05-31-24

INTRODUCTION. Susceptibility testing, identification by DNA gene sequencing and DNA fingerprinting of the rapidly growing mycobacteria and other nontuberculous mycobacteria (NTM) and related aerobic actinomycetes are performed at the UTHSCT Mycobacteria/Nocardia Laboratory. The laboratory is College of American Pathologists (CAP) accredited and holds a CLIA license. We also have a Pennsylvania and California license.

The Mycobacteria/Nocardia Laboratory has been in operation for approximately 40 years and accepts pure culture isolates (not gross specimens) of the above groups of organisms for susceptibility, identification, and DNA fingerprinting. **Please note there will be an additional charge and increased turn-around time for any isolates that are not pure.** If desired, gross specimens should be submitted to the UTHSCT Pathology Laboratory (See Mycobacterium/Mycology Referral; call **903-877-5745** for details. Additional charges will apply).

Please note that if a mixed culture (e.g., more than one colony morphology), unless otherwise specified on the requisition, we will only work up the predominant organism type. If ID/MICs are requested on all colony types, please check/initial the box on the requisition.

SUSCEPTIBILITY TESTING. Susceptibility testing is performed using the CLSI recommended broth microdilution MIC method for the NTM, Nocardia, and other related aerobic actinomycetes. For rapidly growing mycobacteria (RGM), the routine panel of drugs* includes clarithromycin, amikacin, imipenem, linezolid, tigecycline, tobramycin (for *M. chelonae/immunogenum* complex), cefoxitin, moxifloxacin, ciprofloxacin, trimethoprim-sulfamethoxazole, and doxycycline and/or minocycline. Incubation time is 3-5 days at 30^{o}C except for clarithromycin which is held up to 14 days to check for inducible *in vitro* resistance (*erm* gene). However, if isolates of the *Mycobacterium abscessus* subspecies*, M. chelonae, M. immunogenum* and *M. mucogenicum* complex are sequenced in our laboratory, extended incubation will not be necessary and the clarithromycin MICs will be reported after 3-4 days incubation as susceptible or resistant based on sequencing of the *erm* gene, *rpoB* sequence identification, and/or exclusion of a 23S rRNA gene mutation.

Due to recent findings of a new plasmid-mediated *erm* gene [erm (55)], we have now extended clarithromycin incubation for up to 2 weeks for most RGM (Brown-Elliott, et al., J. Clin. Microbiol. 2023).

In isolates with resistant clarithromycin MICs, macrolide mutational resistance can be confirmed by ordering macrolide mutational sequencing 23S rRNA (Test Code 13). Additionally, confirmation of amikacin mutational resistance can be performed in isolates with high level amikacin MICs by ordering 16S rRNA mutational sequencing (Test Code 13).

For Nocardia and other related aerobic actinomycetes, the panel of drugs* includes amoxicillin/clavulanic acid, linezolid, clarithromycin, trimethoprim-sulfamethoxazole, ciprofloxacin, moxifloxacin, ceftriaxone, imipenem, tobramycin, amikacin, doxycycline and/or minocycline. Incubation time is 3-5 days at 35°C for most species. Slowly growing NTM (other than *M. avium* complex and *M. kansasii*) are tested against clarithromycin, amikacin, trimethoprim-sulfamethoxazole, rifabutin, ciprofloxacin, moxifloxacin, minocycline, linezolid, and rifampin.

Current recommendations by the ATS and the CLSI M24, 3rd ed., 2018 advise testing only clarithromycin and amikacin against isolates of *Mycobacterium avium* complex (MAC) as these are the only agents which have been shown to have *in vitro* correlation of MICs with clinical response. Clarithromycin is used as a "class drug" in susceptibility testing of azithromycin and other related macrolides. Thus, isolates of MAC are tested only for susceptibility to clarithromycin and amikacin. Resistance to clarithromycin confers resistance to azithromycin and vice versa. It may be reasonable to test agents such as moxifloxacin and linezolid and these may, in some cases, provide useful adjunctive treatment options. They should not, however, be used as treatment substitutions for any of the standard treatment agents (macrolide, ethambutol, rifampin, rifabutin) and their efficacy for treatment remains unproven. First line TB agents (ethambutol, rifampin, and isoniazid) are not reported with isolates of MAC in accordance with CLSI and ATS recommendations. Recently CLSI recommended breakpoints for amikacin MICs ≥64 µg/mL (IV treatment) and >64 µg/mL (inhaled treatment) as resistant so that resistance would be defined as ≥64 µg/mL depending upon the method of administration of amikacin. Mutational resistance can be confirmed for clarithromycin and/or amikacin by ordering Test Code 13 and specifying amikacin, clarithromycin or both.

In accordance with the American Thoracic Society (ATS) and CLSI recommendations, isolates of *M. kansasii* are reported with rifampin and clarithromycin susceptibility only, if they are rifampin susceptible. Additional agents can be included with special physician requests. Rifampin resistant *M. kansasii* will include a panel of drugs (same as noted for slowly growing NTM other than MAC). Incubation time is 7-14 days at 35°C. Please note that CLSI (M24, 3rd ed., 2018) no longer recommends *in vitro* MIC testing of ethambutol due to technical problems in testing this antimicrobial.

The methods and breakpoints for susceptibility testing of RGM and aerobic actinomycetes have been published by the CLSI. Recommendations for breakpoints for these organisms using the broth microdilution MIC method with nine drugs (amikacin, tobramycin, trimethoprim-sulfamethoxazole (TMP-SMX), cefoxitin, imipenem, linezolid, doxycycline/minocycline, clarithromycin and ciprofloxacin) are shown in Tables 1, 2, and 4. Breakpoints for tigecycline have not been addressed by the CLSI yet for NTM or other aerobic actinomycetes. However, our laboratory will report MICs to tigecycline for RGM without interpretation as has recently been recommended by the CLSI (M24, 3rd ed., 2018; M24S, 2023).

Generally, turn-around-time (T-A-T) for susceptibility testing is approximately **14-30 working days** for RGM (includes macrolide induction testing if sequencing is not 3 ordered), **7–14 working days** for Nocardia and related aerobic actinomycetes, and **21-35 working days** for the slowly growing NTM and some other aerobic actinomycetes.

Please note, if isolates are identified in our laboratory as containing a functional erm gene (i.e., most *M. abscessus* subsp*. abscessus*, *M. abscessus* subsp. *bolletii)*, these isolates will be reported as macrolide resistant and 14d clarithromycin induction will not be required. T-A-T for susceptibility will be reduced to approximately 7-10 working days, provided the culture submitted is a pure culture isolate and no problems occur with growth (Nash, et al., AAC 2006;50:3476; Nash, et al., JAC 2005:55:170; Brown-Elliott et al., 2015;53:1211).

If the isolate is identified in our laboratory as *M. senegalense, M. peregrinum, M. chelonae, M. abscessus* subsp*. massiliense, M. immunogenum, M. mucogenicum* complex or a sequence variant of *M. abscessus* which does not harbor a functional *erm* gene, it will be reported as macrolide susceptible without 14d induction (Brown-Elliott et al., JCM 2015;53:875). Again, T-A-T will be reduced to approximately 7-10 working days with the same provisions as described above. Other RGM species currently will require extended incubation to phenotypically describe macrolide susceptibility.

Isolates of *Mycobacterium haemophilum* are tested using the CLSI recommended agar disk elution method with hemin supplementation (CLSI, 2011). These isolates require longer incubation with an average T-A-T of approximately 6 weeks. The susceptibility panel includes amikacin, ciprofloxacin, clarithromycin, doxycycline and/or minocycline, linezolid, rifampin, and TMP-SMX.

The T-A-Ts given assume receipt of a viable pure culture isolate. Results are sent by FAX and mail. **FOR SUSCEPTIBILITY RESULTS OR TO CHECK RECEIPT OF ISOLATES PLEASE CALL (903) 877-7978.**

Recent studies have shown that previously treated isolates of *M. abscessus* may be difficult to grow adequately for susceptibility testing in broth, probably due to antibiotic pressure and stress on the isolates. Extended incubation, which may be deleterious to some antimicrobials may be required and if so, this will be noted on the report. If the organism does not grow within 5 days, the report will state that susceptibility testing is unable to be performed within the acceptable time period. Molecular sequencing of the 16S rRNA gene and *erm* gene are recommended to determine amikacin and clarithromycin susceptibility/resistance respectively. (Amikacin and clarithromycin are two important agents used for treatment of *M. abscessus*.) Clinical consultation for treatment recommendations and interpretation of these results should be considered.

If the 14-day clarithromycin MIC result is 4 µg/mL (intermediate), the submitter should consider ordering *erm* gene sequencing to more accurately and rapidly determine susceptibility/resistance of the isolate.

More studies are needed to assess the utility of sequencing of the *erm* gene among isolates of the *M. fortuitum* complex except for isolates of *M. senegalense* and *M. peregrinum* which do not contain functional *erm* genes. Until such evaluations are performed, extended (up to 14 days) incubation of isolates to assess macrolide susceptibility/resistance will be required unless the isolate has an initial (3-5 day) MIC of ≥8 µg/mL (suggesting mutational resistance which can be confirmed by ordering Test code 13, for clarithromycin gene mutation).

IDENTIFICATION. The application of molecular techniques for the identification of mycobacteria and nocardia has become the “gold standard” among methods of identification for these species.

The Mycobacteria/Nocardia Laboratory primarily uses *rpoB* sequencing along with *erm* gene sequence for identification of most clinically significant nonpigmented rapidly growing mycobacteria (Adekambi 2003; Steingrube 1995, 1997; Nash 2009). However, for slowly growing mycobacteria, Nocardia and other aerobic actinomycetes, sequence analysis of 500 bp segment of the 16S rRNA gene is also often used. Sequencing of the entire 16S rRNA gene (approximately 1500 bp) is not a practical method for routine test identification and thus sequencing of other target genes may be necessary. If species identification is not possible using the 16S rRNA, our laboratory, in some cases, will perform full 16S rRNA gene sequencing upon request. The interpretation of gene sequences follows the recommendations of the CLSI published in the MM18, 2nd ed. (CLSI, 2018). Isolates which give indeterminate results may be submitted for additional testing which may include full 16S rRNA sequence and/or multi-gene sequence analysis if required.

Recent studies have shown interspecies gene transfer among species and subspecies of RGM especially within the *M. abscessus* subspecies, *M. chelonae* and *M. fortuitum* complexes. This new finding necessitates the use of multiple gene sequences in order to determine definitive identification of species level. This additional step adds a significant amount of work to the identification process, and we will be re-assessing our T-A-Ts (based on working days) as time to finalize test results will be longer. Preliminary test results should, however, be available in approximately 72 hours. Please call us if you have an urgent need for identification of an isolate and we will attempt to identify as soon as possible.

FOR SEQUENCING OR DNA STRAIN TYPING RESULTS, PLEASE CALL (903) 877-5947 or (903) 877-7683.

DNA FINGERPRINTING. Isolates of NTM or other aerobic actinomycetes may also be submitted for DNA fingerprinting. The isolates will first be subcultured to check for purity and then sequenced to determine if strain typing is appropriate. If they are not the same species/subspecies, strain typing is not required. Techniques used for strain typing in cases of outbreaks, pseudo-outbreaks, or epidemics include pulsed-field gel electrophoresis (PFGE). Variable number tandem repeat (VNTR) is also available for

strain typing of *Mycobacterium avium* and *M. intracellulare* (Iakhiaeva et al., 2013; Iakhiaeva et al., 2016). Before sending isolates for restriction fragment length polymorphism (RFLP) analysis, please call for consultation. T-A-T for PFGE is approximately 6-8 weeks and 1-2 weeks for VNTR from receipt of the isolate depending on the type and number of organisms submitted for testing. (See Fees for Laboratory Services)

The PFGE instrumentation is no longer being manufactured. However, we have been working to obtain a used instrument. Please call (903) 877-7685 for availability/status.

FOR TECHNICAL CONSULTATION CALL BARBARA BROWN-ELLIOTT AT (903) 877-7685.

***Subject to Change (See Table 3 for expanded current panels available)**

Table 1. Suggested broth microdilution breakpoints for rapidly growing mycobacteria[a].

	Minimal Inhibitory Concentration (µg/mL) for Category		
Antimicrobial Agent	**Susceptible**	**Intermediate**	**Resistant**
Amikacin[b]	≤16	32	≥64
Cefoxitin	≤16	32-64	≥128
Ciprofloxacin	≤1	2	≥4
Clarithromycin	≤2	4	≥8
Doxycycline/Minocycline	≤1	2-4	≥8
Imipenem/Meropenem	≤4	8-16	≥32
Linezolid	≤8	16	≥32
Moxifloxacin	≤1	2	≥4
Tigecycline[c]	-	-	-
Tobramycin[d]	2	4	≥8
TMP-SMX[e]	≤2/38	-	≥4/76

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[a] Breakpoints from the CLSI M24S, 2023.

[b] Amikacin resistance ≥64 µg/mL applies to IV treatment; >64 µg/mL is considered resistant for inhaled treatment. (Amikacin resistance can also be confirmed by sequencing the 16S rRNA gene for the mutation, Test Code 13).

[c] There are no interpretive criteria for tigecycline established with nontuberculous mycobacteria currently. MICs for tigecycline are given without interpretation of values.

[d] Tobramycin MIC is only reported for *M. chelonae* complex.

[e] Trimethoprim-Sulfamethoxazole

Table 2. CLSI Suggestions for susceptibility testing of the *M. abscessus* subspecies, *M. chelonae*, and the *M. fortuitum* complexes by broth microdilution[a].

Drug	Comment
Tobramycin	If the initial MIC is >4 µg/mL, the test should be repeated. If the repeat result is >4 µg/mL, the MIC should be reported with a comment.[b]
Sulfonamides	MIC is read at 80% inhibition of growth.
Doxycycline	Breakpoints are 8 µg/mL (resistant).
Cefoxitin	Breakpoints are 128 µg/mL (resistant).
Imipenem	If MIC for *M. fortuitum* complex, *M. smegmatis* complex, or *M. mucogenicum* complex is >8 µg/mL, test should be repeated with incubation period of no more than 3 days. If the repeat result is >8 µg/mL, the MIC should be reported with comment.[b]
Amikacin	Isolates of *M. abscessus* for which MIC is >64 µg/mL should be retested. If the repeat result is >64 µg/mL, the MIC should be reported with a comment.[b]
Clarithromycin	Isolates of *M. fortuitum* complex with a trailing endpoint should be considered resistant. Extended incubation up to 14 days should be performed to detect the inducible *erm* gene.

[a] For laboratories that infrequently isolate rapidly growing mycobacteria, sending isolates to an experienced reference laboratory is recommended. For laboratories that perform MIC testing, (i) proficiency testing by comparison of test results with those of an experienced reference laboratory is necessary upon initial validation and at regular intervals thereafter and (ii) identification of isolates to the species level or, at a minimum, differentiation of the *M. fortuitum* complex from the *M. chelonae* complex and *M. abscessus* subspecies is recommended.

[b] Comment: (i) the MIC is greater than expected for this species and (ii) if the drug is being considered for therapy, the laboratory should be notified so the isolate can be sent to a reference laboratory for confirmation of resistance. Please note that isolates of *M. immunogenum* are usually resistant to tobramycin (MIC >4 µg/mL) in contrast to isolates of *M. chelonae* which usually have tobramycin MICs ≤2 µg/mL. Therefore, sequence identification may also be helpful.

Table 3. Antibiotic Panels Available

Slowly Growing Mycobacteria*[1,2]	**Rapidly Growing Mycobacteria**	**Nocardia**
Amikacin	Amikacin	Amikacin
Ciprofloxacin (Augmentin)	Cefoxitin	Amoxicillin-Clavulanic Acid
Clarithromycin[3]	Ciprofloxacin	Ceftriaxone
Doxycycline or Minocycline	Clarithromycin	Ciprofloxacin
Linezolid[4]	Doxycycline	Clarithromycin[3]
Moxifloxacin[4]	Clarithromycin[3]	Doxycycline
Rifabutin	Imipenem	Imipenem
Rifampin	Linezolid	Linezolid
TMP-SMX[5]	Minocycline	Minocycline
	Moxifloxacin	Moxifloxacin
	TMP-SMX	TMP-SMX
	Tigecycline[6]	Tobramycin
	Tobramycin[7]	

[1] Please note that the MIC testing of ethambutol (which is considered to be inconsistent and unreliable by many investigators) has recently been removed from testing by the CLSI.

[2] Isolates of *Mycobacterium avium* complex (MAC) are routinely tested for clarithromycin and amikacin susceptibility only, and rifampin susceptible *M. kansasii* are tested for susceptibility to rifampin and clarithromycin only.

[3] Class drug for newer macrolides (i.e., azithromycin and clarithromycin) .

[4] Upon request, isolates of MAC may be tested for susceptibility to moxifloxacin and linezolid but no first line TB drugs are reported.

[5]TMP-SMX = Trimethoprim-Sulfamethoxazole

[6] MIC reported without interpretation since no breakpoints yet established.

[7] MIC only reported for *M. chelonae* complex.

**Upon physician request, isolates of rifampin susceptible *M. kansasii* may be tested for susceptibility to other agents.

NOTE: Antimicrobials tested are subject to change.

For availability of susceptibilities to agents other than listed above, please call (903) 877-7685, or (903) 877-7978.

Special requests: Bedaquiline, clofazimine, eravacycline, omadacycline, tedizolid, meropenem, ertapenem. (There are no interpretive criteria or breakpoints established with these antimicrobials, other than meropenem, see Table 1).

Table 4. Suggested broth microdilution interpretive criteria for Nocardia and other aerobic actinomycetes.[1]

	Minimal Inhibitory Concentration (µg/mL) for Category		
Antimicrobial Agent	**Susceptible**	**Intermediate**	**Resistant**
Amikacin	≤8	-	≥16
Amoxicillin/Clavulanic Acid	≤8/4	16/8	≥32/16
Ceftriaxone	≤8	16-32	≥64
Ciprofloxacin	≤1	2	≥4
Clarithromycin	≤2	4	≥8
Imipenem	≤4	8	≥16
Linezolid	≤8	-	-
Minocycline/Doxycycline	≤1	2-4	≥8
Moxifloxacin	≤1	2	≥4
Rifampin	≤1	2	≥4
TMP-SMX	≤2/38	-	≥4/76
Tobramycin	≤4	8	≥16
Vancomycin	≤2	4-8	≥16

[1]Reference for breakpoints: CLSI, M24S, 2023.

TMP-SMX = Trimethoprim-Sulfamethoxazole

Vancomycin and rifampin are added for isolates of *Rhodococcus equi* (CLSI M24, 3rd ed., 2018; M24S, 2023).

Table 5. Suggested broth microdilution breakpoints for slowly growing nontuberculous mycobacteria.[1]

	Minimal Inhibitory Concentration (µg/mL) for Category		
Antimicrobial Agent	**Susceptible**	**Intermediate**	**Resistant**
Amikacin[2,3]	≤16	32	≥64
Rifampin	≤1	-	≥ 2
Ethambutol[4]	≤2	4	≥8
Rifabutin	≤2	-	≥4
Ciprofloxacin	≤1	2	≥4
TMP-SMX[5]	≤32 (2/38)	-	≥64(4/76)
Clarithromycin[3]	≤8	16	≥32
Moxifloxacin	≤1	2	≥4
Linezolid	≤8	16	≥32
Minocycline, Doxycycline	≤1	2-4	>8

[1] CLSI: M24S, 2023.

[2] Amikacin >64 µg/mL is considered resistant for inhaled treatment; ≥64 µg/mL is considered resistant using IV amikacin treatment. Confirmation of amikacin resistance can be done by ordering mutational sequencing of the 16S rRNA gene (Test Code 13).

[3] Only clarithromycin and amikacin are routinely reported for MAC isolates. Clarithromycin serves as a class drug for all newer macrolides (especially azithromycin).

[4] Please note that the MIC testing of ethambutol, which is considered to have inconsistent and unreliable MICs by many investigators, is currently not recommended by the CLSI.

[5] TMP-SMX = Trimethoprim-Sulfamethoxazole

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