

Issues in *Mycobacterium tuberculosis* Complex (MTBC) Drug Susceptibility Testing: Ethambutol (EMB)

BACKGROUND

Ethambutol (EMB) is an antituberculosis drug used as part of the first line treatment against *Mycobacterium tuberculosis* complex (MTBC).¹ A primary role of EMB when used in initial combination antituberculosis therapy is to minimize the risk of drug resistance development to companion first line drugs, particularly isoniazid.² Resistance to EMB will not decrease the effectiveness or increase the length of treatment for MTBC susceptible to the other first-line drugs. Within current dosing recommendations, EMB is bacteriostatic against actively replicating bacteria but can be bactericidal when serum concentrations are over 10 µg/mL.³ Ethambutol acts through disruption of MTBC cell wall synthesis, targeting and inhibiting the function of arabinosyl transferases, encoded by the embCAB operon, and responsible for biosynthesis of the cell wall components arabinogalactan and lipoarabinomannan.⁴⁻⁶ This disruption may lead to increased permeability of the cell wall.^{4,7} Ethambutol has also been found to inhibit RNA biosynthesis.⁸

The recommended dose for EMB is 15-20 mg/kg once daily in combination with other antituberculosis medications. It is used most often in the US in the initial multi-drug treatment regimen if drug susceptibility results are not yet available and background drug resistance in the community exceeds 4% or where isolates from a given case are determined to be resistant to other medications.⁹ Common side effects include optical neuritis, gastrointestinal upset, nausea, fever, dizziness and rash. According to information provided from the NIH on hepatotoxicity, the addition of EMB to the drug regimen does not appear to increase serum aminotransferase elevations.¹⁰ Ethambutol has only been associated with rare instances of acute, symptomatic liver injury.¹¹

PRACTICAL LABORATORY ISSUES

Ethambutol Drug Susceptibility Testing and Test Methods

Current EMB drug susceptibility testing (DST) methods in the United States include the agar proportion (AP) method using the Clinical and Laboratory Standards Institute (CLSI) recommended critical concentration¹² on either 7H10 or 7H11 medium, commercial broth systems with reduced incubation times (MGIT 960, VersaTREK), which are FDA cleared, a commercial microdilution plate for the testing of minimum inhibitory concentration (MIC) (Sensititre), and detection of mutations in drug resistance determining regions of the MTBC genome (laboratory developed on a variety of sequencing platforms, and commercial line probe assays). Accumulating data indicates the genotypic results from molecular testing may be discordant with the phenotypic results observed with AP, commercial rapid broth systems or MIC testing.^{13,14}

Laboratories performing drug susceptibility testing using FDA-cleared assays should strictly follow manufacturer's guidelines for performance of the assays. The current assays on the market appear to be much more technique dependent than the radiometric BACTEC™ 460

method. For more specific methods on ensuring reproducibility see Section “Approaches to improving reproducibility and specificity” below.

Agar proportion testing has its challenges as well. Variability among laboratories may be introduced in the manufacture of drug-containing agar plates. Some laboratories prepare their own drugs while others use commercially available elution disks. Commercially available OADC used in the manufacture process varies in purity among manufacturers. Drug activity may vary from lot to lot of OADC which may affect the performance of the assay.¹⁵ The CLSI guideline reports findings of microcolonies within the EMB quadrants, and that the frequency of microcolonies may vary from one laboratory to another.¹² The significance of microcolonies is unknown, and may represent true resistance, partial resistance or may be a result of drug degradation. All of these issues may lead to inconsistencies with testing and reporting of EMB drug resistance with the AP method.

Sensititre® MYCOTB is a commercial microdilution plate for the determination of MICs. It consists of a 96 well microtiter plate containing twelve antimicrobial agents at appropriate dilutions with EMB being tested at a range of 0.5 µg/mL to 32 µg/mL. There are no established interpretive break points for the assay and mycobacteria endpoints can be difficult to interpret. The manufacturer’s protocol requires growth from solid media, 7H10 Agar, followed by reading plates at 7, 10, 14 and 21 days resulting in significant delays in the reporting of drug susceptibility results.

Molecular-based assays are available to provide more rapid DST results for EMB compared to culture-based methods. These assays are designed to detect mutations in the *embB* gene. Mutations at codon 306 are the most commonly detected point mutations conferring EMB resistance.⁷ It has been reported that 30 to 70% of EMB-resistant strains have a mutation in *embB*.¹⁶⁻¹⁸ Resistance mutations have also been found in *embC* and *embA* genes.¹⁹ However, not all mutations associated with resistance to EMB are known and further research must be conducted to determine the significance of other mutations related to EMB resistance.

Commercially available line probe assays such as the Genotype MTBDRsl (HAIN Lifesciences, Nehren, Germany) are being manufactured and may be available for purchase in the US as research use only products. This assay is not FDA-cleared and as such will require the performance of a validation study as a laboratory developed test prior to adopting the method. Mutations can be detected in the *embB* locus. However, information regarding the specific mutation(s) found with this assay is not available. Mutations in the *embA* and *embC* genes would not be detected with this assay.

Reproducibility of susceptibility testing for EMB

FDA cleared commercial broth susceptibility testing methods are most frequently used in the US because they provide more rapid results than solid media systems, but they have the most challenges for accurately determining EMB susceptibility and resistance. Discordance among the phenotypic testing methods, particularly with EMB, has been documented as more laboratories report DST results for broth susceptibility testing and AP.^{13,20,21} Furthermore, accumulating gene sequencing data also indicates that genotypic results from molecular testing may be discordant with the phenotypic results observed with AP, commercial rapid broth systems or MIC testing.^{7,13,21}

A 2002 study asserted that difficulty with EMB testing may be due to the bacteriostatic nature of the drug itself, reduced activity in a culture medium and/or the narrow range between the MIC's of resistant strains and susceptible strains.²¹ In this study, EMB was compared at concentrations of 2.5 µg/mL and 3.75 µg/mL in the Bactec 460, and results demonstrated that the equivalent concentration for the Bactec 460 method was between 2.5 and 3.75 µg/mL. Increasing the concentration of EMB yielded more results with susceptible findings that were inconsistent with the AP method. A more recent study indicates that the MGIT 960 is about 84% sensitive in detecting EMB resistance compared with other methods. However, the report goes on to state that there is poor to variable agreement among methods when testing for EMB susceptibility, including MGIT 960 at the current critical concentration of 5 µg/mL.²²

Findings in other studies have concluded that much of the discordance can be linked to isolates with EMB MIC values at or around the critical concentration.^{13,14} It has also been noted that there is lower performance for detecting EMB resistance with the MGIT 960 method, as the current tested concentration of EMB (5.0 µg/mL) is not equivalent to the critical concentration of drug initially established with the LJ Proportion and AP methods.^{13,22} Studies suggest the need for re-evaluation of the critical concentration of EMB in the MGIT 960 due to false susceptibility with the test method.²²

Table 1. Culture-based and Molecular Drug Susceptibility Testing Methods

	Culture-based Method	Determination of Resistance
FDA Cleared	MGIT 960	Growth in the presence of EMB at the critical concentration of 5.0 µg/mL
	VersaTREK	Growth in the presence of EMB at the critical concentration of 5.0 µg/mL
Reference	Agar Proportion 7H10	The number of colony forming units (CFU) growing on medium containing EMB at the critical concentration of 5.0 µg/mL compared with the number of CFU growing on the drug-free medium
	Agar Proportion 7H11	The number of colony forming units (CFU) growing on medium containing EMB at the critical concentration of 7.5 µg/mL compared with the number of CFU growing on the drug-free medium
Research Use Only (RUO)	Sensititre	The lowest concentration that reduces visible growth from a range of 0.5-32 µg/mL EMB

	Molecular-based Method	Determination of Resistance
Reference	Sanger Sequencing	Detection of mutations in genetic loci associated with resistance to EMB (<i>embB</i>)
	Pyrosequencing	Detection of mutations in genetic loci associated with resistance to EMB (<i>embB</i>)
Research Use Only (RUO)	Hain GenoTypeMTBDRsl <i>line probe assay</i>	Detection of mutations associated with EMB resistance using 2 "mutation probes" visualized on a test strip

Shaded cells refer to non-phenotypic DST methods

Proficiency Testing

Laboratories are urged to participate in qualified proficiency testing (PT) programs to satisfy regulatory requirements for drug susceptibility performance. In the US, the College of American Pathologists provides proficiency testing for EMB and other antituberculosis drugs; however, their program provides only two challenge isolates per year and includes only pan-susceptible strains. Other commercial PT programs, such as API, do provide PT challenges for MTBC DST. The Centers for Disease Control and Prevention (CDC) offers the Model Performance Evaluation Program (MPEP) for MTBC DST, which is not a formal, graded PT program but could be used as an adjunct to the laboratory's regulatory PT program. The MPEP Program is an educational self-assessment tool offering five MTBC isolates per challenge of which both drug resistant and drug susceptible isolates are included). It provides an opportunity to compare results to those obtained by other participants using the same methods.

Approaches to improving reproducibility and specificity

Limit and Monitor Contamination:

Laboratories performing drug susceptibility testing in liquid media may detect drug resistance to one or more of the first line drugs. Drug resistance may be true drug resistance or may be related to other factors. CLSI recommends checking for purity of the culture to look for the presence of contaminants or Nontuberculous Mycobacteria (NTM).¹² Following the CLSI guidance, if any DST results from the liquid media are considered questionable, repeat testing should be performed. Testing may be repeated using the same method or AP may be used if the laboratory conducts this testing. CLSI indicates that repeat testing for rarely occurring resistant results such as mono-EMB resistance from the initial testing should be considered. Please refer to *Section D: Guidance* below.

EMB DST Inoculum:

Preparation of inoculum may have significant outcome on results of the drug susceptibility test, particularly with the liquid test systems. Laboratorians should closely follow manufacturer's recommendations (MGIT960 and VersaTREK) for performance of the drug susceptibility test and preparation of the inoculum. The MGIT960 manual contains a full protocol for standardizing inoculum specifying fresh growth, sufficient vortexing and allowing the suspension to settle before inoculating. Failure to closely adhere to testing protocols may lead to false resistance in the drug susceptibility test. VersaTREK also has specific inoculum preparation instructions for both McFarland equivalent cell suspensions made from solid media and seed bottles. Other recommendations include subculturing to 7H10 or 7H11 and blood agar to verify the purity of the seed bottle and staining all bottles that are signal positive.

IMPACT ON CLINICAL OUTCOMES

Controlled clinical trials have shown the effectiveness of EMB in the treatment of tuberculosis, especially in combination with other antituberculosis drugs.^{23,24} Accurate laboratory methods for EMB susceptibility testing are needed so that patients are appropriately treated, especially in multidrug-resistant (MDR) cases. Research indicates EMB penetrates and accumulates in macrophages, as those seen in inflammation sites of human lungs, in concentrations up to ten-fold that seen in serum or plasma.^{8,25}

Banu and colleagues in their study of discordance across several drug susceptibility methods in a single laboratory conclude that while false susceptibility to EMB is of less consequence in the setting of drug susceptible MTBC, it is a major concern with MDR-TB.²⁰ Data from their laboratory, located in a high MDR-TB setting, indicated that if they were to use only MGIT 960 SIRE (streptomycin, isoniazid, rifampin, and ethambutol) kits, as much as 49% of all EMB susceptible results would be falsely susceptible.²⁰

AREAS OF ONGOING RESEARCH

Many studies have focused on determining resistance through the detection of mutations leading to resistance.²⁰ Early molecular data indicated that strains resistant at both 2.5 and 7.5 µg/mL exhibited *embB* mutations.¹⁶ Multiple investigators have found that mutations at codon 306 of *embB* are the most commonly detected point mutations conferring EMB resistance^{4,14,26} but mutations in codons 313 and 315 were also detected in EMB resistant isolates.^{7,26} Not all strains exhibiting phenotypic resistance harbor *embB* mutations suggesting other resistance mechanisms have yet to be discovered.^{7,14,27} The data reported by Starks et al. indicates that the presence of *embB* 306 mutations is useful for detection of EMB resistance in 50 to 70% of clinical isolates.⁴

Ethambutol resistance determined by the microdilution method may present better correlation with *embB* mutations in MDR-TB isolates but further studies comparing microdilution to the 7H10 AP method rather than the LJ proportion method need to be conducted.⁷ One study of the microdilution method also reports fewer instances of false EMB susceptibility when the breakpoint is set at 4 µg/mL.²⁸ Clinical correlation of these test concentrations has yet to be determined.

GUIDANCE

Laboratories should consider the following:

- All EMB mono-resistant isolates should be questioned because EMB mono-resistance is rare and it is most commonly associated with resistance to INH (up to 96.6% of isolates).²¹
 - CLSI recommends that any results considered questionable in commercial based broth systems be repeated using the same system or standard agar proportion. Ideally, a different culture-based method than the original should be used.
 - The initial resistant DST results should be reported immediately, prior to the availability of confirmatory results. It should be made clear to the healthcare provider that confirmatory testing is being performed.
 - Testing for mutations using molecular methods is potentially useful as mutations associated with resistance may be rapidly identified. However, absence of a mutation does not confirm susceptibility to EMB.
 - Quality control issues including contamination should also be considered if EMB mono-resistance is identified.
- Testing of secondary antituberculosis drugs should be performed on all isolates of MTBC that are resistant to Rifampin or any two of the primary drugs.
- For patients not responding to therapy after three months, labs should follow the APHL and CDC Guidelines for Submission of Sputum Specimens²⁹ for MTBC testing for re-evaluation of drug resistance.

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ACKNOWLEDGMENTS

This document was prepared by the APHL Tuberculosis Subcommittee, with significant input from the following:

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This project was 100% funded with federal funds from a federal program of \$2,189,000. This brief was supported by Cooperative Agreement # U600E000103 funded by the Centers for Disease Control and Prevention. Its contents are solely the responsibility of the authors and do not necessarily represent the official views of CDC or the Department of Health and Human Services.

Office of Surveillance, Epidemiology and Laboratory Services (OSELS)
National Center for HIV, Viral Hepatitis, STDs and TB Prevention (PS)
National Center for Zoonotic, Vector-borne, and Enteric Diseases (CK)
National Center for Immunization and Respiratory Diseases (IP)
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