodium) method was performed with glass beads and vortex. Decontamination was done with sodium buffer. Concentration was done by centrifugation (15 min at 3000 g). After the pre-treatment EZN stain was applied for the presence of bacilli.

Sample (0.5 ml) was inoculated into LJ medium and BACTEC MGIT 320 liquid culture bottle (BD BBL™ MGIT™, Ireland). The remaining samples were stored in a -20˚C refrigerator. If there was growth in media, positivity was seen within one week (standard deviation 10 days). Culture positivity was determined by both automated system and visual evaluation. In addition to routine EZN staining, positive culture samples were also stained to determine mycobacterial species.If 6-6'-dimicolate-a-D-trehalose (KORD) factor was seen in microscopy, the bacteria grown in the medium was accepted as TB (Tuberculosis Complex Bacteria). After full proof of TB positivity, mycobacterial susceptibility tests was performed. Tuberculosis culture extended to six weeks in the absence of signal (20).

Samples were given to the molecular unit for TB-DNA study after pre-treatment. DNA extraction was carried out in accordance with the protocol using a DNA isolation kit (Artus® M. tuberculosis RG PCR Kit, QIAGEN, Germany).The primers used in the PCR test are specific for M. tuberculosis (TB-DNA target site). All operations before and after extraction were carried out in a biological safety cabinet. The isolated samples were amplified on the Rotor-Gene RT-PCR (QIAGEN, Germany). M. tuberculosis complex was considered positive when the targeted DNA region was detected.

**Statistical Analysis**

The results obtained in the study were analyzed with the SPSS 21.0 package program. Frequency and percentages were given as descriptive statistics. Roc Curve was used to determine the reliability of the tests in diagnosis. Sensitivity, specificity, positive and negative predictive values of EZN, Culture and PCR tests were calculated manually. Culture was accepted as the gold standard method.

**Ethics Approval**

For this research; Selcuk University Faculty of Medicine Local Ethics Committee approval was obtained (Date: 21.06.2022; number: 2022/13).

**Results**

The results of 1706 samples from various clinical samples were scanned in order to detect the presence of M. tuberculosis complex. The mean age of the patients was 46.49±25.77 years. Of the patients, 1025 (60.1%) were male and 681 (39.9%) were female. Culture, PCR, and EZN positivity rates were; 48 (2.8%) , 40 (2.3%)

and 32 (1.9%) respectively ( Table 1). Repeated results of the same patients were not included in the study. TB-DNA in 10 patients (0.58%) with negative culture results and EZN staining test in five patients (0.29%) were positive. In addition, 30 (1.8%) patients with positive culture results had positive TB-DNA tests and 27 (1.6%) had positive EZN results. 52.8% (n = 900) of the samples were sent from chest diseases clinics and 24.1% (n = 412) from pediatrics (Table 2). Sputum samples constitute the majority of the samples (n = 1169; 68.5%) and followed by bronchoalveolar lavage samples with a rate of 17.7% ( Figure 2).

Table 1. Positivity rates of the methods

| Methods | Positive | | Negative | |
|---|---|---|---|---|
| | n | % | N | % |
| Culture | 48 | 2.8 | 1658 | 97.2 |
| TB-DNA | 40 | 2.3 | 1666 | 97.7 |
| EZN | 32 | 1.9 | 1674 | 98.1 |
| Total | | | 1706 | 100 |

TB DNA* Tuberculosis complex Deoxyribo nucleic acid

EZN* Ehrlich-Ziehl-Neelsen

As a result of the evaluation made by accepting the culture method as the gold standard, the sensitivity of the PCR and EZN methods was calculated as 60.4% and 41.6%, and the specificity as 99.3% and 99.2%, respectively. The positive predictive value of the PCR test was 81.3%, the negative predictive value was 99.3%; the positive predictive value of EZN was 70% and the negative predictive value was 99.2%.

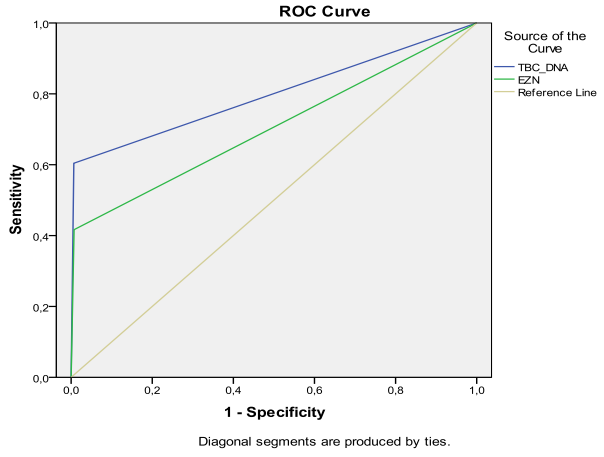


Figure 1. Roc Curve

In our study the area under the curve (AUC) for TBC DNA was 0.79 for EZN, and 1.00 for Culture as a result of ROC analysis when culture was taken as a reference test.

Other*= Nephrology, Orthopedics and Traumatology, Urology, Hematology, Palliative Care Center, Dermatology, Rheumatology, General Surgery, Gynecology and Obstetrics, Anesthesia and Reanimation, Medical Oncology, Plastic, Reconstructive and Aesthetic Surgery, Infectious Diseases .

Table 2. Distribution of clinical samples by hospital units

| Hospital Units | N | % |
|---|---|---|
| Chest diseases | 900 | 52.8 |
| Pediatric | 412 | 24.1 |
| Cardiology | 6 | 0. 4 |
| Thoracic surgery | 19 | 1.1 |
| Neurology | 23 | 1.4 |
| Gastroenterology | 44 | 2.6 |
| Internal diseases | 18 | 1 |
| Other | 284 | 16.6 |
| Total | 1706 | 100.0 |

According to the results of the research, more than half of the samples (52.8% (n=900)) were sent from the chest diseases unit.

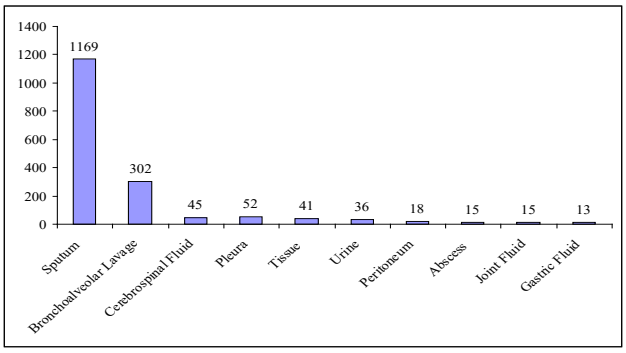


Figure 2. Distribution of clinical samples

Most of the clinical samples examined were sputum samples (n = 1169; 68.5%), followed by bronchoalveolar lavage samples with 17.7%.

## Discussion and Conclusion

Tuberculosis maintains its importance as public health problems. Diagnosis and control of tuberculosis is extremely important in terms of protecting public health as it is an airborne disease (10). EZN staining is a rapid test used in the early diagnosis of tuberculosis. Even if the initial diagnosis of pulmonary and extra-pulmonary tuberculosis is made with clinical data, it is essential for the definitive diagnosis to be detected by laboratory tests (11).

Prior to the introduction of molecular methods into diagnostic mycobacteriology, the only way for rapid diagnosis of tuberculosis was the evaluation of EZN smears, preferably prepared from sputum, by direct microscopy. In microscopic examination, 5,000-10,000

bacilli per milliliter must be present in the patient sample (12). Although it is an inexpensive test and results can be obtained within one day, the staining technique applied to the samples of culture-confirmed tuberculosis cases, the low sensitivity (45-80%), which varies depending on the speed of the centrifugation process, the experience of the person evaluating the smear preparation, and the prevalence of tuberculosis in the studied population. Since it cannot be excluded and has a low positive predictive value (50-80%), microscopy is insufficient in the diagnosis of TB (13, 14).

In our study, EZN positivity was detected in 32 (1.9%) of 1706 samples. The sensitivity and specificity of EZN were found to be 41.6% and 99.2 respectively. Since M.tuberculosis and non-tuberculosis Mycobacteria cannot be distinguished by microscopic examination, culture is still considered as the gold standard (Table 1). If ≥10 live tuberculosis bacilli are found in the clinical sample, it can be demonstrated in culture. It is a conventional method with a sensitivity of 80-85% and a specificity of approximately 98%. Since M. tuberculosis divides and reproduces approximately every 18 hours, an average of 2-8 weeks is required for the agent to be identified in culture media (10,11,14).

Efforts are made around the world to make the routine use of rapid and reliable diagnostic tests with high specificity and sensitivity, at certain standards on which intensive research has been carried out. Many diagnostic systems and test techniques have been developed and put into use by the manufacturers, especially in the last 20 years that can be applied directly from patient samples and/or culture environments, and can provide fast results for the diagnosis of tuberculosis. Among these, molecular genetic techniques are promising (10, 15,16).

Nucleic acid amplification based molecular diagnostic tests are still being developed to support clinical and laboratory diagnosis, and research on their study performance continues uninterruptedly (13). Parallel with our results Malburny et al (17) conducted a study for the detection of M.tuberculosis by PCR (Xpert MTB/RIF) from a total of 180 samples, 91 of which were respiratory and 89 were non-respiratory, taken from 132 patients with suspected tuberculosis. PCR sensitivity and specificity were 100% and 100% and 85.7% and 97.3% for respiratory and non-respiratory samples, respectively. Kit-based commercial tests are used in the rapid diagnosis of tuberculosis. In a meta-analysis of sixty studies, the mean sensitivity and specificity of these tests were 96% and 85% in smear-positive samples, respectively; it was found to be 66% and 98% in smear-negative respiratory samples (18).

In our study the area under the curve (AUC) for TBC DNA was 0.79 for EZN, and 1.00 for Culture as a result of ROC analysis when culture was taken as a reference test (Figure 1). In addition, according to the results of the research, 52.8% (n = 900) of the samples were sent from the chest diseases unit (Table 2).The majority of clinical samples studied were sputum samples (n = 1169; 68.5%), followed by bronchoalveolar lavage samples with 17.7% (Figure 2).

In the study conducted by Kivihya-Ndgugga et al (19), the effectiveness of the PCR method and the routine procedures used in the diagnosis of tuberculosis were compared. As a result of the study, it was reported that the PCR method is superior to the routine procedures in the diagnosis of tuberculosis.

As a result, a test that can diagnose and detect drug resistance in a short time is needed for the effective and accurate treatment of tuberculosis , which causes approximately two million deaths each year. Microscopy cannot meet this need due to its low sensitivity despite its high specificity values. PCR method is reliable, fast and superior to the routine procedures in the diagnosis of tuberculosis but in the same time it is very expensive. Culture methods, which are still used as the gold standard, cause delays in diagnosis and treatment due to their high sensitivity and high specificity. Considering the samples with EZN (+) and culture (–),Non-M.tuberculosis mycobacteria (MOTT) was not determined in the study. Finally, it should be emphasized that the use of culture, PCR and EZN staining methods together in the microbiological diagnosis of tuberculosis is very important for early diagnosis and diagnosis of the disease all over the world.

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**Presentation Statement:** This study was not presented orally or as a poster in any congress.

**Declaration Of Interest Statement:** All authors declare that they have no conflict of interest.

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