

Rapid Purity Check Method for Susceptibility Testing of *M. tuberculosis* Complex with the MGIT 960 System

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Abstract. The Bactec MGIT 960 system is a rapid and reliable automated method for drug susceptibility testing of *Mycobacterium tuberculosis* complex (MTBC) that yields a high percentage of agreement with the standard method. The microscopic cord morphology of *M. tuberculosis* in liquid medium is characteristic, and readily differentiates MTBC from nontuberculous mycobacteria (NTM). The goals of this study were to describe the microscopic and macroscopic growth morphology of MTBC in antimicrobial-containing MGIT tubes and to evaluate the usefulness of the growth appearance during purity checking. The macroscopic cotton wool-like appearance of MTBC isolates in isoniazid (INH), streptomycin (SM), rifampin (RMP), and ethambutol (EMB)-containing tubes was observed in 97, 90, 93, and 71% of the isolates, respectively. The percentage of typical cord, loose, or frayed rope microscopic features in smears prepared from MTBC-positive cultures of INH, SM, RMP, and EMB-containing tubes was 96, 86, 97, and 71%, respectively. The sensitivity of the macroscopic morphology for predicting the purity of drug-containing MGIT tubes was 93%, while the microscopic morphology predicted the purity with a sensitivity rate of 92%. We found that simply examining the macroscopic morphology of the antimicrobial-containing MGIT tubes of drug-resistant MTBC isolates is useful in preventing false resistant results of susceptibility testing by the MGIT 960 system.

Keywords: *M. tuberculosis* complex, MGIT, antimicrobial susceptibility testing

Introduction

The emergence of multidrug-resistant strains of *Mycobacterium tuberculosis* complex (MTBC) emphasizes the importance of rapid identification and timely detection of drug resistance for optimal management of patients with tuberculosis. The prompt detection of drug resistance is critical not only for the effective treatment of patients, but also for public health programs to prevent or reduce the spreading of drug-resistant tuberculosis (TB).

The Bactec Mycobacteria Growth Indicator Tube (MGIT 960) System has been widely validated as a rapid and reliable method for detecting and testing the susceptibility of MTBC isolates to first-line drugs such as streptomycin (SM), isoniazid (INH), rifampicin (RMP), ethambutol (EMB), and pyrazinamide (PZA) [1-9]. To provide antimicrobial susceptibility results, the MTBC should be recovered, confirmed by identification, and subjected to susceptibility testing. To confirm that a microorganism recovered in a positive MGIT tube is MTBC, the tube should be subcultured on an agar plate to obtain pure colonies for identification. This process requires an average of 1 to 3 weeks, which significantly decreases the benefit of

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using rapid detection and susceptibility testing systems. Therefore, the culture confirmation is a key step to reduce the time needed for reporting the final susceptibility testing results when the MGIT 960 system is used for both recovery and susceptibility testing.

A nucleic acid amplification-based method, such as BD ProbeTec ET system (Becton-Dickinson Diagnostic Systems, Baltimore, MD), is a reliable method for identification of MTBC isolates [10,11]. It reduces the turnaround time to one working day. The limitation of such a method is its inability to detect the presence of non-MTBC organisms. In the cases where MTBC are mixed with bacteria or nontuberculous mycobacteria (NTM), resistance caused by NTM will be mis-interpreted as being associated with MTBC and the susceptibility testing results may indicate false resistance [12]. Turbid appearance of MGIT tubes with/without drugs or growth on purity check plates signals the presence of bacteria or nontuberculous mycobacteria (NTM) at the time the growth unit (GU) value of the control tube reaches 400 and the susceptibility tests are ready to be interpreted. However, information about interference of normal respiratory flora, particularly slow growing mycobacteria, in the susceptibility testing by the MGIT 960 system is very limited in the published literature.

In our previous experience, the distinctive morphology of some mycobacteria when grown in liquid culture was shown to be a cost-effective method for rapid and presumptive identification of MTBC [13]. In MGIT broth, MTBC has a

characteristic colony clumping appearance that we call "cotton wool-like." Our present objectives are to describe the microscopic and macroscopic growth appearance of MBC in drug-containing MGIT tubes and to evaluate the usefulness of the growth appearance in purity checking.

Materials and Methods

A total of 13,618 specimens collected from 5,157 patients at the Kaohsiung Veterans General Hospital, Kaohsiung, Taiwan, were processed from June to November 2005. Specimens were digested and decontaminated with N-acetyl-L-cysteine and 2% NaOH for 15 min. Then the reaction was truncated by addition of phosphate buffer and the specimens were centrifuged at 3,000 g for 15 min. Tissues and normally sterile body fluids were inoculated directly into the culture media including inoculation into one MGIT tube (Becton-Dickinson, Sparks, MD) and one Lowenstein-Jensen (L-J) agar slant. The MGIT tube was automatically monitored continuously for positive growth and reported as negative after 42 days of incubation in the Bactec MGIT 960 instrument. L-J agar slants were incubated at 35°C in 5% CO₂ and observed weekly for 10 wk. Positive cultures were examined microscopically by Kinyoun acid-fast staining without previous vortexing [13]. Cultures with microscopic results indicative of the presence of mycobacteria were identified with the BD ProbeTec ET system, performed according to the manufacturer's instructions. If the microscopic appearance of the smear prepared from the positive culture was observed to be cord-like and tested positive with BD ProbeTec ET, the broths served as test inoculums and were subjected directly to susceptibility testing with the MGIT 960 system. The rest of the positive culture broth was sub-cultured to 7H11 plates and identified before susceptibility testing was performed. The identification was made by color and morphology of the colonies, biochemical tests, or other identification methods such as PCR-restriction analysis (PRA) [14-15]. Any MTBC identified by such

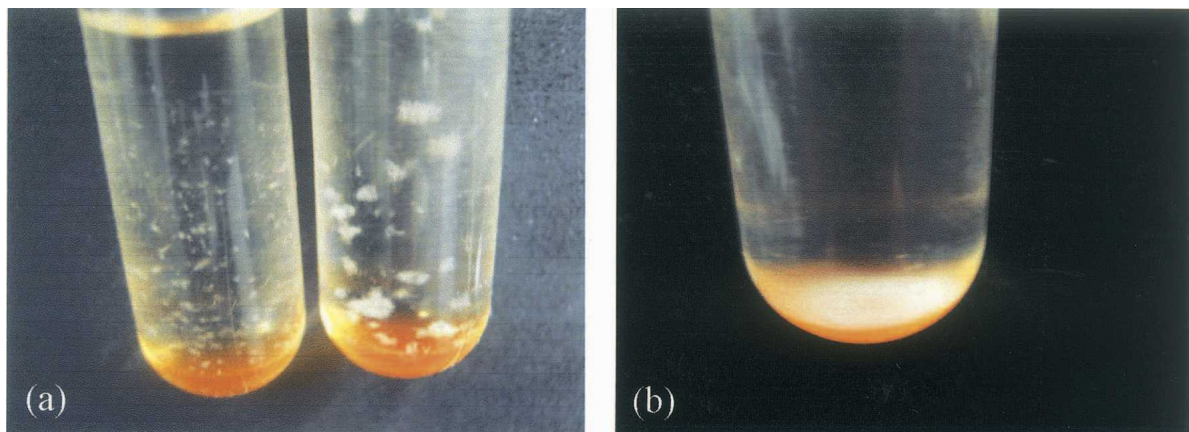


Fig. 1. Macroscopic morphology of MTBC described as (a) granular (left) or cotton-wool-like (right) and as (b) film-like (without swirling the tube).

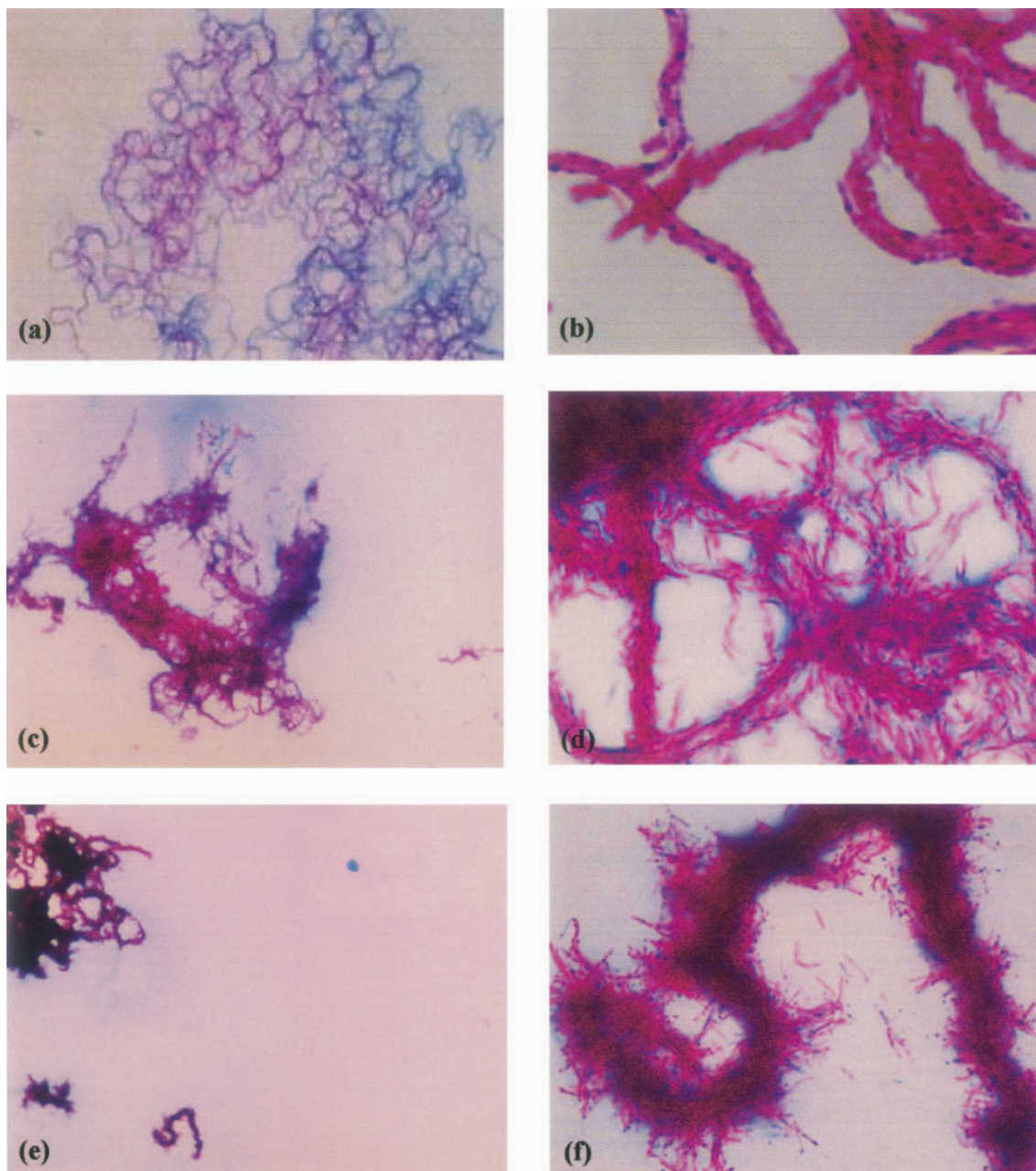


Fig. 2. Microscopic morphology of MTBC (Kinyoun stain) described as (a) cording, magnification $\times 100$; (b) cording, magnification $\times 1000$; (c) loose aggregation, magnification $\times 100$; (d) loose aggregation, magnification $\times 1000$; (e) frayed, magnification $\times 100$; and (f) frayed, magnification $\times 1000$.

procedures was subjected to susceptibility testing using bacterial suspensions from the colonies.

Susceptibility testing was performed using the MGIT 960 system with the following final drug concentrations: 1.0 and 6.0 $\mu\text{g/ml}$ for SM; 0.1 $\mu\text{g/ml}$ for INH; 1.0 $\mu\text{g/ml}$ for RMP; and 5.0 $\mu\text{g/ml}$ for EMB. All strains were tested at critical (low) and high concentrations for SM, and at critical

concentrations for the remaining 3 drugs. All mycobacterial suspensions showing drug resistance were checked for purity on sheep blood agar and Middlebrook 7H11 agar plates.

Microscopic and macroscopic evaluation of the morphology of drug-resistant MTBC isolates was performed. The isolates were subcultured on 7H11 plates and the colonies were identified by PRA. Bacterial DNA was prepared using

Table 1. Macroscopic and microscopic features of antimicrobial-resistant isolates in MGIT tubes.

	No. (%) of tubes with the following culture results in various drugs				
	Isoniazid (n = 80)		Streptomycin (n = 29)	Rifampin (n = 30)	Ethambutol (n = 14)
	MTBC (n = 73)	NTM (n = 7)	MTBC (n = 9)	MTBC (n = 30)	MTBC (n = 14)
Macroscopic morphology					
Cotton wool	71 (97.3%)	0	26 (89.7%)	28 (93.3%)	10 (71.4%)
Granular	2 (2.7%)	2 (28.6%)	3 (10.3%)	2 (6.7%)	4 (28.6%)
Film	0	5 (71.4%)	0	0	0
Microscopic morphology					
Cord	55 (75.3%)	0	20 (69.0%)	23 (76.7%)	5 (35.7%)
Loose aggregation	15 (20.5%)	0	3 (10.3%)	4 (13.3%)	3 (21.4%)
Frayed	0	0	2 (6.9%)	2 (6.7%)	2 (14.3%)
Needle	1 (1.4%)	0	0	0	0
Dot	0	3 (42.9%)	0	0	0
Other	2 (2.7%)	4 (57.1%)	4 (13.8%)	1 (3.3%)	4 (28.6%)

MTBC: *Mycobacterium tuberculosis* complex; NTM: nontuberculous mycobacteria.

QIAamp DNA Mini Kit (Qiagen Inc, Valencia, CA) and a 5 µl aliquot of purified DNA was used for PCR. Amplification and restriction enzyme analysis were performed [14-15].

The macroscopic appearance of every resistant MTBC isolate was observed in an antimicrobial-containing MGIT tube by gently swirling the tube intermittently after it had been sitting for >30 min. The morphology of colony clumps was described as cotton wool, granular, or film-like (Fig. 1).

Five morphological patterns were commonly observed microscopically in acid-fast smears prepared from antimicrobial-containing MGIT tubes: namely, cording, loose aggregation, fraying, dot, and needle. Cording, loose aggregation, and frayed growth appearance were observed in tubes with MTBC. Cording is defined as formation of 2-3 µm long, serpentine cords or rope-like aggregates in which the long axis of the bacteria is parallel to the long axis of the cord (Figs. 2a, 2b) [16]. Loose aggregation is the formation of 2-3 µm long, rope-like aggregates in which the long axis of the bacteria is also parallel to the long axis. It resembles a cord but shows less tight aggregation and is usually broader at 100× magnification and looks like frayed rope (Figs. 2c, 2d). It branches at 45° angles along the axis at 1000× magnification. A frayed appearance (Figs. 2e, 2f) denotes an aggregate that looks like frayed rope at both 100× and 1000× magnifications. Dots are irregular 1-2 µm short bacilli associated with coccoid forms that are arranged as single, dispersed cells or in small clumps. Needles are 2-3 µm bacilli that are dispersed or form irregular clumps. Morphologies of some MTBC-containing tubes are termed "non-specific" or "not otherwise classified."

Results

A total of 534 individual MTBC clinical isolates from June to November 2005 were directly

subjected to drug susceptibility tests. Of these, 106 isolates were resistant to at least one of the tested antimicrobial agents. A total of 153 culture-positive MGIT tubes were included in this study and resistance to SM, INH, RMP, and EMB was found in 29, 80, 30, and 14 of these MGIT tubes, respectively. Table 1 lists the appearance, acid-fast smear results, and microorganisms recovered from the tubes.

Of the 80 strains recovered from INH-containing tubes, 73 were MTBC, and 7 were NTM. Among the 73 INH-resistant MTBC isolates, 71 (97.3%) exhibited cotton wool-like clumps. The 7 NTM-containing tubes included 3 *M. kansasii*, 2 *M. goodnae*, 1 *M. intracellulare*, and 1 *M. szulgai* isolates and demonstrated granular or film-like clumps macroscopically.

All of the 29, 30, and 14 isolates subcultured from SM, RMP, and EMB-containing tubes grew MTBC. Macroscopic cotton wool-like appearance were observed in 26/29 (89.7%), 28/30 (93.3%), and 10/14 (71.4%) of the SM, RMP, and EMB-containing tubes, respectively. Simply observing the appearance of the positive resistant tubes as cotton wool-like appearance gave an 88.2% (135/153) accuracy in predicting the presence of MTBC. The ability of cotton wool-like appearance to predict the purity of MTBC in drug-containing MGIT tubes was 100% (Table 2).

Table 2. Prediction of purity of isolates in antimicrobial-containing MGIT tubes by macroscopic and microscopic morphology.

	No. (%) of tubes with culture results MTBC (n = 146)	NTM (n = 7)	Sensitivity	Positive predictive value
Macroscopic morphology				
Cotton wool	135 (100%)	0	92.5%	100%
Microscopic morphology				
Cording	103 (100%)	0	70.5%	100%
Cording + Loose aggregation + Frayed	134 (100%)		91.8%	100%

MTBC: *Mycobacterium tuberculosis* complex; NTM: nontuberculous mycobacteria

Typical cording, loose aggregation, or fraying in smears prepared from MTBC positive cultures of drug-containing MGIT tubes was present in 70/73 (95.9%), 25/29 (86.2%), 29/30 (96.7%), and 10/14 (71.4%) of the INH, SM, RMP, and EMB-containing tubes, respectively (Table 1). The sole presence of typical cording from MTBC positive cultures of drug-containing MGIT tubes was not conclusive.

The sensitivity of macro- and microscopic morphology to predict the purity of drug-containing MGIT tubes was 92.5% and 91.8%, respectively. The positive predictive values of both methods was 100% (Table 2).

Discussion

Although rapid detection of drug resistance has been made possible with the nucleic acid-based method, resistance to most anti-MTBC drugs, with the exception of RMP, cannot be attributed to a single locus in a substantial percentage (>90%) [17]. Multiple gene loci to be investigated for diagnosis of the drug resistance render most approaches tedious and resource-intensive for routine clinical laboratory services. Phenotypic susceptibility testing therefore remains the method of choice.

The MGIT 960 system is an efficient system not only for MTBC detection but also for susceptibility testing. Reducing the time between MTBC recovery and the subjection of MTBC to susceptibility testing is the key step in reducing the turnaround time. The BD ProbeTEC ET procedure is a high throughput and reliable method for the identification of MTBC organisms. We view this

as a significant step toward a complete work flow using the MGIT 960 system. Using the BD ProbeTEC ET system to confirm the presence of MTBC in an instrument positive MGIT tube and using the tube as the test inoculum directly for susceptibility testing could shorten the turnaround time. However, possible problems caused by the presence of slowly growing NTM should be eliminated.

Our previous work showed that presence of cords in MGIT broth is a reliable criterion for rapid, predictive identification of MTBC [13]. However, the growth appearance of MTBC in drug-containing liquid medium could be loose or frayed rope-like, as presented herein. The sensitivity of the acid-fast morphology to document purity in the resistant tubes was not as high as the macroscopic appearance. The present limited data reveal that erroneous results were obtained when the slowly growing NTM was present only for testing susceptibility of MTBC to INH. The appearance of MTBC in the EMB-resistant tube was more dispersed, and the proportion of acid-fast morphology that was typically cord-like was only 35.7%. Perhaps this is because antimicrobial effects of ethambutol inhibit the transfer of mycolic acids to the cell wall-linked arabinogalactan that prevents the formation of the outer leaflet of trehalose dimycolate (cord factor) [18]. This phenomenon does not appear in INH-resistant tubes, although the antimicrobial effects of INH also target the cell wall.

The presence of NTM could be easily excluded by the cotton wool-like appearance, which is the typical macroscopic morphology of MTBC grown in the MGIT tube. The ability of the cotton wool-

like appearance to predict the purity of MTBC in drug-containing MGIT tubes was 100%.

NTM can be introduced into the sputum exogenously or endogenously. NTM is normally present in the environment, especially in water, and could be introduced into the sputum when patients rinse their mouth with tap water. It could be introduced into the sputum endogenously by the NTM colonized in the respiratory tract.

In our previous investigation of the effect of sputum quality on the culture rate and contamination rate, the specimens submitted to our laboratory exhibited a high colonization rate of NTM. The sputum adequacy was determined by using the quality score (Q score) examined by Gram stain. A total of 4,701 sputa were evaluated and a total of 106 (5.4%) of 1,977 Q2-Q3 sputa, and 106 (3.9%) of 2,724 Q0-Q1 sputa, grew NTM (unpublished data). The NTM positivity rates were not significantly different, indicating that most of the NTM was from endogenous colonization.

NTM colonization in the respiratory tract may also cause interference in other identification or susceptibility tests. In the Bactec 460 system, in the presence of NTM and MTBC, NTM is not inhibited by NAP and increases the growth index, masking the presence of MTBC. In addition, in biochemical tests for identification, inaccurate results are obtained if a mixed culture is not identified.

The importance of purity check plates cannot be over-emphasized. Rapidly growing mycobacteria, which are most frequently recovered in respiratory specimens, can be detected on the purity check plates at the time of positivity. The package insert of the MGIT 960 SIRE drug susceptibility kit states that only a blood agar plate is required. We suggest that at least a 7H11 plate be used to detect larger quantities and more species of NTM.

The MGIT 960 method is reliable for rapid detection of MTBC and antimicrobial susceptibility testing. The BD ProbeTEC ET system is user-friendly and reduces the time between identification and the subjection of MTBC to susceptibility tests, allowing patient management decisions to be more rapid. However, the presence of NTM must be excluded to prevent inaccurate susceptibility results, leading to incorrect treatment. Such inaccurate

results can occur especially in areas where the colonization rate of NTM of the respiratory tract is high. We conclude that it is essential to check the appearance of the resistant tubes as well as the purity check plates before the susceptibility results are reported. The resistant tubes with macroscopic characteristics other than cotton wool-like appearance should not be reported until purity is confirmed after organisms are grown on the purity check plates.

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