Wisconsin State Laboratory of Hygiene

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Why are We Concerned with Non-Tuberculous Mycobacteria?

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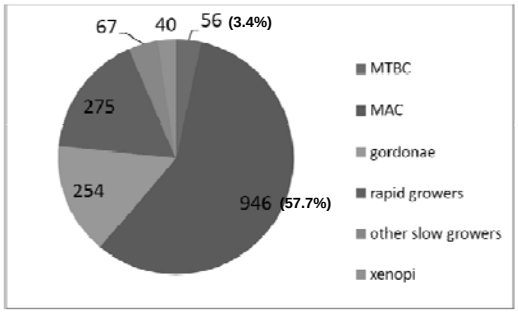
Outline

- Incidence of Non-tuberculous mycobacteria (NTM) in Wisconsin
- Clinical significance of NTM
- Identification of NTM
- Susceptibility testing of NTM

2012 Wisconsin State-wide Surveillance Mycobacteria Isolates, n=1638



Clinical Significance of Mycobacteria

- Identification of *Mycobacterium tuberculosis* complex (MTBC) is the most important finding in the laboratory
- Finding of MTBC has serious clinical and public health consequences
  - Isolation almost always signifies disease
    - Exception: A single patient specimen in the absence of clinical indications may be a false positive
  - MTBC is not found in the environment

Clinical Significance of Non-Tuberculous Mycobacteria (NTM)

- NTM are free-living mycobacteria, usually found in association with water or soil habitats.
- Although not components of the microbiota of humans, NTM may be isolated as "bystanders" from the skin, upper respiratory tract, intestinal tract and genital tract in asymptomatic individuals
- Not all NTM isolations are clinically significant as sources of human disease
- Due to their ubiquitous nature, the question of clinical significance is important and often difficult to answer.

Clinical Significance of NTM

- Considerations for clinical relevance
  - Clinical setting and host
  - Organism species and its pathogenic potential
  - Source of the culture isolate & likelihood for contamination/colonization
  - Quantification of organisms detected both in culture and AFB smears
  - Number of positive cultures

Clinical Significance of NTM

- Likely significant if collected surgically or aseptically: sterile body fluids, tissue biopsies
- For respiratory specimens:
  - Multiple culture-positive specimens
  - 2 positive sputa or 1 bronch
  - 1 trans-bronch or lung biopsy
  - Single positive sputum specimen not likely to be significant
- Laboratory clues:
  - Abundant growth on primary culture
  - More than one culture positive
  - Liquid and solid media positive

Clinical Relevance of Mycobacteria Isolated from Respiratory Specimens

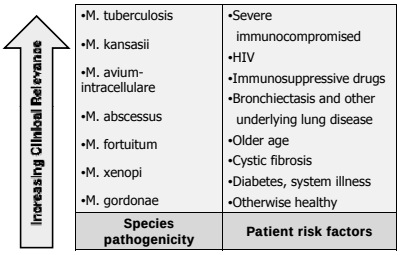


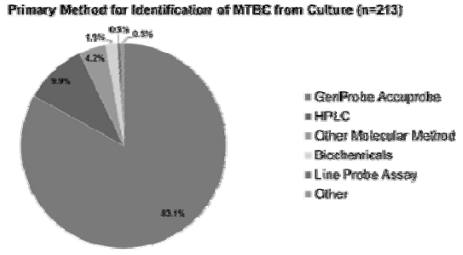
Figure adapted from Clinical and Laboratory Standards Institute (CLSI), Laboratory Detection and Identification of Mycobacteria; M48–A, 2008

Common NTM Diseases

NTM	Disease
M. abscessus	Skin and soft tissue infections following traumatic injury and surgical or medical procedures, pulmonary disease in CF patients
M. avium complex	Tuberculosis-like pulmonary disease, chronic pulmonary disease, disseminated disease in patients with AIDS, lymphadenitis in children
M. chelonae	Skin and soft tissue infections following traumatic injury and surgical or medical procedures
M. fortuitum group	Skin and soft tissue infections following traumatic injury and surgical or medical procedures, nail salon infections
M. gordonae	Rarely a pathogen, contamination from tap water
M. kansasii	Chronic pulmonary disease resembling tuberculosis
M. marinum	Cutaneous infections ("fish tank granuloma")
M. xenopi	Lung infections

Identification of Mycobacteria

National TB Laboratory Services Survey



APHL. National TB Laboratory Services Survey Report. 2012

Wisconsin Mycobacteriology Laboratory Network (WMLN)

- 32 labs around the state that provide some level of mycobacteriology testing
  - 30 clinical labs
  - Milwaukee City Health Department Lab
  - Wisconsin State Lab of Hygiene (WSLH)
- 7 labs perform identification of mycobacteria
  - GenProbe Accuprobe
  - Biochemical reactions
  - DNA sequencing
  - MALDI-TOF

Identification of Mycobacteria

CLASSICAL METHODS

Growth Characteristics

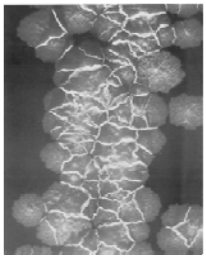
- Growth Rate
  - Can be observed on the primary solid media, but dependent on appropriate incubation temperature and number of organisms in the primary specimen
  - To perform a standardized growth test from subculture
    - Inoculate a defined suspension of mycobacteria on solid media
    - Incubate at 30 C and 35–37 C
    - Observe for growth at 5–7 days and weekly thereafter
  - Rapidly Growing Mycobacteria: form visible colonies within 7 days of incubation (usually 3–4 days)
  - Slowly Growing Mycobacteria: require more than 7days for visible colonies to form

Growth Characteristics (2)

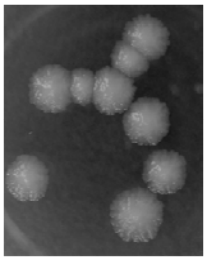
- Colony Morphology

  Observe on solid media, use stereoscope to visualize young and small colonies
  - Colony texture and consistency
    - Rough, smooth, dry, granular, mucoid, translucent
  - Colony pigment
    - Color range: Non-pigmented, buff, yellow, orange, pink
      - Photochromogen: require light to form pigment
      - Scotochromogen: form pigment in either light or dark
      - Non-photochromogens: no pigment

Growth Characteristics (3)



M. tuberculosis complex



M. gordonae

Pictures from Manual of Clinical Microbiology 10th Edition, p 475, 510

Conventional Biochemical Testing

- Classical approach to identification
- Requires sufficient amount of bacterial cells and several weeks of incubation
- New mycobacterial species cannot be reliably identified by biochemical and other phenotypic tests
- Current recommendations are for rapid methods

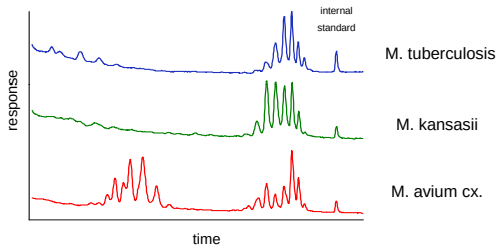
High Performance Liquid Chromatography (HPLC)

- Cell wall mycolic acids are extracted and derivatized to fluorescent or UV-adsorbing esters and then separated by chromatography
- A pattern of peaks (chromatogram) is generated as mycolic acids are detected
- Identification is based on comparison of isolate's pattern to a database or library of chromatograms

Considerations for HPLC

- Advantages
  - Some methods can identify *Mycobacterium tuberculosis* complex (MTBC) and nontuberculous mycobacteria (NTM) from broth culture and directly from clinical specimens
  - Cost of individual sample testing is relatively inexpensive
  - FDA-cleared system commercially available
- Limitations
  - Initial equipment costs are high
  - Some methods require mature solid medium growth
  - Problematic for identification of rapidly-growing mycobacteria; limited ability to resolve some NTM groups/complexes
  - Extraction uses hazardous chemicals; hazardous waste production

Fluorescence-HPLC Patterns



Identification of Mycobacteria

MOLECULAR AND EMERGING METHODS

GenProbe Accuprobe

- In-solution hybridization assay for identification of growth on solid or liquid media
  - Nucleic acids are extracted after organisms are lysed and made non-viable
  - Single-stranded labeled DNA probes (in tubes) are allowed to anneal to target RNA
  - If present, RNA:DNA hybrids are detected by chemiluminescence
- Commercially available kits for identification of MTBC, *M. avium* complex, *M. gordonae, M. kansasii*

Considerations for Accuprobe

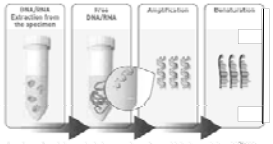
- Advantages
  - Identifies four frequently-isolated mycobacteria; three clinically significant
  - Performed routinely by many laboratories
  - Relatively easy to use
  - FDA-cleared
- Limitations
  - No nucleic acid amplification occurs during this assay; sufficient culture growth is necessary for identification
  - Beware of relative light units (RLU) values that are near the cutoff; "high negative" values could indicate that the target organism is present in low numbers

Line Probe Assay

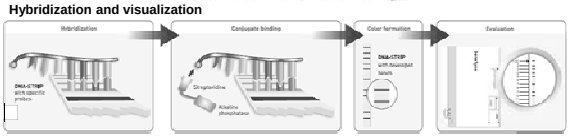
- Platform is nitrocellulose strip used for identification of MTBC and commonly-isolated NTM by reverse hybridization
  - Hybridization of denatured DNA to probes on the membrane strip
- Commercially developed assays
  - HAIN GenoType Mycobacterium CM (MTBC + 24 NTM) and GenoType Mycobacterium AS (19 NTM)
  - Innogenetics INNO-LiPA Mycobacteria v2 (MTBC + 7 NTM)

Line Probe Assay (2)

Sample preparation

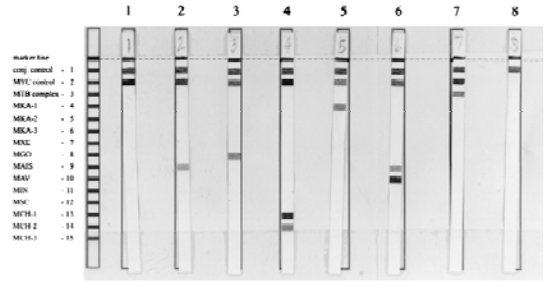


Hybridization and visualization



http://www.innogenetics.de/en/technologies/dnastrip.html

INNO-LIPA



Miller, N et al. Evaluation of the LiPA MYCOBACTERIA assay for identification of mycobacterial species from BACTEC 12B bottles. J. Clin. Microbiol. 2000;38:1915-1919

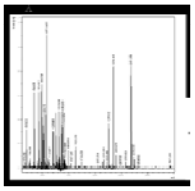
Considerations for Line Probe Assay

- Advantages
  - Nucleic acid amplification for increase sensitivity
  - Some assays detect mutations associated with MTBC drug resistance
  - Relatively low implementation costs
- Limitations
  - Not FDA approved
  - Can be difficult to differentiate bands with visual inspection
  - Sometimes difficult to identify species within *Mycobacterium fortuitum* complex, *M. chelonae/abscessus* group

MALDI-TOF

- "Matrix-Assisted Laser Desorption Ionization Time-of-Flight"
- A form of mass spectrometry
- Used to analyze proteins and other macromolecules
- Recent advances have allowed application to the clinical realm
- Charged particles are accelerated by a laser. Time of flight is proportional to the ion's mass. Patterns are compared to a library for identification.

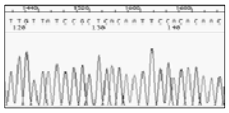
http://www.bruker.com/



Considerations for MALDI-TOF

- Advantages
  - Small sample size
  - Rapid identification
  - Can also be used for identification of many bacteria and fungi in the laboratory
- Limitations
  - Difficult heat inactivation/cell disruption for Mycobacteria
  - Database limitations
  - Initial cost investment high
  - Cannot identify to species within the MTBC

DNA Sequencing



- Determining the precise order of nucleotides
- DNA sequence is compared to a database of sequences from known/characterized organisms
- 16S rRNA, rpoB and hsp65 genes are commonly sequenced for identification

http://seqcore.brcf.med.umich.edu/doc/dnaseq/interpret.html

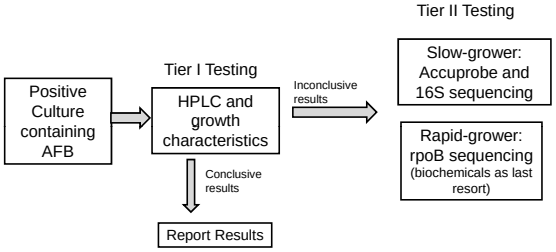
Limitations of 16S sequencing

- Cannot differentiate between
  - *Members of the TB complex*
  - *M. marinum* vs*. M. ulcerans*
  - *M chelonae* vs*. M. abscessus*
  - *M. kansasii* vs*. M. gastri*

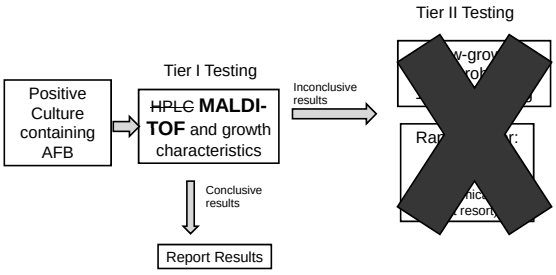
Considerations for Molecular and Emerging Methods

- Advantages
  - Quicker turnaround time (TAT)
  - Uses liquid or solid media cultures, possibly smear-positive clinical specimens
  - Ability to recognize new strains
- Limitations
  - Cost, in particular the initial setup cost
  - Computer and Database management
  - Specialized equipment
  - Requires new expertise and training
  - New methods do not always replace standard methods

WSLH Algorithm for Identification



WSLH Algorithm for Identification



Mycobacteria Taxonomy and Nomenclature

- More than 130 currently validated (fully characterized) species of NTM
- Molecular techniques increase discrimination for better identification to species and appropriate grouping of genetically similar organisms
- Nomenclature and taxonomy are constantly changing.

Slowly Growing Mycobacteria

Group or Taxon	Species within the group or taxon
M. avium complex[2]	*M. avium* subsp. *avium*, *M. avium* subsp. *silvaticum*, *M. avium* subsp. *paratuberculosis*, *M. avium* subsp. *hominissuis*, *M. intracellulare*, *M. chimaera*, *M. colombiense*, *M. vulneris*, *M. marseillense*, *M. timonense*, *M. bouchedurhonense*.
M. simiae clade[1]	Over 150 species: *M. triplex*, *M. genevense*, *M. florentinum*, *M. lentiflavum*, *M. palustre*, *M. kubicae*, *M. parascrofulaceum*, *M. heidelbergense*, *M. interjectum*, *M. simiae*, *M. longobardum*
M. terrae complex	*M. nonchromogenicum*, *M. terrae*, *M. trivale*, *M. arupense*

(1) Tortoli et al., IJSEM 2012
(2) Manual of Clin. Micro 10th Edition

Major Groups of Rapidly-growing Mycobacteria

Group or Taxon	Species within the group or taxon
M. fortuitum group	*M. fortuitum*, *M. peregrinum*, *M. senegalense*, *M. setense*, *M. septicum*, *M. porcinum*, *M. houstonense*, *M. boenickei*, *M. brisbanense*, *M. neworleansense*
M. chelonae/ M. abscessus group	*M. chelonae*, *M. immunogenum*, *M. abscessus* subsp. *abscessus*, *M. abscessus* subsp. *bolletii*, *M. salmoniphilum*
M. mucogenicum group	*M. mucogenicum*, *M. aubagnense*, *M. phocaicum*
Early pigmented rapidly-growing mycobacteria	*M. neoaurum*, *M. canariasense*, *M. cosmeticum*, *M. monacense*
Late Pigmented rapidly-growing mycobacteria	*M. smegmatis*, *M. goodii*, *M. mageritense*/ *M. wolinskyi*

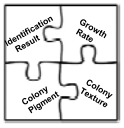
Adapted from Manual of Clin. Micro 10th Edition

Mycobacteria Identification Reporting

- Report identification as TB or not TB as soon as possible (≤ 21 days)
  - Preliminary information (colony morphology) can be helpful to health care providers)
- If sending to a reference laboratory, ensure that this lab is meeting the expected turn-around times.

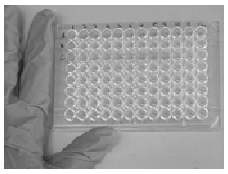
Mycobacteria Identification

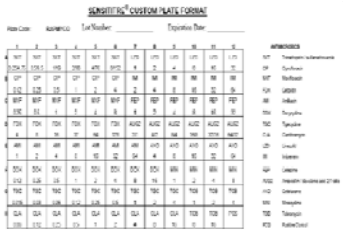
- Identification to species or group is important to help determine clinical significance and treatment
- Use a multi-faceted approach
  - Ensure that identification result matches phenotypic results (e.g., colony morphology and growth rate) before issuing final report
  - Biochemical reactions no longer recommended
  - May need molecular methods for accurate ID
  - Send tricky isolates to reference lab with expertise



Susceptibility testing of Non-tuberculous Mycobacteria

NTM Susceptibility Testing:
Broth microdilution method





M. avium complex Susceptibility Testing

- Which isolates to test?
  - Initial isolates to establish baseline value
  - Isolates from patients on prior macrolide therapy
  - Isolates from patients who develop bacteremia on macrolide prophylaxis
  - Isolates from patients who relapse on macrolides
- Testing should be repeated in 3 months for patients with disseminated disease and 6 months for patients with no improvement of chronic pulmonary disease.

CLSI M24-A2

M. avium complex: Clinically active drugs used for therapy

- macrolide
  - clarithromycin
  - azithromycin
- ethambutol
- rifamycins
  - rifampin
  - rifabutin
- aminoglycosides
  - streptomycin
  - amikacin

M. avium complex Susceptibility Testing

- Routine testing of clarithromycin testing only (broth based)
- Secondary drugs: moxifloxacin and linezolid
- No correlation between in vitro susceptibility results for anti-tuberculous agents (rifampin/rifabutin, ethambutol) with clinical outcome
- No 1st line anti-tuberculous agents should be reported

CLSI M24-A2

M. avium complex Susceptibility Testing Available

- WSLH
  - Broth microdilution, clarithromycin only
- National Jewish, Denver, CO
  - 8-drug panel: amikacin, ciprofloxacin, clofazimine, clarithromycin, ethambutol, rifabutin, rifampin, streptomycin, rifampin + ethambutol synergy
  - 12-drug panel: 8 drug panel plus cycloserine, ethionamide, kanamycin moxifloxacin, rifampin + ethambutol synergy

M. marinum Susceptibility Testing

- Routine susceptibility testing not recommended (all untreated strains have the same drug pattern)
- MICs performed at 3 months if still culture positive
- Clinically active drugs used for therapy: clarithromycin, rifampin, doxycycline/minocycline, trimethoprim-sulfamethoxazole, rifampin + ethambutol

CLSI M24-A2

M. marinum Susceptibility Testing Available

- WSLH
  - Susceptibility testing not performed.
- National Jewish, Denver, CO
  - 10 drug panel plus 3 single drugs:
    - Agar proportion: isoniazid, rifampin, ethambutol, ethionamide, streptomycin, amikacin, kanamycin, capreomycin, cycloserine, PAS
    - radiometric MIC: clarithromycin, ciprofloxacin, rifabutin
  - Susceptibility testing of single drugs

M. kansasii Susceptibility Testing

- Clinically active drugs used for therapy: clarithromycin, ethambutol, rifampin/rifabutin, INH
- Routine testing of rifampin and clarithromycin as primary agents
- Test secondary agents only if rifampin resistant (amikacin, ciprofloxacin, levofloxacin, ethambutol, linezolid, moxifloxacin, rifabutin, trimethoprim-sulfamethoxazole

CLSI M24-A2

M. kansasii Susceptibility Testing Available

- WSLH
  - Susceptibility testing not performed
- National Jewish, Denver, CO
  - 10 drug panel plus 3 single drugs:
    - Agar proportion: isoniazid, rifampin, ethambutol, ethionamide, streptomycin, amikacin, kanamycin, capreomycin, cycloserine, PAS
    - radiometric MIC: clarithromycin, doxycycline, Bactrim/SXT
  - Susceptibility testing of single requested drugs

RGM: Which Isolates to Perform Susceptibility Testing on?

- Follow ATS criteria for respiratory specimens:
  - Multiple culture-positive specimens
  - 2 positive sputa or 1 bronch
  - 1 trans-bronch or lung biopsy
- Clinically significant isolates from blood, sterile body fluids, skin and soft tissue
- Repeat susceptibility in 6 months if cultures remain positive

Rapidly-Growing Mycobacteria Susceptibility Testing

- Agents that should be tested: amikacin, cefoxitin, ciprofloxacin, clarithromycin, doxycycline (minocycline), imipenem, linezolid, moxifloxacin, trimethoprim/sulfamethoxazole, tobramycin
- No anti-tuberculous agents reported
- Clarithromycin MIC's
  - Read at 3-5 days for mutational resistance
  - Final reading at 14 days to detect inducible resistance due to *erm* gene

CLSI M24-A2

erm Gene

- Clarithromycin resistance in *M. fortuitum* and *M. abscessus*
- Patients with isolates containing the erm gene have delayed treatment response and possible failures compared to those patients whose isolates do not contain functional erm gene
- *M. abscessus* subsp. *abscessus* (erm +)
- *M. abscessus* subsp. *bolletii* (erm negative)
- No *erm* gene in *M. chelonae*

Rapid Grower Susceptibility Testing Available

- WSLH
  - 9 drugs by broth microdilution: amikacin, cefoxitin, ciprofloxacin, clarithromycin, imipenem, linezolid, doxycycline, SXT, tobramycin
- National Jewish, Denver, CO
  - 15 drug panel: amikacin, kanamycin, imipenem, ciprofloxacin, tobramycin, trimethoprim/sulfa, linezolid, augmentin, azithromycin, clarithromycin, gentamycin, ceftriaxone, cefepime, cefotaxime, minocycline
  - Susceptibility testing of single requested drugs

NTM Susceptibility Testing Summary

- Not all drugs used for patient therapy have standardized protocols for *in vitro* susceptibility testing or guidelines for interpretation for results.
  - Correlation between *in vitro* susceptibility tests and clinical response has not demonstrated in controlled clinical trials
  - Not adequately studied, breakpoints have not been established
  - Technical difficulties with drug testing
    - Poor drug solubility in assays
    - Poor reproducibility of testing

NTM Susceptibility Testing Summary

- Perform testing on clinically significant isolates
- WSLH performs testing per CLSI recommendations for MAC and RGs
- Further testing can be performed at National Jewish (Denver, CO)

To Request Add-on Susceptibility Testing for Clinically Significant Isolates (WSLH)

Test	Test Codes (old/new)	CPT code	Price
M. avium complex susceptibility (clarithromycin)	652MAC/ MM00202	87186 X 1	$135
Rapid grower susceptibility (9 drugs)	652RG/ MM00207	87186 X 9	$145

To Request Add-on Susceptibility Testing for Clinically Significant Isolates (WSLH)

- Call WSLH Mycobacteriology for help, 608-262-1618.
- WSLH Reference manual and fee schedule: www.slh.wisc.edu
- WSLH requisition Other Tests: "Isolate already at WSLH", indicate desired test codes
- Fax requisition form to 608-890-4891.

To Request Add-on Susceptibility Testing for Clinically Significant Isolates (National Jewish)

Test	Test Code	CPT code	Price
M. avium complex susceptibility (8 drugs)	Panel 3G	87188 X 8	$305.70
M. avium complex susceptibility (12 drugs)	Panel 3H	87188 X 12	$427.98
Rapid grower susceptibility (15 drugs)	Panel 3I	87186 X 15	$265.05
M. Marinum (13 drugs)	Panel 3A + 3 drugs	87190 X 10 87188 X 3	$234
M. Kansasii (13 drugs)	Panel 3A + 3 drugs	87190 X 10 87188 X 3	$234

To Request Add-on Susceptibility Testing for Clinically Significant Isolates (National Jewish)

- Call WSLH Mycobacteriology for help, 608-262-1618.
- WSLH requisition Other Test: "Isolate already at WSLH, send to National Jewish for panel ___"
- Fax requisition form to 608-890-4891.
- $90 shipping and handling fee

Summary

- Non-tuberculous mycobacteria make up the majority of mycobacteria isolations in Wisconsin.
- NTM can cause clinically significant disease, sometimes mimicking tuberculosis
- Identification of mycobacteria requires a multi-faceted approach
- Susceptibility testing for clinically significant NTM is available

For More Information

- WSLH Mycobacteriology Lab: 608-262-1618
- WSLH Customer Service: 1-800-862-1013
- Julie Tans-Kersten

Wisconsin State Lab of Hygiene
(608) 263-5364
Fax: (608) 890-2548
julie.tanskersten@slh.wisc.edu

References

- An Official ATS/IDSA Statement: Diagnosis, Treatment, and Prevention of Nontuberculous Mycobacterial Diseases in American Journal of Respiratory Critical Care Med Vol 175. pp 367-416, 2007.
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- Clinical and Laboratory Standards Institute (CLSI), Susceptibility Testing of Mycobacteria, Nocardiae and other Aerobic Actinomycetes; M24–A2, 2011
- www.slh.wisc.edu

WSLH Laboratory Team


Nate


Dave


Youngmi and Ana


Julie B.


Don


Julie TK