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Comparing mycobacterium tuberculosis complex susceptibility methods, a developing country's experience

Yasemin Ay Altintop[1], Duygu Percin[2]

[1]*Kayseri Training and Research Hospita, Department of Microbiology, Kayseri, Turkey*
[2]*Erciyes University Medical Faculty, Department of Microbiolog, Kayseri, Turkey*

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Abstract
In spite of current diagnosis and treatment, *Mycobacterium tuberculosis* complex is still an important cause of mortality and morbidity. Uneffective drug combinations and patient incompliance lead to increasing drug resistance. This study by managing four different -non molecular- susceptibility methods was carried out to determine the susceptibility patterns of *Mycobacterium tuberculosis* complex isolates in central part of Turkey and to compare the methods. The susceptibility testing of 33 isolates were initially performed using the agar proportion method, the results of which were compared with the other three methods; BACTEC 460TB, MGIT 960, and Etest prospectively. According to the "gold standard" agar proportion method, none of the strains were resistant to STR or ETM, while two isolates were found to be resistant to both isoniazid and rifampicin. BACTEC 460 TB remains the reference method of choice, and for low-volume centers. The MGIT 960 system is more suited for high-volume laboratories but has a high risk of contamination. Etest may seem expensive and produces cumbersome results. Regardless of the test used, validation with the gold standard method should be performed periodically as well as in the event of a discordant result. There remains a need for further studies to establish whether available rapid tests will eventually become the gold standard.

Keywords: Mycobacterium tuberculosis, susceptibility methods, drug resistance, Turkey

Objective

Tuberculosis is an important public health problem, both in developing and developed countries. The standard treatment recommended by the World Health Organization (WHO) is a combination of rifampicin (RIF), isoniazid (INH), pyrazinamide (PZD) and streptomycin (STR) or ethambutol (ETM) [1]. Centers for Disease and Control (CDC) proposes managing susceptibility testing to every culture positive sample; although suitable therapy given, still culture and smear EZN positive samples and samples has no response to standard therapy [2]. Susceptibility testing confirms the initial form of drug therapy, guides the choice of further therapy and estimate the prevalence of drug resistance [3,4]. According to the CLSI M24-A2 document, the agar proportion method, which is the most widely used method globally, is considered to be the gold standard [5].

The aim of this study was to determine the susceptibility of *M.tuberculosis* complex strains to the four basic anti-tuberculosis drugs, STR, ETM, INH and RIF, using the BACTEC 460TB, MGIT 960 and gradient test (Etest) methods and to compare the results with the gold-standard agar proportion method. Thus give a modest comment to the susceptibility acting laboratories those who have difficulties on approaching molecular susceptibility tests.

***Coresponding Author:** Yasemin Ay Altintop, Department of Microbiology, Kayseri Training and Research Hospital, Kayseri, Turkey
E-mail: dryaltintop@hotmail.com

Design

***Mycobacterium tuberculosis* strains:**
This study was undertaken in the Hospital of Erciyes University Faculty of Medicine with the approval of the local ethics committee on 33 strains of *M.tuberculosis* isolated from samples obtained from patients with a preliminary diagnosis of tuberculosis. This study was supported by Erciyes University Scientific Research Projects Coordination Unit. ERU/BAP. The isolates were obtained from sputum (% 24), cerebrospinal fluid (%18), bronchoalveolar lavage(% 15), pleural fluid (% 12), abscess (% 9), tissue (% 9), urine (% 6), peritoneal fluid (% 3) and intraabdominal fluid (% 3).

The isolated mycobacterial rods were identified as *M. tuberculosis* complex by NAP testing in MGIT 960 in addition to standard identifying methods. Each strain was stored in two different media due to be tested at the end of the study; in milk cream (Oxoid, UK) at -70°C and in M7H9 broth at +4 °C which required passage of the isolates every 6 months. Prior to antibiotic susceptibility testing, strains were revived by being subcultured in Löwenstein-Jensen solid media and M7H9 broth.

Blood agar was used to check for bacterial contamination and Middlebrook 7H10 agar medium was used to guarantee purity of the mycobacterial cultures.

For each antibiotic-susceptibility method to be tested, *M. tuberculosis* H37Rv (ATCC 27294) was used as the quality-control strain. For isolates demonstrating resistance, INH-resistant (ATCC 35822) and RIF-resistant (ATCC 35838) strains were used for quality control.

The susceptibility testing of all isolates were initially performed using the agar proportion method, the results of which were compared with susceptibility patterns obtained with the other three methods; BACTEC 460TB, MGIT 960, and gradient test(Etest).

Agar proportion method
The standard method described by Kubica et al [6]. was adopted using the standard critical concentrations recommended in the CLSI M24-A2 document [5]. According to this method, Middlebrook 7H10 (GBL Rosmedia) agars were prepared by adding powdered forms of the antibiotics to be studied. Critical concentrations used were 2.0 µg/mL for STR, 0.2 and 1.0 µg/mL for INH, 1.0 µg/mL for RIF and 5.0 µg/mL for ETM.

Bactec 460
Susceptibility tests were performed using the BACTEC 460 (Becton Dickinson) radiometric system, according to manufacturer instructions. Subcultures were prepared by transferring 100 µL of the contents of MGIT 960 bottles, which are routinely used for primary isolation, into 12B tubes. Stock solutions for STR, INH, RİF and ETM obtained from the BACTEC SIRE kit were prepared. Critical concentrations for each antibiotic were achieved by adding 5 ml of sterile deionized water into the lyophilized flacons. When GI achieved ≥30 in the control tube any growth in the antibiotic tubes were considered as resistant and no growth in the antibiotic tubes were considered as susceptible.

Mgit 960
One or two day old positive cultures were used in the MGIT 960 fluorometric system. Susceptibility tests were performed according to manufacturer instructions. To check for bacterial contamination, dilutions on blood agar were inoculated, and in the event of growth with "unwanted" microorganisms, the test was repeated. The test was completed when the automated device provided a result. For strains that were deemed resistant to any of the anti-tuberculosis drugs, the test was repeated with the highest possible concentration of the drug in question (4.0 µg/mL for STR, 0.4 µg/mL for INH, 1.0 µg/ml for RIF, which is the same as the initial concentration).

Etest
Prior to susceptibility testing with the Etest, two-week old colonies that were grown on Löwenstein-Jensen culture medium were scraped and emulsified in M7H9 broth containing 6-8 glass beads. Thirty minutes was allowed for large particles to settle after which the supernatant was transferred by a sterile Pasteur pipette in to an empty sterile glass tube. Liquid culture medium was then added to the tube until McFarland turbidity no. 3 was achieved (approximately 9×10^8 CFU/mL). The resulting suspension was then inoculated radially onto a petri dish containing M7H10 solid culture medium, before being stored in an incubator set to 37°C, with 5-10% CO_2. After approximately 24 hours, Etest strips for STR, INH, RIF and ETM were placed into the petri dish which was covered by a plastic paraffin film to prevent drying. Petri dishes were then stored in an incubator with the same settings (37°C, 5-10% CO_2) for 5-10 days. At the end of the incubation period, the resulting ellipse was evaluated, and the point at which it intersected the MIC value scale (in µg/ml), where the concentration of the antibiotic tested inhibited microorganism growth, was recorded. The cut-off MIC values for the determination of susceptibility or resistance were set taking into consideration recommended critical concentrations established by the agar proportion method in previous studies; an isolate with a MIC value >2µg/mL for STR, >0.2µg/mL for INH, >1.0 µg/mL for RIF and >5µg/mL for ETM was considered resistant.

Pyrazinamid (PRZ) is one of the important first line drugs but through all methods included in this study there weren't completly standardised methods for PRZ.

Statistical analysis
Bayes' theorem was used to compare the susceptibility results of the different methods tested. The difference between methods in term of test duration was evaluated using Tukey's analysis.

Results

The susceptibility and resistance patterns of the isolates using all four methods have been summarized in table 1. According to the "gold standard" agar proportion method, none of the strains were resistant to STR or ETM, while two isolates were found to be resistant to both isoniazid and rifampicin.

The specificity, sensitivity and compatibility values for the evaluated methods in comparison to the agar proportion method have been summarized in tables 2 and 3.

The results obtained with BACTEC 460 TB were 100 % compatible with the agar proportion method for all antibiotics. The MGIT 960 provided mostly compatible results, except for 1 isolate for which resistance to streptomycin was observed. MIC values could only be obtained for 15 of the 33 isolates using the Etest due to fungal contamination, despite the test being repeated twice for each isolate. Out of 2 isolates that appeared to be resistant to both isoniazid and rifampicin, but one isolate

was confirmed to be susceptible to both antibiotics using the standard method. The results of susceptibility tests were interpreted according to Bayes' theorem.

Mean test durations are shown on table 4. There was no significant difference of durations between methods other than agar proportion.

The characteristics of the resistant isolates are shown on table 5.

Table 1. Results of susceptibility testing for all isolates by agar proportion (AP), BACTEC 460, MGIT 960 and Etest (n/%)

	AP		BACTEC		MGIT		ETEST	
	Susceptible (%)	Resistant (%)	Susceptible (%)	Resistant (%)	Susceptible (%)	Resistant (%)	Susceptible (%)	Resistant (%)
STR	33(100)	0	33(100)	0	32(96.9)	1(3.1)	15(100)	0
INH	31(93.9)	2(6.1)	31(93.9)	2(6.1)	31(93.9)	2(6.1)	14(93.3)	1(6.7)
RIF	31(93.9)	2(6.1)	31(93.9)	2(6.1)	31(93.9)	2(6.1)	14(93.3)	1(6.7)
ETM	33(100)	0	33(100)	0	33(100)	0	15(100)	0

(STR : Streptomisin, İNH: İsoniazid, RİF: Rifampisin, ETM: Ethambutol)

Table 2. Specificities and sensitivities of BACTEC 460, MGIT 960 and Etest using the agar proportion method as the gold standard.

BACTEC (%)		MGIT (%)		ETEST (%)	
Sensitivity	Specify	Sensitivity	Specify	Sensitivity	Specify
*	100	*	96.8	*	100
100	100	100	100	50.0	92.8
100	100	100	100	50.0	92.8
*	100	100	100	*	100

*Could not be calculated due to the absence of resistant strains

Table 3. Error and compatibility rates for anti-tuberculosis drugs tested using BACTEC 460, MGIT 960 and Etest, with the agar proportion method as the gold standard.

	STR (n/%)			İNH (n/%)			RİF (n/%)			ETM (n/%)		
	VME	ME	C	VME	ME	C	VME	ME	C	VME	ME	C
BACTEC (n=132)	0	0	33/100	0	0	33/100	0	0	33/100	0	0	33/100
MGIT (n=132)	0	1(0.8)	32/97	0	0	33/100	0	0	33100	0	0	33/100
ETEST (n=60)	0	0	15/100	1 (2)	0	14/93	1(2)	0	14/93	0	0	15/100

(VME: Very major Error, ME: Major Error, C: Compatibility)

Table 4. Test durations

TESTS	AP	BACTEC	MGIT	ETEST
Mean test durations (days)	21	6.39	6.93	7.06

Table 5. The characteristics of the resistant isolates

Isolate no.	Sample	Drug	AP	BACTEC	MGIT	ETEST (µg/ml)
30	Sputum	STR	S	S	R	0.064 S
32	Tissue	İNH	R	R	R	0.016 S
		RİF	R	R	R	0.016 S
33	Intra abdominal fluid	İNH	R	R	R	32.0 R
		RİF	R	R	R	2.0 R

S: Susceptible R: Resistant

Discussion

Among the susceptibility methods available, the CLSI considers the agar proportion as the "reference method" for determining susceptibility [5]. In our study, the BACTEC 460 radiometric method had 100% specificity and sensitivity for INH and RIF. Although this method was 100% specific for both STR and ETM, sensitivity could not be calculated due to the lack of resistant strains. General compatibility of this method with the agar proportion method was 97.3%; 96.5% compatibility for INH and 98.8% compatibility for RIF. Similar results had been gained in several studies [3,7,8].

In our study, we did not encounter ETM resistance in any of strains tested. CLSI guidelines state that isolated resistance to ETM is not a likely occurrence, and that such a result warrants retesting for confirmation. Adjers et al [9] reported that both the MGIT and BACTEC 460 systems were quick and reliable for evaluating resistance to INH and RIF, but concluded that testing for ETM and STR resistance required further research.

The BACTEC 460TB has recently become a standard method for evaluating susceptibility due to its reliability and quick results [10]. However, its cost, and the difficulties encountered regarding the disposal of tubes containing radioactive material are issues that need to be addressed [11].

Although MGIT 960 system is expensive, it is an alternative system that has no radioactive waste [12]. In essence, this is an automated method based on proportion that does not have a problem of waste disposal, and works on a computer program network thus relieving workload. Although this method is not better than BACTEC 460TB in terms of duration required to produce a result, it has

been shown to minimize the likelihood of human error by laboratory personnel, since the actual test is performed and interpreted by a computer program. Other advantages of MGIT 960 over the BACTEC system are that bottles are plastic and are continuously monitored [13]. However, compared to the BACTEC method, there is a higher risk of contamination and hence obtaining an erroneous result. The enriched media of liquid culture systems make them the most suitable culture media for the growth of microorganisms with growth times shorter than *M.tuberculosis* complex. Additionally, the bottles used in the MGIT system are capped, and use of pipettes increases the risk of contamination [12,14]. In contrast, tubes in the BACTEC system have a plastic top, and the small injectors used for transfer of material have been designed to minimize the risk of contamination [9,10]. Investigators reported on a general contamination rate of 2.3%, and recommended that all isolates deemed resistant by the MGIT system should be evaluated for purity of the culture and likelihood of contamination.

Other studies have also reported on more erroneous results with MGIT compared to BACTEC 460TB [12,15,16]. MGIT seems to be associated with higher rates of major and very major errors.

In the 1990s, investigators started performing susceptibility studies using the Etest, a simple method based on MIC value measurements, which is particularly useful for rapidly growing mycobacteria. Being a quantitative test, interpretation of results was left to the discretion of the investigator, which makes this method ideal for the evaluation of new anti-tuberculosis drugs.

After Wanger and Mills demonstrated that the Etest could also be used for slowly growing Mycobacteria, several comparative studies were undertaken [17]. The Etest is recommended for use as a screening test in developing countries where MDR strains of *M. tuberculosis* are a rising problem, mainly because it provides a rapid result while also allowing for antibiotic testing at different concentrations using varying dilutions. Based on the notion that resistance to RIF implies multi-drug resistance, use of the RIF Etest alone in areas were resistant strains are endemic is advocated as a low-cost and effective method to provide a general idea about resistance and potential treatment strategies.

The gold standard method is based on interpretation of 1% resistance, and despite the MIC values being different from those of the Etest, studies have generally shown good compatibility between the two methods [18,19]. In our study, MIC values could only be obtained for 15 of the 33 isolates evaluated, despite susceptibility for each isolate being tested twice using McFarland no. 3. Fungal contamination of the petri dishes also proved to be a significant problem. Although culture purity was insured in our study, some strains could not be retrieved despite several attempts at obtaining a subculture. The contamination rate observed was also higher than that of other studies. Furthermore, tests in this study needed to be repeated several times to obtain a result. Due to the the probability of selecting some susceptible and resistant isolates, the Etest method couldn't be found as convenient as the other methods - at least in this study. Just as other investigators have reiterated, our results show that arriving at a conclusion regarding multi drug resistant (MDR) tuberculosis using a single Etest strip is not quite possible. On the other hand, utilization of multiple Etest strips for every isolate, followed by confirmation using the standard agar or liquid systems is impractical and expensive.

There are certain issues regarding performance, interpretation and standardization of the Etest. For slow-growing bacteria such as *M.tuberculosis* complex, determination of the point of intersection on the Etest strip with a short duration of growth, requires use of a denser inoculum. McFarland no. 4 and McFarland no. 3 have both been used interchangeably in several studies [13,17,20]. However, the general recommendation is the use of McFarland no. 1-3, since the higher the density of inoculum used the faster an intersection point will be discernible. With the Etest, when the critical concentrations used in the agar proportion method for the anti-tuberculosis medications (5.0 μg/ml for STR, 0.2 μg/ml for INH, 1.0 μg/ml for INH and 2.0 μg/ml for ETM) are set as the cut-off MIC values, some susceptible strains may mistakenly by identified as resistant.

In terms of test duration, Tukey analysis revealed the presence of a statistically significant difference only between BACTEC and Etest ($p<0.05$). As with previous studies, our results show that susceptibility tests other than the standard method do not have a particular advantage over each other, except perhaps regarding test duration. Although very few resistant strains were included in this study, the number of MDR isolates was significant. If the resistance pattern of these isolates is assumed to be primary resistance, then the high resistance rate observed suggests that urgent action is needed to review current tuberculosis control measures. There is a need for further multicentric studies with a larger number of isolates, representative enough to help establish the resistance profile of the general population.

Conclusion

Regardless of the test used, validation with the gold standard method should be performed periodically as well as in the event of a discordant result. There remains a need for further studies to establish whether available rapid tests will eventually become the gold standard.

M. tuberculosis complex remains shrouded in mystery regarding the way it has evolved to protect itself against host defense mechanisms, especially structural

modifications in the cell wall. This study was an attempt to provide insight into susceptibility testing.

Foremost, an attitude that "Accuracy is more important than speed" needs to be adopted. Non-standardized tests with poor reproducibility, the results of which that are difficult to interpret, should not be used in a routine laboratory setting just for the sake of providing a quick result.

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