



CLINICAL AND
LABORATORY
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3rd Edition

M24

Susceptibility Testing of Mycobacteria, *Nocardia* spp., and Other Aerobic Actinomycetes

This standard provides protocols and related quality control parameters for antimicrobial susceptibility testing of mycobacteria, *Nocardia* spp., and other aerobic actinomycetes.

A standard for global application developed through the Clinical and Laboratory Standards Institute consensus process.

Susceptibility Testing of Mycobacteria, *Nocardia* spp., and Other Aerobic Actinomycetes

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Abstract

Clinical and Laboratory Standards Institute standard M24—*Susceptibility Testing of Mycobacteria, Nocardia spp., and Other Aerobic Actinomycetes* includes susceptibility testing procedures for *Mycobacterium tuberculosis* complex (MTBC), clinically significant slowly and rapidly growing mycobacterial species, *Nocardia* spp., and other aerobic actinomycetes. Also included in this standard are recommendations for selecting agents for first-line and second-line drug testing, organism group-specific methodologies, reporting recommendations, and organism quality control criteria. Recommendations regarding agent selection for testing mycobacteria are based primarily on published guidelines. For testing MTBC, M24 recognizes agar proportion as the reference methodology on which all other methodologies are based. In addition, this standard includes recommendations for using commercial broth susceptibility methods with shorter incubation times, which are now in widespread use for MTBC susceptibility testing, and information on molecular methods for detecting drug resistance and their integration with culture-based methods.

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Foreword

This standard includes recommendations for testing *Mycobacterium tuberculosis* complex (MTBC), certain nontuberculous mycobacteria (NTM), *Nocardia* spp., and other aerobic actinomycetes. Currently, sufficient data exist to support recommendations for antimicrobial susceptibility testing (AST) of MTBC, *Mycobacterium avium* complex (MAC), *M. kansasii*, *M. marinum*, the rapidly growing mycobacteria (RGM), *Nocardia* spp., and certain other aerobic actinomycetes. Breakpoints for some NTMs, *Nocardia* spp., and other aerobic actinomycetes are based on organism population distributions, clinical data, breakpoints used for other organisms, and the experience of experts in the field. M24 was revised in response to new developments in mycobacterial susceptibility testing and comments from laboratorians who perform routine mycobacterial and/or aerobic actinomycete testing. Additional revisions are anticipated as more relevant data become available.

Overview of Changes

This standard replaces the previous edition of the approved standard, M24-A2, published in 2011. Several changes were made in this edition, including:

- Removed information related to the short-incubation, liquid-radiometric testing system, because this system is no longer available
- Expanded the description of molecular testing for both MTBC and NTM to determine antimicrobial susceptibility or resistance
 - For MTBC, Table 3 (Considerations for Molecular or Repeat Testing After Initial Testing on MTBC Using a Commercial Short-Incubation Broth System) and text are included to describe the integration of molecular and culture-based test results for the best possible prediction of the expected drug efficacy.
 - For NTM, text is included to describe integration of molecular techniques to assist in determining efficacy of macrolides and amikacin in the treatment of infections caused by MAC and various RGM.
- Added a description of recently discovered challenges to MTBC AST accuracy with use of rapid broth systems and/or the agar proportion method, particularly limited sensitivity in detection of low-level resistance to rifampin and ethambutol
- Added information in Appendix A regarding the relationship of pharmacokinetics and pharmacodynamics in determining breakpoints and interpretive criteria
- Updated all breakpoint and quality control tables and moved them to a newly created informational supplement, CLSI document M62¹

NOTE: The content of this standard is supported by the CLSI consensus process and does not necessarily reflect the views of any single individual or organization.

Key Words

Aerobic actinomycetes, antimicrobial susceptibility testing, antimycobacterial drugs, antituberculous drugs, *Mycobacterium tuberculosis* complex, *Nocardia* spp., nontuberculous mycobacteria

Susceptibility Testing of Mycobacteria, *Nocardia* spp., and Other Aerobic Actinomycetes

Chapter 1: Introduction

This chapter includes:

- Standard's scope and applicable exclusions
- Background information pertinent to the standard's content
- Standard precautions information
- "Note on Terminology" that highlights particular use and/or variation in use of terms and/or definitions
- Terms and definitions used in the standard
- Abbreviations and acronyms used in the standard

1.1 Scope

M24 includes antimicrobial susceptibility testing (AST) protocols for three major categories of mycobacterial species:

- *Mycobacterium tuberculosis* complex (MTBC)
- The slowly growing nontuberculous mycobacteria (SGM)
- The rapidly growing mycobacteria (RGM)

Also provided are:

- AST recommendations for *Nocardia* spp. and other aerobic actinomycetes
- Guidance on selecting first-line and, for some organisms, second-line antimicrobial agents for testing and reporting
- Instructions for performing the standard agar proportion (AP) method for MTBC and broth microdilution for mycobacteria and aerobic actinomycetes
- Molecular methods for detecting mutations associated with MTBC drug resistance
- QC protocols for each organism category

Testing and reporting recommendations and QC procedures apply to both reference methods and commercial shorter-incubation broth systems that have been regulatory organization cleared or approved for testing MTBC. This standard does not cover identification methods, nor does it provide an in-depth discussion of molecular test procedures. This standard is intended for use by hospital, public health, and referral laboratories that perform AST on MTBC, nontuberculous mycobacteria (NTM), *Nocardia* spp., and/or other aerobic actinomycetes.

1.2 Background

Because mycobacteria grow slowly, culture-based antimicrobial susceptibility testing (cAST) takes from several days (for RGM) to as long as three weeks (for MTBC). Molecular methods, which can detect genetic changes associated with drug resistance, can provide results more quickly. Molecular methods can also be useful for situations in which cAST is less reliable (eg, with pyrazinamide [PZA]) or for quick confirmation of resistance detected by culture-based methods. Through use of specific nucleic acid primers for amplification, it is possible to directly detect mycobacterial DNA sequences and drug resistance–associated mutations in patient samples or in mixed or contaminated cultures. This technique enables more rapid detection of drug resistance. Molecular methods have become routinely integrated into mycobacteriology laboratory practice.

Laboratory tests for evaluating the antimicrobial susceptibility of mycobacteria and aerobic actinomycetes can confirm the initial drug treatment choice and any emerging drug resistance when a patient fails to respond satisfactorily to treatment. Additionally, AST may provide additional information for treatment choices with different drugs. MTBC AST can also be used to estimate the prevalence of intrinsic and acquired drug resistance, defined as “drug resistance among new cases” and “drug resistance among previously treated patients,” respectively, in a community.² For these purposes, using a reliable technique to perform the susceptibility test is essential.

Currently, first-line therapy for tuberculosis (TB) includes isoniazid (INH), rifampin (RIF), ethambutol (EMB), and PZA. To provide clinicians with comprehensive information about this multidrug regimen, initial isolates from all patients should be tested for susceptibility to all four agents. AST should be repeated if the patient is culture positive after two to three months of appropriate therapy or earlier if the patient shows clinical evidence of failure to respond to therapy or is unable to tolerate the treatment regimen. To detect possible resistance as early as possible, a commercial, shorter-incubation system should be used in conjunction with rapid methods for primary culture and identification (see Subchapter 3.2). As a result, first-line susceptibility test results for most MTBC isolates should be reported within 17 days of MTB identification by the laboratory.^{3,4} Because of the nature of MTBC, the use of pharmacokinetics and pharmacodynamics for antitubercular agents differs from that commonly applied to conventional antibacterial agents. For additional information, refer to Appendix A.

Although not a common problem in most developed countries, multidrug-resistant tuberculosis (MDRTB) (ie, resistant to INH and RIF, two of the most powerful first-line drugs) is a serious threat to global TB control. Extensively drug-resistant TB (disease caused by isolates resistant to the two most powerful first-line agents, INH and RIF, plus at least one fluoroquinolone and at least one of the three injectable second-line drugs [amikacin, kanamycin, or capreomycin]) is associated with extremely poor outcomes and high mortality rates, especially in patients with concomitant HIV infection, and has heightened the need for global TB control. In addition, first-line drug intolerance or resistance necessitates testing second-line drugs (see Appendix B). Consequently, the importance of testing susceptibility to second-line anti-TB agents has increased.

NTM and aerobic actinomycete AST should be performed on clinically significant isolates⁵ (eg, those from blood, cerebrospinal fluid, skin and soft tissue lesions, or tissues) and on species known to exhibit variability in susceptibility to clinically useful antimicrobial agents and/or to have significant risk of acquired mutational resistance to one or more of these agents. To determine the clinical significance of NTM recovered from respiratory cultures, refer to published guidelines.⁶ Key microbiological criteria include:

- Cultures of at least two positive sputum specimens or one bronchial wash or bronchial lavage sample
- A transbronchial or lung biopsy specimen with mycobacterial histopathological features and positive NTM culture

EXAMPLE*:

Interpretive Category	Breakpoints [†]	
	MIC, µg/mL	Critical Concentration, µg/mL [‡]
Susceptible	≤4	1 (no growth)
Intermediate	8–16	–
Resistant	≥32	1 (growth)

* The example shown applies to a commercial short-incubation broth system.

[†] Formerly “interpretive criteria.”

[‡] Breakpoint depends on the method used and whether the organism grows at a single concentration.

- **susceptible (S)** – a category defined by a breakpoint that implies that isolates with an MIC at or below the susceptible breakpoint are inhibited by the usually achievable concentrations of antimicrobial agent when the dosage recommended to treat the site of infection is used, resulting in likely clinical efficacy; **NOTE:** For **critical concentration**, a level of susceptibility that is not significantly different from that of wild-type strains from patients who have not been treated with the drug is needed, so that the strain is likely to show clinical responsiveness to the drug.
- **intermediate (I)** – for MICs only, a category defined by a breakpoint that includes isolates with MICs within the intermediate range that approach usually attainable blood and tissue levels and for which response rates may be lower than for susceptible isolates; **NOTE:** The intermediate category implies clinical efficacy in body sites where the drugs are physiologically concentrated or when a higher-than-normal dosage of a drug can be used. This category also includes a buffer zone, which should prevent small, uncontrolled, technical factors from causing major discrepancies in interpretations, especially for drugs with narrow pharmacotoxicity margins.
- **resistant (R)** – a category defined by a breakpoint that implies that isolates with an MIC at or above the resistant breakpoint are not inhibited by the usually achievable concentrations of the agent with normal dosage schedules and/or that demonstrate MICs that fall in the range in which specific microbial resistance mechanisms are likely, and clinical efficacy of the agent against the isolate has not been reliably shown in treatment studies; **NOTE:** For **critical concentration**, resistance is defined as diminished susceptibility of a strain that differs from wild-type strains from patients who have not been treated with the drug, so that the strain is unlikely to show clinical responsiveness to the drug.

measurand – quantity intended to be measured.¹⁰

minimal inhibitory concentration (MIC) – the lowest concentration of an antimicrobial agent that prevents visible growth of a microorganism in an agar medium or broth dilution susceptibility test.

nontuberculous mycobacteria (NTM) – all species of mycobacteria other than *Mycobacterium leprae* and those in the *Mycobacterium tuberculosis* complex.

precision (measurement) – closeness of agreement between indications or measured quantity values obtained by replicate measurements on the same or similar objects under specified conditions.¹⁰

quality assurance (QA) – part of quality management focused on providing confidence that quality requirements will be fulfilled¹³; **NOTE:** QA is a comprehensive set of policies, procedures, and practices used to monitor the laboratory’s entire testing process and ensure that the testing site’s results are reliable.

quality control (QC) – part of quality management focused on fulfilling quality requirements.¹³

investigated.^{62,63} Within INH-resistant but phenotypically RIF-susceptible strains, a higher proportion of these strains could be detected by RRDR genetic analysis.^{60,61,64,65}

The clinical implication of strains with RRDR mutations is unclear. Several reports suggest that these strains may show reduced clinical response to standard treatment.^{60,64,66} Additional clinical observations are necessary to estimate the relevance of these specific *rpoB* gene mutations. If a probe-based molecular method detects a mutation, RRDR sequence analysis should be performed as soon as possible to identify the mutation's presence. The observation should be discussed with the treating physician. Because strains with RRDR mutations occur rarely, a general RRDR molecular analysis is not recommended for susceptible MTBC strains. For INH-resistant strains, no general recommendation can be given. However, laboratories may consider using molecular methods for quick confirmation of INH resistance with an understanding that lack of mutations does not rule out INH resistance. If the molecular methods performed include analysis for both INH and RIF, these infrequent *rpoB* mutations will have a chance to be detected when present.

3.2.5 Managing Test Inconsistencies for Pyrazinamide

PZA AST is particularly affected by inoculum size, such that an inoculum that is too large can lead to a pH increase, resulting in PZA inactivation and false resistance. The target inoculum for the PZA-containing tube or vial should be 10^6 CFU/mL.⁶⁷ Initial resistant results should be confirmed by repeat testing using the same method or preferably by starting from a solid media culture, which necessitates using homogeneous cell suspensions and standardized inoculums. Published studies have recommended confirming PZA resistance using a reduced inoculum volume to avoid making the medium too alkaline, thereby rendering the PZA drug less active.⁶⁸ Phenotypic resistance may be confirmed by *pncA* sequence analysis, in which isolates with nonsynonymous mutations would mostly be considered resistant, except for a few mutations.^{69,70} For isolates with silent mutations or without mutations, laboratories can consider repeating the phenotypic testing. If an isolate is resistant only to PZA, determining the MTBC species should be considered. Specifically, *M. bovis* is always resistant to PZA, whereas most MTBC isolates are susceptible to PZA.

3.3 Broth Microdilution Method for Determining *M. tuberculosis* complex Minimal Inhibitory Concentrations

MICs for antimicrobial agents used to treat MTBC infections can be determined by the broth microdilution method. Broth microdilution method use has increased in medical and research laboratories with the introduction of a commercially available microtiter plate formatted specifically for MTBC. The format uses lyophilized antimicrobial agents for first- and second-line drugs across a range of concentrations in a 96-well plate. As of this standard's publication, PZA is not available on commercial broth microdilution panels, and the commercial broth microdilution panels are not cleared or approved by any regulatory organization. The steps for performing the broth microdilution test are listed below.

Step	Action	Comment
1.	Select several isolated colonies from pure growth on an agar plate.	
2.	Add the growth material to a tube containing saline, polysorbate 80, and glass beads.	
3.	Vortex the tube.	Let the large particulates to settle for 10–15 minutes. Then carefully remove the supernatant and adjust to a 0.5 McFarland standard.
4.	Transfer 100 μ L of the inoculum to 11 mL Middlebrook 7H9 medium containing OADC.	