UTHealth
The University of Texas Health Science Center at Tyler

Laboratory Diagnosis and Susceptibility Testing of Nontuberculous Mycobacteria: The Latest Tips for Clinical Microbiologists

Barbara A. Brown-Elliott, MS, MT(ASCP) SM
The University of Texas Health Science Center at Tyler
Associate Professor, Microbiology
Supervisor, Mycobacteria / Nocardia Laboratory

1

SCASM - 2019

1

LABORATORY DIAGNOSIS

OF NTM

2

SCASM - 2019

2

Numbers of NTM Exploding

1880–1978 = 46 species (98 years)

1979–1995 = 23 species (16 years)

1996–2019 = 123 species (~23 years)

Total ~ 192 species (September 2019)

93 Slow Growers, 99 Rapid Growers

More species ID within the past 25 years than the >100 years previously!

Enzeby 2019.

3

SCASM-2019

3

Slowly Growing Mycobacteria

(SGM)

4

SCASM - 2019

4

Isolation of the Clinically Significant SGM NTM

- Generally grow at 35° C, >7 days after subculture
- Grows on Middlebrook 7H10/11 but not routine media
- Do not require CO_2

5

5

M A C
(11 Species Currently)

M. arosiense	*M. marseillense*
M. avium	*M. timonense*
M. bouchedurhonense	
M. chimaera	*M. vulneris*
M. colombiense	*M. yongonense*
M. intracellulare	*M. paraintracellulare*

6

6

M. simiae complex

- *M. europaeum*
- *M. sherrisii*
- *M. simiae*
- *M. stomatepiae* (fish)
- *M. triplex*

M. kansasii complex

- *M. kansasii*
- *M. persicum*
- *M. innocens*
- *M. pseudokansasii*
- *M. attenuatum*

7

7

M. terrae complex

- *M. arupense*
- *M. engbaekii*
- *M. heraklionense*
- *M. hiberniae*
- *M. kumamotonense*
- *M. longobardum*
- *M. minnesotense*
- *M. nonchromogenicum*
- *M. senuense*
- *M. terrae*
- *M. virginiense*
- *M. algericum* (goat/fish)

8

8

ID of Clinically Significant NTM

- Phenotypic (except pigment, growth)/Bios insufficient
- HPLC – ID not definitive for most species
- MAC ID by probe (except not for "MAC-X")
- Other slowly growing NTM usually ID by partial 16S sequence
  - DNA Line Probe assays 16S-23S rRNA spacer region and 23S rRNA
    Multiple (approx. 20) species/groups on one strip assay

9

9

Molecular Methods of ID of NTM

Nucleic Acid Probes

INNO LiPA (Innogenetics; Ghent, Belgium) reverse hybridization
16S-23S ITS (internal transcribed spacer region)

GenoType (Hain Lifescience GmbH, Nehren, Germany) – 23S rRNA gene

- Identification of a large number of species by single PCR
- Good specificity and sensitivity but some cross reactions

10

10

Molecular Methods of ID of NTM (cont'd)

16S rRNA Sequence Analysis

Background

- Routine sequencing of 1500 NT sequence of the 16S rRNA gene not feasible for most labs.
- 500 bp – Commercially available method
- Many NTM require multi-gene sequencing (e.g., *rpoβ, erm*)
- Supplementation with in-house database

11

11

ID of Clinically Significant NTM (cont'd)

Same 16S rRNA gene partial sequence (1st 500 bp)

- *M. kansasii / M. gastri*
- *M. marinum / M. ulcerans*
- Other species generally ID by 16S rRNA gene sequence

12

12

Matrix-Assisted Laser Desorption Ionization Time of Flight (MALDI-TOF)

- Rapid testing analysis of protein spectra
- Requires inactivation of organisms
- Requires extraction-purification of protein
- Less expensive per test than sequencing
- Identifies large number of NTM species
  ~60% ID to highest taxonomic level for both manufacturers.

13

13

MALDI Can Not Differentiate

- *M. intracellulare / chimaera*
- *M. marinum / shottsii / pseudoshottsii*
- *M. terrae* complex
- *M. gordonae / paragordonae*

14

14

MALDI
Can Not Differentiate (cont'd)

MAC (*M. avium, M. intracellulare, M. colombiense / chimaera, M. vulneris, M. paraintracellulare, M. yongonense, M. timonense, M. arosiense, M. marseillense, M. bouchederhonense)*

15

15

Rapidly Growing Mycobacteria

(RGM)

16

16

Isolation of the Clinically Significant RGM

- Generally 30° C, <7 days after subculture
- Agar-based culture media: Middlebrook 7H10/7H11
- Grow on routine culture media (Blood, Chocolate, TSA)
- Do not require CO_2
- New media (RGM) not yet commercially available ↑ yield *M. abscessus* ↓contamination

17

17

Identification of RGM

- Phenotypic testing/Bios not useful (except growth rate/pigment/colony type)
- HPLC generally only for group ID – not species specific
- AST patterns helpful but not confirmatory

18

18

Rapidly Growing Mycobacteria

Group 1

- **Non-pigmented Pathogens**

***M. fortuitum* group**

M. porcinum	*M. brisbanense*
M. houstonenense	*M. neworleansense*
M. boenickei	*M. septicum*
M. fortuitum	*M. senegalense/ conceptionense*
M. peregrinum	

Schinsky, et al., IJSEM 2004

19

Rapidly Growing Mycobacteria

Group 2

- **Non-pigmented Pathogens**

***M. abscessus* complex**

M. abscessus* subsp. *abscessus

M. abscessus* subsp. *massiliense

M. abscessus* subsp. *bolletii

20

Rapidly Growing Mycobacteria

Group 2 (cont'd)

***M. chelonae* complex**

M. chelonae

M. immunogenum

****M. salmoniphilum, M. franklinii***

***Fish pathogen**

Adekambi, et al., J. Clin. Microbiol. 2003
Brown-Elliott, et al., CMR, 2002
Adekambi, et al., J. Clin. Microbiol. 2004
Whipps, et al., IJSEM, 2007
Adekambi, et al., IJSEM 2006
Leao, et al., J. Clin. Microbiol 2009
Simmon, Brown-Elliott, EID, 2010

Rapidly Growing Mycobacteria

Group 3

- **Non-pigmented Pathogens**

***M. mucogenicum* group (formerly *M. chelonae*-like Organism)**

M. mucogenicum
M. aubagnense
M. phocaicum

Adekambi, et al., IJSEM, 2006
Springer, et al., J. Clin. Microbiol., 1995.

Rapidly Growing Mycobacteria

Group 4

- **Non-pigmented Pathogens**

***M. mageritense/wolinskyi* group**

Group 5

- **Late Pigmenting Pathogens**

***M. smegmatis* group**

  - ***M. smegmatis* (formerly *M. smegmatis* sensu stricto)**
  - ***M. goodii***

Brown BA, et al., IJSB, 1999.

Rapidly Growing Mycobacteria

Group 6

- **Early Pigmenting Species**

***M. bacteremicum**
***M. canariasense**
***M. cosmeticum**
***M. elephantis**
***M. iranicum**
*"M. lacticola"**
***M. monacense**
***M. neoaurum**
***M. thermoresistibile**
M. flavescens
M. phlei
M. psychrotolerans
M. vaccae

*** Proven pathogens**

Tortoli, E., FEMS Immunol. Med Microbiol. 2006.
Shojaei, H., et al. IJSEM. 2012.

Molecular Methods of ID of RGM

16S rRNA Sequence Analysis (500 bp)

- **Can not discriminate:**
  - ***M. chelonae* from *M. abscessus***
  - ***M. houstonense* from human isolates of *M. senegalense***
  - ***M. peregrinum* from *M. septicum***

25

25

Molecular Methods of ID of RGM (cont'd)

- ***M. chelonae, M. abscessus* require sequencing outside first 500 bp as they are same in those regions (4 bp difference in complete 16S)**
- **RGM except for *M. chelonae, M. abscessus* contain two copies of 16S rRNA operon**
- **1st 500 bp 16S sequence not definitive for ID of most *M. fortuitum* group**

Tortoli, *Clin. Microbiol. Rev.* 2003.

26

26

Molecular Methods of ID of RGM (cont'd)

Sequencing Secondary Gene Targets

- Heat shock protein (*hsp*65)
- *rpo* ß gene
- 16S to 23S rRNA internal transcribed spacer (ITS)
- 32 kDA protein
- Superoxide dismutase gene (*sod*)
- *dnaJ* gene
- *sec*A 1 gene
- *rec*A gene
- Short sequence analysis of 30 bp in
- Hypervariable Region

27

27

MALDI can not differentiate:

- *M. abscessus* subsp. *massiliense,* subsp. *abscessus,* subsp. *bolletii*
- Species within *M. fortuitum* group and *M. mucogenicum* group
- *M. chelonae/salmoniphilum*

Brown-Elliott, et al,. Am. J. Clin. Pathol. 2019

28

28

ANTIMICROBIAL SUSCEPTIBILITY TESTING

CLSI M24, 3rd Ed. 2018
CLSI M62, 1st ED. 2018

Guidelines for AST of NTM

- Slowly growing NTM
  - MAC
  - *M. kansasii*
  - *M. marinum*
- Rapidly Growing Mycobacteria

Indications for Performing Antimycobacterial Susceptibility Tests (AST)

- Clinically significant isolates
  - Blood, sterile body fluids
  - Tissue
  - Skin/soft tissue lesions
  - Sputum/Respiratory isolates in large numbers, smear (+), or multiple positives
- Failure to eradicate RGM from site after 6 months of appropriate treatment necessitates repeat species confirmation and/or AST

31

31

Susceptibility Testing: CLSI Guidelines

- Broth microdilution "Gold standard"
- Match 0.5 McFarland turbidity standard
- 2-fold serial dilutions in CAMHB + OADC in 96-well microtiter plates
- Organism concentration ~ 10^5 CFU/mL
- Incubation 30° C / 3 days/room air (RGM)
  35° C / 7 days/room air (SGM)

32

32

Antimicrobial Susceptibility Testing (AST) of SGM

AST of MAC

- ***In vitro*** **MIC data for EMB, RMP, RBT show poor correlation with clinical response**
- **Insufficient data using moxifloxacin, linezolid to establish correlation of *in* vitro results to clinical response**
- **Azithromycin is technically difficult to test, so use clarithromycin as "Class drug"**

CLSI, M24 3rd ed., 2018.

MAC Amikacin Susceptibility

- New study shows correlation of amikacin MICs with clinical response
- Amikacin MICs >64 µg/mL are associated with mutation in 16S rRNA gene and prior amikacin Rx (position 1408 A→G)
- Amikacin MICs ≤64 µg/mL not associated with mutation in 16S rRNA gene

35

SCASM-2019

35

Antimicrobial Susceptibility Breakpoints for *Mycobacterium avium* complex

Antimicrobial	MIC (µg/mL)		
First Line	S	I	R
Clarithromycin	≤8	16	≥32
Amikacin (IV)	≤16	32	≥64
Amikacin (liposomal inhaled)	≤64		≥128
Second Line	S	I	R
Moxifloxacin	≤1	2	≥4
Linezolid	≤8	16	≥32

SCASM-2019

36

36

AST of *M. kansasii*

- Routine testing of Rifampin (RMP) and clarithromycin (CLARI) only
- RMP susceptibility ≤ 1 µg/mL
- If RMP susceptible, will be rifabutin susceptible (HIV patients on protease inhibitors)
- Test 2º agents only if RMP resistant (treatment failure generally seen only with RMP resistance; testing other TB drugs can be problematic)
- Repeat MICs at 3 months if still culture (+)

37

SCASM-2019

37

Antimicrobial Agents and Breakpoints for Testing Slowly Growing NTM other than MAC

Antimicrobial	MIC (µg/mL)		
	S	I	R
Clarithromycin*	≤8	16	≥32
Rifampin*	≤1	–	≥2
Amikacin	≤16	32	≥64
Ciprofloxacin	≤1	2	≥4

*First Line for *M. kansasii* (RMPs)
Note EMB removed
CLSI M62 1st ed. 2018

SCASM-2019

38

38

Antimicrobial Agents and Breakpoints for Testing Slowly Growing NTM other than MAC (cont'd)

Antimicrobial	MIC (µg/mL)		
	S	I	R
Doxycycline/Minocycline	≤1	2 - 4	≥8
Linezolid	≤8	16	≥32
Moxifloxacin	≤1	2	≥4
Rifabutin	≤2	–	≥4
TMP-SMX	≤2/38	–	≥4/76

CLSI M62 1st ed. 2018

SCASM-2019

39

39

AST of Other slowly growing NTM

- Includes Primary and Secondary agents as for RMP^{R}
  M. kansasii
  - *M. simiae*
  - *M. malmoense*
  - *M. xenopi*
  - Too few isolates to recommend specific method of AST

CLSI M24 3rd ed., 2018.

40

SCASM-2019

40

Fastidious Species of NTM
No current standardized AST method

M. haemophilum

Requires hemin/iron compounds

- Agar disk elution/"X" strips
- Broth microdilution/ferric ammonium citrate

Extended incubation 2-3 weeks 28-30°C

Usually S to AMK, RMP, SXT, CLARI, LZD, R to EMB, V to DOXY/MINO, CIP/MOXI

41

41

Fastidious Species of NTM
No current standardized AST method

M. genavense

Requires Mycobactin J supplementation

Extended incubation ≥ 6 weeks

Treatment multidrug ~ MAC

M. ulcerans

Extended incubation 4-6 weeks

Clarithromycin useful for treatment

42

42

Antimicrobial Susceptibility Testing (AST) of RGM

43

AST OF RGM
CLSI Reporting: RGM MICs

- Amikacin with *M. abscessus* complex
- If MIC ≥64 µg/mL – repeat/confirm
- Ciprofloxacin / Levofloxacin MICs are interchangeable but both are less active *in vitro* than moxifloxacin
- Sulfamethoxazole/TMP-SMX MICs are read at 80% inhibition

44

AST OF RGM
CLSI Reporting: RGM MICs
(cont'd)

- Doxycycline / Minocycline MICs are interchangeable
- Imipenem with *M. fortuitum* group
  - If MIC >8 µg/mL – repeat / confirm
  - Report MIC with *M. abscessus/chelonae*
- Tobramycin with *M. chelonae*
  - If MIC >4 µg/mL – repeat / confirm ID

CLSI M24 3rd ed., 2018

45

SCASM-2019

45

Erythromycin methylase (*erm*) gene

- The major mechanism of macrolide resistance in *M. abscessus* is a chromosomal inducible *erm* gene [*erm* (41)]

- Phenotypic detection of inducible macrolide resistance in the presence of the drug requires extended incubation (up to 14 days)

Nash, et al, AAC, 2009; Brown-Elliott, et al., JCM 2015.

46

SCASM-2019

46

MACROLIDE RESISTANCE IN RGM

- rRNA erythromycin methylase genes
- Confers inducible macrolide resistance

  erm(38) *M. smegmatis* group
  erm(39) *M. fortuitum* group
  erm(40) *M. mageritense/M. wolinskyi* group
  erm (41) *M. abscessus*
  No *erm* gene in *M. chelonae,*
  M. senegalense, M. peregrinum

- 23S rRNA mutation for macrolide resistance

(Resistance at 3 days)

Nash, et al., Antimicrob. Agents Chemother. 2009.

47

47

M. abscessus Taxonomy

M. abscessus complex

subsp. *abscessus*

- 80% of all clinical isolates (U.S.)
- 15% have inactive *erm* gene

subsp. *massiliense*

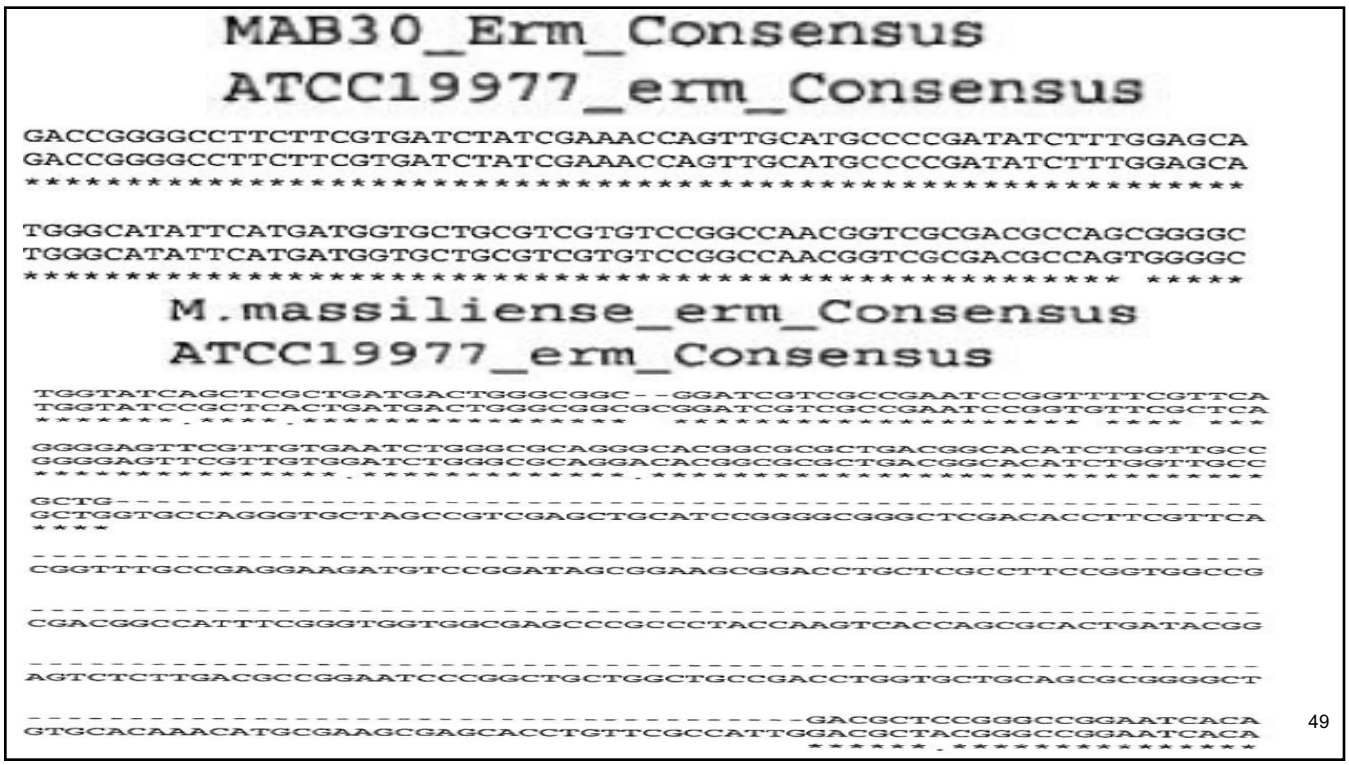
- 15% all clinical isolates (U.S.)
- 100% truncated (non-functional *erm* gene)

subsp. *bolletii*

- Rare in U.S.; Clari^{R} (functional *erm* gene)

48

48



49

Recommendation for Molecular Clarithromycin Susceptibility Testing

- **Perform *erm* gene sequence**
- **If isolate has no functional *erm* gene (*M. chelonae, M. abscessus* subsp. *massiliense, M. immunogenum, M. mucogenicum, M. peregrinum, M. senegalense*), 14d incubation not necessary. (Macrolide S)**
- **If isolate has functional *erm* gene, extended incubation not needed. (Macrolide R)**

50

50

Recommendation for Extended Clarithromycin Susceptibility Testing

- **CLSI recommends reporting resistance upon day of occurrence (3-14d)**
- **3 day resistance implies mutational resistance (Confirm by 23S rRNA gene mutation)**

CLSI 3rd ed., 2018.

51

51

23S rRNA Gene

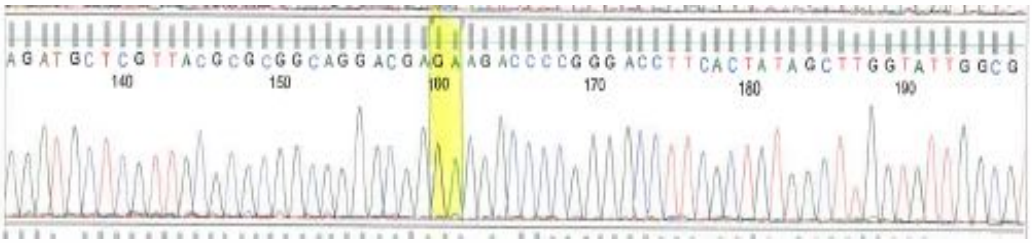
- **Macrolide resistance at 3d**
- **"*rrl*" mutations = mutational macrolide resistance which involves single point mutation (methylation) in peptidyl transferase region (Domain V –where macrolides bind to ribosome) at adenine position 2058 (38%) or 2059 (62%) in the 23S rRNA gene.**

Wallace, et al., AAC 1996; 40:1676-1681.
Nash, et al., AAC, 2009.
Brown-Elliott, et al., JCM, 2015.

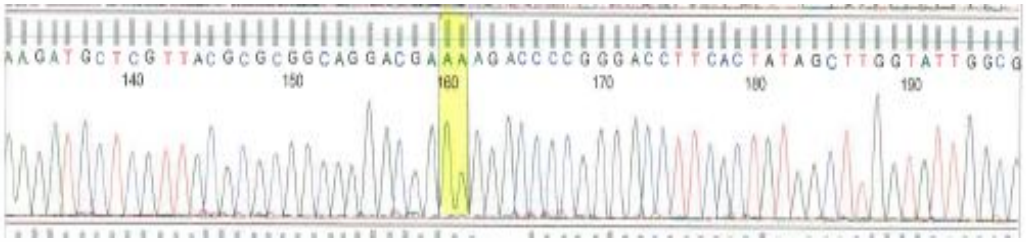
52

52

Clarithromycin Mutation A – G in 23S



Clarithromycin Wild Type



53

SCASM-2019

53

Broth Microdilution MIC Breakpoints (µg/mL) for RGM			
Antimicrobial	Susceptible	Intermediate	Resistant
Amikacin	≤16	32	≥64
Cefoxitin	≤16	32-64	≥128
Cipro/Levofloxacin	≤1	2	≥4
Clarithromycin	≤2	4	≥8
Doxy/Minocycline	≤1	2 - 4	≥8
Imipenem/Meropenem	≤4	8 - 16	≥32
Linezolid	≤8	16	≥32
Moxifloxacin	≤1	2	≥4
TMP-SMX	≤2/38	–	≥4/76
Tobramycin	≤2	4	≥8
CLSI M62 1st ed., 2018	Tigecycline added without breakpoints.		

ID Week 2019

54

54

Tigecycline

- First glycylcycline used for NTM
- No tigecycline breakpoints established
- Almost all RGM MIC_{50} ≤0.12 µg/mL; MIC_{90} ≤0.25 µg/mL including Doxycycline/ Minocycline – resistant isolates
- Slowly growing NTM generally resistant

Wallace, Brown-Elliott, et al., AAC, 2002.

55

55

CLSI: Caveats for Reporting MICs of NTM

- If results of an MIC for an organism are not as expected for that species, the MIC should be repeated.
- If repeat results are still in the unexpected range, the isolate should be sent to a qualified reference lab for re-testing.

56

56

Susceptibility Testing Caveats (cont'd)

- If a laboratory does not handle a sufficient number of isolates to maintain proficiency, referral to a qualified reference laboratory is recommended.