Issues in *Mycobacterium tuberculosis* Complex Drug Susceptibility Testing: Ethambutol

HIGHLIGHTS

- Due to inherent difficulties with growth-based DST for detection of EMB resistance, rapid molecular methods based on the detection of genetic mutations may prove more valuable for determining EMB resistance.[5]
- All EMB monoresistant isolates should be reviewed since EMB monoresistance is rare and is most commonly associated with resistance to INH (up to 96.6% of isolates).[27]
- Mutations at codon 306 of *embB* are the most commonly detected point mutations and confer resistance in 50–70% EMB resistant TB isolates.

BACKGROUND

Ethambutol (EMB) is an antituberculosis drug used as part of the first-line treatment against *Mycobacterium tuberculosis* complex (MTBC) to minimize development of drug resistance to companion first-line drugs, particularly isoniazid (INH).[1,2] EMB is bacteriostatic against actively replicating bacteria but can be bactericidal when serum concentrations are over 10 µg/mL.[3] Research indicates EMB penetrates and accumulates in macrophages—within inflammation sites of human lungs—with measured EMB concentrations up to ten times higher in comparison to those seen in serum or plasma levels.[4]

EMB acts through disruption of MTBC cell wall synthesis, targeting and inhibiting the function of the arabinosyl transferases encoded by the *embCAB* operon, comprised of three adjacent genes (*embC*, *embA*, and *embB*) responsible for biosynthesis of the cell wall components arabinogalactan and lipoarabinomannan.[5–7] EMB mainly interrupts polymerization steps in the biosynthesis of the arabinan component of cell wall arabinogalactan.[6,7] This disruption may lead to increased permeability of the cell wall.[1,8]

EMB resistant strains tend to have a minimum inhibitory concentration (MIC) ranging from 7.5 µg/mL to 40 µg/mL.[8–11] The test concentration of 5 µg/mL (using the Mycobacteria Growth Indicator Tube, MGIT) allows discrimination between the majority of susceptible and resistant strains. In addition to traditional growth-based drug susceptibility testing (DST), molecular detection of DNA mutations can provide valuable information to predict drug resistance. While the mechanism of resistance of MTBC to EMB is not well-defined nor genomic targets well-documented,[12] many researchers have focused investigations on the role of the *embCAB* operon, specifically the *embB* gene. Multiple investigators have found that mutations at codon 306 of *embB* are the most commonly detected point mutations, with 50–70% of isolates containing the mutation conferring EMB resistance.[5,8,11,13–16] However, additional mutations in *embB*, as well as mutations in *embC* and *embA*, have also been

The World Health Organization (WHO) has published a second edition of the "Catalogue of mutations in *Mycobacterium tuberculosis* complex and their association with drug resistance, 2nd edition" (2023). This document provides a common, standardized reference for the interpretation of resistance to first- and second-line drugs. The catalogue includes over 30,000 variants, their frequency and association with resistance. The mutations are further classified into two tiers. Tier 1 comprises genes considered most likely to contain the resistance mutations. Tier 2 comprises genes that are reasonably likely to contain resistance mutations, with the additional, literature-defined promoter sequences. The WHO document is an important resource with detailed information on numerous mutations associated with drug resistance and may include mutations not specifically addressed in this white paper. We recommend readers review both documents if they are interested in molecular detection of drug resistance.

detected in EMB resistant isolates.[14,17] More information about alternative mechanisms or markers of resistance are discussed in the section Areas of Ongoing Research.

PRACTICAL LABORATORY ISSUES

Growth-based Phenotypic EMB DST and Test Methods

Current growth-based DST methods for EMB in the US include the agar proportion (AP) method using the Clinical and Laboratory Standards Institute (CLSI) recommended critical concentrations on either 7H10 or 7H11 medium, commercial automated broth systems BACTEC™ MGIT™ 960 (MGIT) with the BACTEC™ SIRE Drug kit (MGIT assay, Becton Dickinson), VersaTREK™ Automated Microbial Detection System (VersaTREK) with the VersaTREK™ Myco Susceptibility kit (VersaTREK assay, TREK Diagnostic Systems, Thermo Scientific™) and a microdilution plate method, Sensititre™ *Mycobacterium tuberculosis* MYCOTB MIC Plate (Thermo Scientific™) (Table 1). Only the MGIT and VersaTREK assays are FDA-cleared to test for susceptibility to EMB. The Sensititre™ MYCOTB is labeled for research use only (RUO).

The AP method compares the growth of an isolate on solid agar medium with and without drug at various concentrations. An isolate is determined to be resistant if the number of colonies that grow in the presence of the drug is ≥1% of the number of colonies of the same isolate that grows in the absence of the drug. The agar proportion method also allows the determination of the proportion or percentage of resistant organisms within the sample.This method remains the accepted reference method but is not without its challenges.[10] For example, variability among laboratories may be introduced with the production of drug-containing agar plates and the oleic albumin dextrose catalase (OADC) commercial growth supplement used in the preparation of the agar medium. The OADC may vary in purity from lot-to-lot which impacts drug activity. Few clinical laboratories use the AP method because of the complexity of preparing the drug plates and the long turnaround time compared to commercial automated broth systems.

The commercially available MGIT assay is based on growth of the MTBC isolate in a drug-containing tube compared with a drug-free tube (growth control). The MGIT 960 instrument continuously monitors tubes for increased fluorescence. Analysis of fluorescence in the drug-containing tube compared with the fluorescence of the growth control tube is used by the instrument to determine susceptibility results. The VersaTREK assay utilizes a standardized suspension of MTBC to inoculate into drug-containing VersaTREK Myco bottles and a drug-free control bottle which are monitored for growth. Growth is detected by a change in gas pressure in the headspace of the bottle due to the consumption of oxygen by the mycobacterial isolate. Commercial automated broth systems are the most commonly used method for first-line DST in clinical laboratories.[18] This is preferred to the AP method due to the shorter turnaround time. The most commonly utilized system for growth-based DST is the MGIT assay performed on the BACTEC™ MGIT™ 960. The current critical concentration per CLSI for EMB is 5 µg/mL.[10] The VersaTREK assay is FDA-cleared to test EMB at both 5 µg/mL and 8 µg/mL. Regardless of the system used, CLSI suggests that these systems may be less effective at identifying EMB resistance than AP.[10]

Sensititre® MYCOTB is a broth microdilution assay consisting of a 96-well microtiter plate containing 12 antimicrobial agents at appropriate dilutions. EMB is evaluated in the concentration range of 0.5 µg/mL to 32 µg/mL. The manufacturer has no established interpretive break points for the assay, and mycobacteria endpoints can be difficult to interpret. CLSI has published a suggested breakpoint but notes that the significance of the MIC value of 4µg/mL is unknown because clinical correlation is not available; however, comparisons to the AP method have been evaluated.[10,19,20] The manufacturer's protocol requires growth from solid media and MIC plate incubation times of 7, 10, 14 and 21 days with interpretation as early as day 7 of incubation.

Molecular-based Genotypic EMB DST and Test Methods

Molecular-based assays detect mutations in drug resistance determining regions of the MTBC genome and are able to provide more rapid DST results for EMB compared to growth-based methods. There is at least one commercially available method, GenoType MTBDRsl VER 1.0 (HAIN Lifesciences) and a number of laboratory developed tests (LDTs) utilizing targeted and whole genome sequencing methods, none of which are FDA-cleared at this time. EMB resistance is most often associated with mutations in *embB* at codon 306, specifically Met306Val. However, other less common mutations are still considered "Tier 1" mutations by WHO including mutations at codons 319, 354, 406 and 497 as well as mutations in *embC* and *embA* which have also been detected in EMB-resistant isolates.[4,10,14,17,21,22] Not all mutations associated with resistance to EMB are known, and genes other than *embB* have been implicated [See Areas of Ongoing Research].

Line probe assays (LiPA) allow for detection of MTBC DNA from isolates or directly from clinical samples. The procedure involves a DNA extraction step followed by PCR, labeling and hybridization. The GenoType MTBDRsl VER 1.0 (HAIN Lifesciences), are available for purchase in the US as RUO products for the identification of MTBC and EMB resistance. This assay is not FDA-

cleared and therefore, requires the performance of a validation study prior to adopting the method. The GenoType MTBDRsl VER 1.0 detects mutations in the *embB* locus, but the specific nucleotide mutations are not delineated in the Manufacturer's Instructions for Use. Mutations in the *embA* and *embC* genes are not detected with this assay. *Note: EMB resistance is not evaluated in the Genotype MTBDRsl VER 2.0 (HAIN Lifesciences).*

There are two general types of sequencing based assays; targeted sequencing such as sanger sequencing, pyrosequencing (PSQ) and targeted next generation sequencing (tNGS) and non-targeted or whole genome sequencing. The targeted sequencing methods mentioned vary in their methodology but they are able to detect specific "targeted" changes in a gene or loci. PSQ and tNGS can be performed on clinical specimens due to the PCR amplification included in the method and isolates whereas whole genome sequencing is most commonly performed on isolates. Depending on work flows, targeted sequencing methods can have a shorter turnaround time and be more cost-effective. See Areas of Ongoing Research: Sequencing Technologies to Identify Resistance for more details.

With all molecular methods, particularly sequence-based methods used as a stand-alone test or to confirm other DST results, the region(s) tested and the analysis pipeline must be able to differentiate mutations that are known to confer resistance to EMB from those that are not known to predict resistance. For example, certain mutations such as the Glu378Ala polymorphism in *embB* have been detected in isolates that were EMB-susceptible by AP and may represent lineage markers, but are not associated with EMB resistance.[23]

Table 1. Growth-based and Molecular-based Drug Susceptibility Methods for EMB[a]

Growth-based Method	Determination of Resistance	Commercially Available Test Systems-US (Regulatory Status)
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	N/A
Agar Proportion 7H11	The number of 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	N/A
Automated Broth System	Growth in the presence of EMB at the critical concentration of 5.0 µg/mL and/or higher concentration of 8.0 µg/mL	BACTEC™ MGIT™ 960 SIRE Drug Kit (FDA cleared), VersaTREK™ Myco Susceptibility Kit (FDA cleared)
Microdilution Plate Method	The lowest concentration (range 0.5-32 µg/mL) that shows no visible growth	Sensititre™ Mycobacterium tuberculosis MIC Plate (RUO)[b]
Molecular-based Method	**Determination of Resistance**	**Commercially Available Test Systems-US (Regulatory Status)**
Line Probe Assay (LPA)	Detection of mutations in *embB* associated with EMB resistance by lack of hybridization to wild-type sequence probes and/or hybridization to probes containing known mutations visualized on a test strip[c]	GenoType MTBDR[sl] VER 1.0 (RUO)[b]
Sanger sequencing	Detection of specific mutations within the genetic loci associated with resistance to EMB (*embB* (most common), *embA* and *embC*)	LDT only
Pyrosequencing (PSQ)	Detection of specific mutations within the genetic loci associated with resistance to EMB (*embB* (most common), *embA* and *embC*)	LDT only
Targeted next generation sequencing (tNGS)	Detection of specific mutations within the genetic loci associated with resistance to EMB (*embB* (most common), *embA* and *embC*)[d]	Deeplex Myc-TB (RUO), Ion AmpliSeq™ TB Panel (RUO)[b]
Whole genome sequencing (WGS)	Detection of mutations within *embB* (most common), *embA* and *embC* and analyzing the entire genome for other genetic predictors of drug resistance[d]	LDT only

a. Critical concentrations included in the table are from CLSI M24 unless noted otherwise.[10]

b. Assays are commercially available as RUO but can be validated for clinical testing.

c. GenoType MTBDRsl VER 1.0 also detects specific mutations in *gyrA* and *rrs*, please review the instructions for use for complete details.

d. Mutations detected in other targets (e.g. *embR*, *ubiC*, *iniA-C* and others) may contribute to EMB resistance, but the data is not conclusive at this time.

Considerations for EMB DST

A notable concern regarding EMB DST is the level of inconsistency reported among and between growth-based and molecular-based assays. Discordance among the EMB growth-based DST methods, commercial rapid broth systems, AP, and microdilution method are well-documented.[23–29] Various studies have asserted that the 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 MICs of resistant strains and susceptible strains.[11,25,27,30] In a systematic review, 66 studies that evaluated EMB DST had poor and variable agreement for all DST methods, including the MGIT assay and suggested the need for re-evaluation of the critical concentration of EMB.[31]

Accumulating data also indicates that molecular-based results, such as data from WGS and LiPAs (i.e., GenoType MTBDRsl), may be discordant with the growth-based assay results. Zhang et al. demonstrated that EMB resistance determined by the microdilution method may present better correlation with *embB* mutations in multidrug-resisant TB (MDR TB) isolates as compared to sequence analyses, but additional studies comparing microdilution to other molecular methods need to be conducted.[8]

Approaches to Improving Reproducibility and Accuracy of Growth-based Methods

Preparation of inoculum may noticeably impact results of DST, particularly with the rapid commercial broth systems. Laboratorians should closely follow manufacturer's recommendations (MGIT assay and VersaTREK assay) for performance of DST and preparation of the inoculum. The MGIT assay manual contains a protocol for standardizing inoculum specifying fresh growth, sufficient vortexing and settling of the suspension before inoculating. Failure to closely adhere to testing protocols may contribute to a false resistant result. The VersaTREK assay provides 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 signal positive.

Quality Assurance

CLIA certified laboratories must participate in a CLIA-approved proficiency testing (PT) program to satisfy regulatory requirements for DST performance. If a qualified program is not available a suitable alternative, such as inter-laboratory comparison, must be implemented. In the US, the College of American Pathologists offers regulatory PT for antituberculosis drugs; however, their program provides only two challenges per year and includes only pan-susceptible strains. Other commercial PT programs, such as the American Proficiency Institute, also provide regulatory PT challenges for MTBC DST. The US Centers for Disease Control and Prevention (CDC) offers the Model Performance Evaluation Program (MPEP) for MTBC DST. MPEP 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. MPEP is not a formal, graded PT program, but could be used as an adjunct to the laboratory's regulatory PT program.

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.[17,32] In the US, EMB is included in the first two months of treatment along with rifampin (RIF), isoniazid (INH) and pyrazinamide (PZA). In special situations such as pregnancy or hepatic disease, treatment for drug susceptible TB (including with EMB) may be longer than two months. Patients with INH-resistant TB may be treated with a six-month regimen of daily RIF, EMB and PZA plus a later generation fluoroquinolone (e.g., moxifloxaxin or levofloxacin). For the treatment of MDR TB, EMB is included only when other more effective drugs cannot be assembled to achieve a total of five drugs in the regimen.[13] Isolated resistance to EMB is rare and does not have much impact on the duration or efficacy of the treatment regime.

Accurate laboratory methods for EMB DST are needed so patients are treated appropriately, especially in MDR TB cases. There are documented concerns that tolerance to the drug may develop in patients previously infected with EMB-susceptible strains of MTBC that subsequently relapsed. These isolates remained characterized as genotypically susceptible to EMB.[33,34]

Banu and colleagues, in their single-laboratory study of discordance across several DST methods, concluded 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. Data from their laboratory, representing a high prevalence MDR TB setting, indicated that if only the MGIT assay was used, as many as 49% of all EMB susceptible results may be discordant with other methods.[28]

AREAS OF ONGOING RESEARCH

Other EMB Resistance-Associated Mutations

Many studies have focused on determining resistance through the detection of mutations leading to resistance.[28] While the data reported by Starks et al. indicate that the presence of mutations in *embB*, codon 306, are useful for detection of EMB resistance in 50 to 70% of clinical isolates,[5] not all strains exhibiting phenotypic resistance harbor *embB* mutations, suggesting other resistance mechanisms have yet to be fully characterized.[8,20,35] Other mutations associated with EMB resistance have also been detected within the *embCAB* operon, including within *embC* and *embA*. Several studies have suggested that mutations in the upstream promoter region of *embA* at positions −8, −11, −12, −16 and −43 may be associated with EMB resistance even in the absence of *embB* mutations.[36,37] In an assessment of the DeeplexMyc-TB test, Jouet et, al. found that 4.7% of discordant samples predicted to be susceptible by this method (absence of resistance associated mutations) were phenotypically resistant and harbored mutations in the *embA* promoter region including C-12T and C-16T mutations.[37] Analysis of NGS data by Andres et al. identified mutations in the *embCAB* operon up- or downstream from a wildtype codon 306 of *embB* in 94% (16/ 17) of phenotypically resistant strains.[30] Overall, the levels of sensitivity and specificity of sequencing as compared to growth-based DST varied greatly by gene target and strains analyzed within each laboratory.[37–40]

EMB resistance associated with mutations in *embR*, which encodes a transcriptional regulatory protein for the *embCAB* operon, *iniA-C* (isoniazid-inducible) genes, and *ubiA*, a gene which is required for MTBC growth, have been investigated using sequencing analyses to determine a correlation with EMB resistance.[38–40] However, most EMB resistant isolates with mutations in *embA*, *embC*, *embR* and *ubiA* were found to also contain mutations in *embB*, suggesting the individual roles of these other mutations requires further study.[39]

Safi et al. presented a detailed description of the arabinan biosynthesis and utilization pathway and proposed that the development of EMB resistance may be complex, involving mutations in several genes that interact to determine the eventual MIC of EMB.[35] These investigators asserted that their allelic exchange studies demonstrated that mutations at location Rv3806c in *ubiA* combined with mutations in *embB* and *embC* would produce high-level EMB resistance.[35] More recently, evidence strongly correlating nonsynonymous mutations in the *ubiA* gene with high-level resistance to EMB has been published.[15]

Sequencing Technologies to Identify Resistance

WGS and tNGS assays have provided insight into the impact of specific mutations and their utility in predicting resistance to antituberculosis drugs. In a large international study, WGS was used to correctly predict phenotypic resistance of four first-line antituberculosis drugs. Accurate predictions of EMB phenotype were obtained in 89.9% (n=9,794) of isolates and EMB resistance was detected with 94.6% sensitivity, 93.6% specificity and a positive predictive value (resistance) of 75.1%.[38] Given the inconsistencies in phenotypic DST and that the mechanisms of resistance are not well defined, the positive predictive value was the lowest of the drugs examined. Conversely, the negative predictive value (susceptible) was 98.8%.[38]

In addition to WGS, there are also tNGS approaches including commercially-available methods such as the Ion AmpliSeq TB Panel. This assay includes full-length gene analysis for a number of genes including embB. A study by Park et al. including 30 MDR TB isolates found reasonable concordance between genotypic prediction and phenotypic (absolute concentration method using LJ solid media).[41] Another commercial assay, Deeplex-MycTB assay involves the amplification and sequencing of 22 mycobacterial loci (18 gene targets) associated with 13 antituberculosis drugs known to be involved in MTBC resistance to first- and second-line drugs.[42]

A recent study by Jajou et al. examined the significance and clinical relevance of mutations associated with MTBC resistance including mutations conferring EMB resistance (as well as other antituberculosis drugs). They concluded that if WGS is applied in a setting with low TB incidence and low prevalence of resistance, WGS can more accurately predict susceptibility than growth-based DST, even though there was still discordance with EMB resistance when low-level resistance was identified by MGIT.[43] WHO published a technical guide regarding the application of WGS to predict TB drug susceptibilities and genomic epidemiology, and other centers (CDC, European CDC) are currently evaluating and validating its utility.[44,45] This document does not specifically address EMB, but is a good general reference for use of WGS to predict TB DST results. While the technical components of WGS for MTBC has vastly improved in the past few years, the utilization of different analyisis pipelines can impact the prediction of resistance. Efforts are underway to create standardized approaches, and some laboratories in the US and Europe have begun using these methods to report drug resistance for clinical use. Understanding that a more comprehensive approach may be necessary to accurately predict EMB resistance, Andres et al. proposed a diagnostic algorithm using both growth-based DST and tNGS results to guide additional testing and clinician communications in a low incidence/high resource setting where growth-based DST is routinely performed.[30]

GUIDANCE

Laboratories should consider the following:

- All EMB monoresistant isolates should be reviewed since EMB monoresistance is rare and is most commonly associated with resistance to INH (up to 96.6% of isolates).[27]
    - The initial detection of growth-based resistance should be reported immediately, prior to the availability of confirmatory results. However, it should be made clear to the healthcare provider that confirmatory testing is being performed.
    - If EMB monoresistance is detected, additional testing should be performed to confirm DST results and quality control should be examined to rule out issues such as potential contamination.
    - CLSI recommends repeating DST, either with AP if the initial result was from a rapid commercial broth system or if using the same method, ensuring that the inoculum is carefully prepared to avoid clumping.[10]
        - ♦ When preparing the inoculum, be sure to follow instructions and assure adequate vortexing to break up clumps of MTBC and allow sufficient time for settling of clumps in the suspension before inoculation of the drug tubes. This will help to avoid potential issues with false-positive or negative results.
    - Testing for mutations using molecular-based methods is potentially useful as mutations associated with EMB resistance may be rapidly identified. However, laboratories and clinicians should be aware of which gene or loci were examined for mutations and that the absence of a mutation does not confirm susceptibility to EMB.
- If your laboratory is unable to perform molecular testing, samples can be referred to a jurisdictional public health laboratory for testing and/or those laboratories can refer to the following services:
    - CDC Molecular Detection of Drug Resistance Service (MDDR) provides rapid algorithm-based testing and other comprehensive testing services at no cost to public health laboratories.
    - The National Public Health Laboratory Drug Susceptibility Testing Reference Center for *Mycobacterium tuberculosis* provides rapid algorithm-based testing at no cost to enrolled public health laboratories. Enrollment is restricted to public health laboratories performing TB DST on fewer than 50 isolates/year.

ABBREVIATIONS

AP	Agar proportion
CDC	US Centers for Diseases Control and Prevention
CFU	Colony forming units
DST	Drug susceptibility testing
FDA	US Food and Drug Administration
CLIA	Clinical Laboratory Improvements Amendment
CLSI	Clinical and Laboratory Standards Institute
EMB	Ethambutol
INH	Isoniazid
LDT	Laboratory developed test
LiPA	Line probe assay
MDR TB	Multidrug-resistant tuberculosis
MGIT	Mycobacterium growth indicator tube
MIC	Minimum inhibitory concentration
MPEP	Model Performance Evaluation Program
MTBC	*Mycobacterium tuberculosis* complex
OADC	Oleic albumin dextrose catalase
PSQ	Pyrosequencing

PT	Proficiency testing
PZA	Pyrazinamide
RIF	Rifampin
RUO	Research use only
TB	Tuberculosis
tNGS	Targeted next generation sequencing
WGS	Whole genome sequencing
WHO	World Health Organization

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