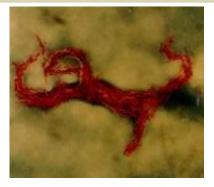


Wisconsin State Laboratory of Hygiene UNIVERSITY OF WISCONSIN-MADISON





Laboratory 101

Nate Simon TB Laboratory Program Coordinator Wisconsin State Laboratory of Hygiene



Objectives

- Specimen collection
- Specimen storage and transport
- Testing offered at WSLH
- Result reporting and interpretation
- Expected turn around times for results



Specimen Types

Almost any source is acceptable for AFB culture and smear

- <u>Sputum</u>
 - Induced
 - Expectorated
- Bronchial washing/BAL
- Gastric aspirate
- Fresh tissue
- Bone
- Blood

- Bone Marrow
- CSF
- Body fluids
- Abscess
- Stool
- Urine
- Skin





Sputum

- Recently discharged material from the bronchial tree, with minimal amounts of oral or nasal material
 - Expectorated: from deep productive cough
 - Induced: use of nebulization to increase fluid in the airway and ease clearance of sputum



Purposes of sputum collection:

- To establish an initial diagnosis of TB
 To monitor the infectiousness of the patient
- To determine the effectiveness of treatment





Specimen collection

- Supervise patient for at least the first specimen, until ability to properly collect the specimen has been demonstrated
- Patient should be in a negative pressure room
- Anyone in the room should wear a fit tested N-95 respirator
- All specimens are collected into sealed leak proof containers
- Label specimen with two patient identifiers, collection date/time and specimen type.



Specimen collection

- Optimal: collect a diagnostic specimen before the initiation of drug therapy
 - Collect a series of three sputum specimens, 8-24 hours apart, at least one of which is an early morning specimen
- Monitoring of therapy: Obtain sputum specimens for culture at least monthly until cultures convert to negative for TB



Specimen collection kits

WSLH Kit #8: Sputum collection Order: 1-800-862-1088 **KITS ARE FREE!**



- Sterile tube with label
- Absorbent pad
- Specimen transport bag
- Cold pack
- Instruction sheet
- Insulated mailer with labels



Storage and Transport

- Sputum samples should be refrigerated if they cannot be transported immediately
- Deliver specimens to the laboratory as soon as possible—try not to batch!
- Recommended: Include a cold pack during specimen transport.



Storage and Transport

Why is this important?

- Minimize overgrowth of normal flora
- Viability of AFB
- Rapid turn-around times
 - Isolation precautions
 - Start/Stop treatment



Submission of Specimens to WSLH

Wisconsin State Laboratory of Hygiene University or Wisconsin-Madison 2601 Agriculture Drive Madison, WI 53718 (Please type or print using black pen)			Vgiene http IN-MADISON CD Pho Fax Kits	D.F.J. Kurtycz, M.D., Medical Director http://www.slh.wisc.edu CDD Customer Service Phone: 800-862-1013 Fax: 844-390-6233 Kits and Supplies: 800-862-1088								
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- Requisition form A
- Order: 1-800-862-1088
- Preprinted with account information
- One form per specimen



Submission of Specimens to WSLH

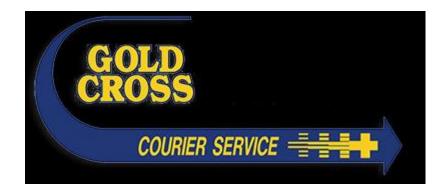
Code		Test Description
MM00250		Mycobacteria (AFB) Smear and Culture
MM00253	•••	Mycobacteria Isolate Identification
MM02881		Mycobacterium tuberculosis Isolate Genotyping
MM00204		Mycobacterium tuberculosis Susceptibility-1st Line Drugs
MM00202		Mycobacterium avium Complex (MAC) Susceptibility
MM00260		Mycobacterium avium Complex PCR Decontaminated? Yes No Smear Result
MM00256		Mycobacterium tuberculosis PCR Decontaminated? Yes No Smear Result



Courier information

Service is offered at no charge to submitters

Call to set up an account and schedule a pickup: 763-233-0099





Assessing Sputum Quality

Test results are used as an aid in patient diagnosis and treatment.

Test results are directly related to the quality of the specimen.



Assessing Specimen Quality

Collection Date/Time:

- CDC MMWR 2005: 54, RR-17: "Persons requiring sputum collection for smear and culture should have at least three consecutive sputum specimens obtained, each collected in 8-24 hour intervals, with at least one being an early morning specimen"
- Specimens >7 days old will be rejected



Assessing Specimen Quality

Sputum Quality

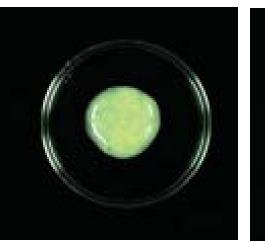
- Specimens are thick and contain mucopurulent material
- 3-5 ml in volume, but smaller quantities accepted if the quality is satisfactory
 - <1 ml sputum will be rejected
- Poor quality specimens are thin and watery—Saliva and nasal secretions are unacceptable
- Induced sputum should be indicated on requisition form to avoid rejection





Assessing Specimen Quality

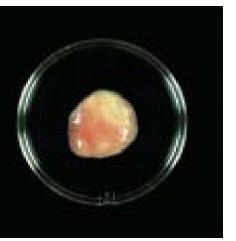
Thick Mucopurulent





Watery (induced?)

Hemoptysis





Salivary

http://www.theunion.org



Mycobacteriology at WSLH

- Full-service mycobacteriology laboratoryBSL-3 facility
- Roles:
 - Primary Diagnostic Facility
 - Reference laboratory
 - Public Health Laboratory





Mycobacteriology at WSLH

- 22 laboratories around the state perform smear and culture
 - 5 labs perform some level of identification
 - Most others send to WSLH for identification
- WSLH receives clinical specimens from:
 - 2 large local hospitals
 - Health Departments (state-wide)
 - Milwaukee City TB Clinic
 - Madison Dane County Public Health

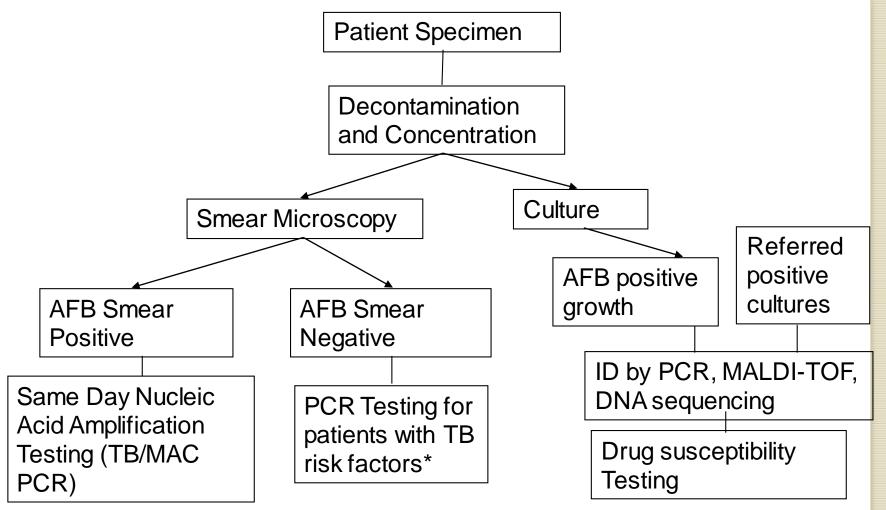


Mycobacteriology at WSLH

- Smear Microscopy
- PCR for direct detection
- Culture
- Identification
- Drug Susceptibility Testing
 - Molecular
 - Conventional



Mycobacteriology Testing at WSLH





Smear Microscopy

 Small amount of concentrated patient specimen is placed on slide and stained with Auramine O fluorescent stain

Rapid and inexpensive screening tool

- First indication of mycobacterial infection and possible TB disease
- Must be accompanied by additional testing including culture for confirmatory diagnosis





Auramine O Smear

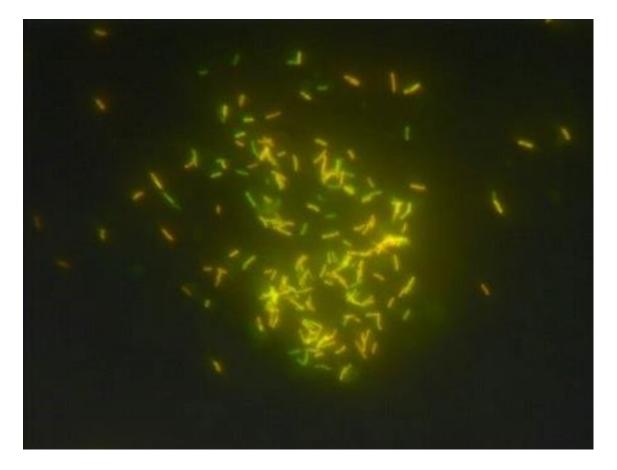


Photo Credit: laboratoryinfo.com



Smear Microscopy: Result Interpretation

WSLH Report	Graded Scale	Qualitative Scale	Interpretation
Negative	Negative	Negative	Potentially infectious
Rare (1-9 AFB per 100 fields)	1+	Positive	Low-level infectious
Few (1-9 AFB per 10 fields)	2+	Positive	Moderately
Moderate (1-9 AFB per field)	3+	Positive	infectious
Many (>9 AFB per field)	4+	Positive	Highly infectious



Microscopy Results Guide Decisions

Clinical Management

- Patient therapy may be initiated for TB
- Changes in smear status important to monitor response to therapy
- Public health interventions
 - Smear status and grade useful for identifying the most infectious cases
 - Contact investigation priority based on smear result
 - Decisions regarding respiratory isolation



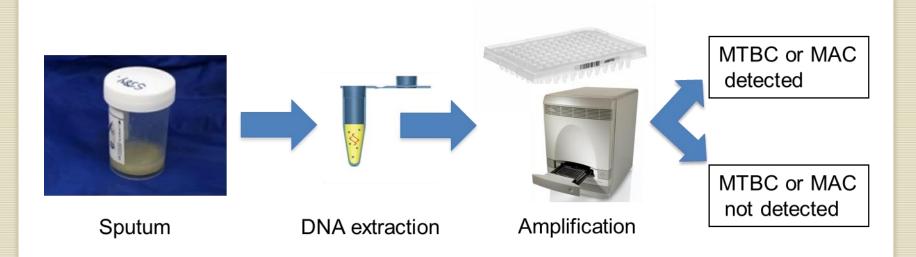
Smear Microscopy: Limitations

- Does not distinguish between viable and dead organisms
- Limited sensitivity
 - High bacterial load: 5,000-10,000 AFB/mL is required for detection
 - Misses >45% of U.S. TB cases
- Limited specificity
 - All mycobacteria are acid fast
 - Does not provide species identification
- Cannot be done without a culture

Direct Detection using PCR



AKA: Nucleic Acid Amplification Testing (NAAT)





WSLH PCR testing

- Detect *M. tuberculosis* complex (MTBC) and *M. avium* complex (MAC) directly from sputum (or other specimen) sediment
 PCR test developed at WSLH
 - 1 other laboratory in the state is performing NAAT (Cepheid GeneXpert)
 - Testing takes about 2 hours

Unable to distinguish live and dead bacilli



PCR testing (cont.)

- Automatically performed on new smear positive patients
- Fee-exempt testing for smear positive specimens and patients suspected of having active TB (approved by WI TB Program)
 Sensitivity
 - >95% for AFB smear-positive, cultureconfirmed TB patients
 - 55-75% of AFB smear-negative, cultureconfirmed TB patients



TB/MAC PCR Specimen Types

Test	Specimen Type	Smear Result
TB PCR	Respiratory	Smear positive and smear negative*
TB PCR	Non-respiratory	Smear positive only
MAC PCR	Respiratory and non- respiratory	Smear positive only

*Smear-negative TB PCR requires approval from the TB program for fee-exempt testing. Submitters may choose to pay for TB PCR testing on smear-negative respiratory specimens.



Who should be tested?

- CDC recommendation: First sputum of all patients suspected to have TB for whom the test result would alter case management or TB control activities
 - Should not be routinely ordered when clinical suspicion of TB is low.

 PCR testing is diagnostic only! Not to be used in place of smear to remove patients from isolation!

Updated Guidelines for the Use of Nucleic Acid Amplification Tests in the Diagnosis of Tuberculosis SEP MMWR 2009; 58 (01); 7-10



Wisconsin TB PCR Criteria (Fee-exempt testing)

- Patient must have signs and symptoms of pulmonary TB
- Patient must be reported to the local or state public health department as a suspect TB case as required by Wisconsin Statute Chapter
 252.05 and Wisconsin Administrative Code Chapter HFS 145.04 (3)(a).
- Patient must be in respiratory isolation



Wisconsin TB PCR Criteria (Cont.)

- Patient must not have been diagnosed with TB or a non-tuberculous mycobacterial infection within the last 12 months
- Patient must have received ≤7 days of antimycobacterial therapy or no such treatment within the last 12 months



Interpretation of PCR Results

WSLH Lab Report	Interpretation
" <i>Mycobacterium tuberculosis</i> complex DNA detected"	Positive for TB
" <i>Mycobacterium avium</i> complex DNA detected"	Positive for MAC
"No <i>Mycobacterium tuberculosis</i> complex DNA detected"	Negative for TB
"No <i>Mycobacterium avium</i> complex DNA detected"	Negative for MAC
"Inhibitory substances that prevent nucleic acid amplification were detected"	Test is of no diagnostic help



Advantages of PCR Testing

- More rapid diagnosis
- Diagnosis in smear negative patients
- Initiation of earlier treatment
- Cost savings for patient isolation
- Faster reporting to TB programs
- Fewer transmissions



TB/MAC PCR Goals

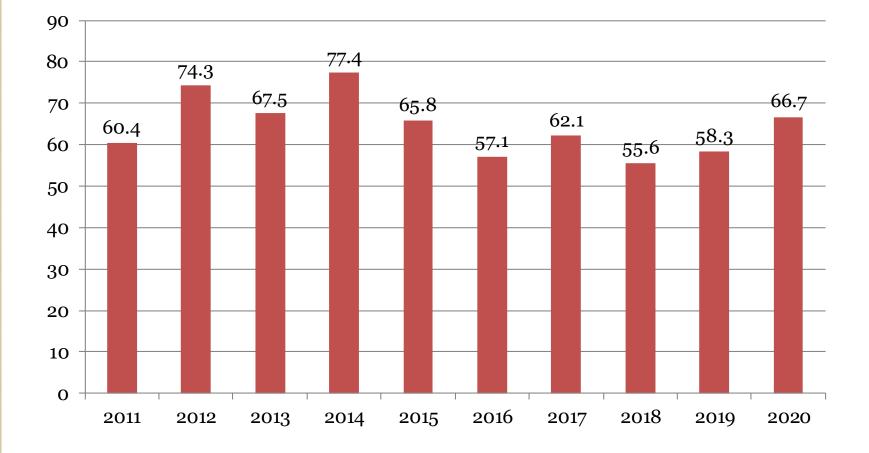
Identify smear-positive TB patients within 48 hours (Target: 77%)

- Respiratory isolation
- Start therapy
- Identify smear positive MAC patients
 - Release from isolation
 - Alter therapy decisions

Presumptive rapid results for about 60% of smear-positive patients



Percentage of Culture-Confirmed Pulmonary TB Cases Detected within 48 hours by NAAT in Wisconsin





Mycobacterial Culture

- Used to detect viable mycobacteria from patient specimens
- Most sensitive method for mycobacterial detection ("Gold Standard")
 - ~ 10 viable bacilli/ml for culture compared to >5000 bacilli/ml for microscopy
- Slowest method
 - Average time to detection for MTBC = 15 days
 - Range for detection of MTBC = 8-30+ days



Mycobacterial Culture

Media	Incubation
Broth "MGIT" tube— <u>M</u> ycobacteria <u>G</u> rowth <u>Indicator T</u> ube	 In automated instrument, read hourly for 42 days O2 consumption detected through fluorescent pad
Solid Middlebrook 7H11 plate (only on non-respiratory and known TB patients)	• Visually inspected once per week for 6 weeks



Bactec MGIT 960



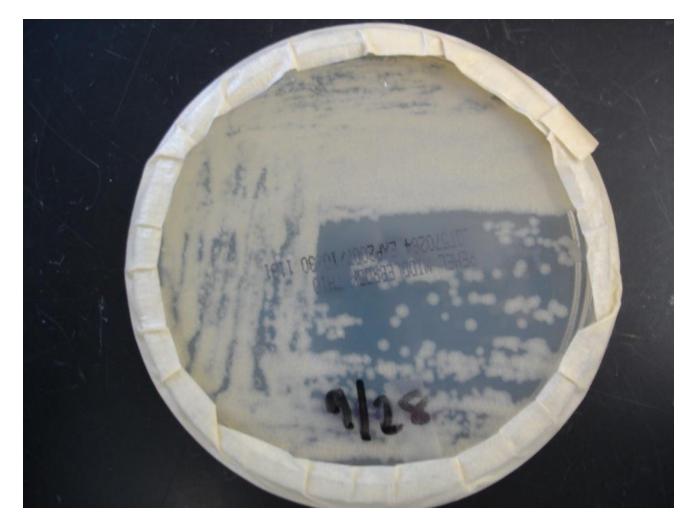








7H11 Plate





Mycobacteria ID Methods at WSLH

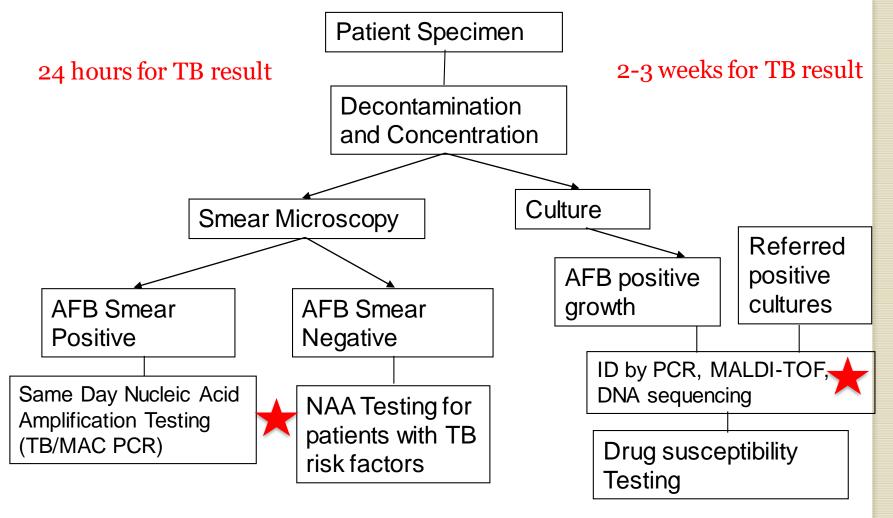
Method	Benefits	Limitations
TB or MAC PCR	 ID from scant growth Rapid ID for >60% of new positive MGIT tubes 	• Can only ID MTBC and <i>M. avium</i> complex
MALDI- TOF	 Good identification from solid media Good ID of rapid growers 	 Need good, pure growth Extraction method Poor ID from positive MGIT broth
DNA Sequencing	• "Gold standard", good ID to species level	 Labor intensive Slow

Significance of MTBC culture results

- MTBC identification is the most important finding in the clinical mycobacteriology laboratory
 - MTBC is not found in the environment
 - Isolation of MTBC almost always signifies disease
- Necessary for species identification, drug susceptibility testing, genotyping
- Monitor patient response to treatment



Mycobacteriology Testing at WSLH





Drug Susceptibility Testing (DST) for MTBC

- Automatically performed on all new cultureconfirmed TB-patients in WI
- Used as a guide in choosing treatment plan provide the best chance of a cure
- Stop transmission of TB by ending infectious period as quickly as possible
- Initiate appropriate treatment for contacts



Culture-based Drug Susceptibility Testing

- WSLH is the only laboratory in the state that performs culture-based TB drug susceptibility testing
- Rarely, DST for a WI TB patient is performed at Mayo
- WI Statutes require that an isolate from each culture-positive TB patient be submitted to WSLH for DST, genotyping, and repository.



Culture-based Drug Susceptibility Testing

A.K.A. phenotypic or conventional DST

Principle: Incubate a known concentration of MTBC isolate with a known concentration of a drug and observe for growth, or inhibition of growth



First Line Drugs

MGIT 960 broth system

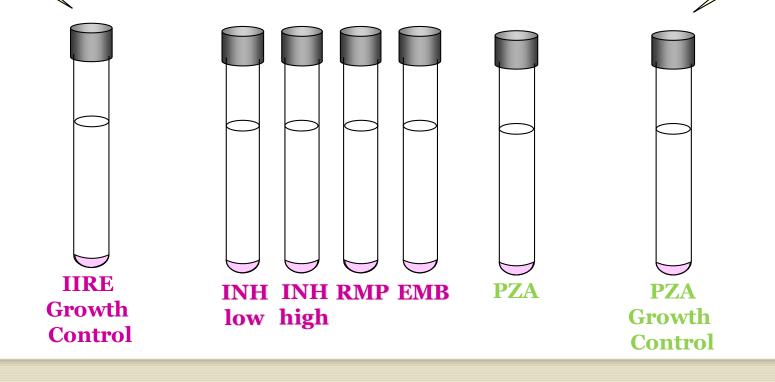
- INH (0.2 ug/ml)
- INH (1.0 ug/ml)
- rifampin (1.0 ug/ml)
- ethambutol (5.0 ug/ml)
- PZA (100 ug/ml)
- Confirm resistance by repeat testing



MGIT Method

Organism suspension added to drug-free tube: diluted Organism suspension added to drug-free tube: diluted

Organism suspension added to drug-containing tubes: undiluted





Critical Concentration

Critical concentration is the lowest concentration of a drug that:

Inhibits growth of all susceptible strains AND Allows growth of all resistant strains

Growth of MTBC at critical concentration = **RESISTANT**

No Growth of MTBC at critical concentration = SUSCEPTIBLE



Interpretation of Drug Susceptibility Results

Result	Interpretation
Susceptible	Strain is likely to show responsiveness to the drug
Resistant	Strain is unlikely to show responsiveness to the drug
Indeterminate	Test is of no help in prediction of responsiveness to the drug

2-4 weeks after positive culture—How do we get quicker results?



Molecular Detection of Drug Resistance (MDDR) Testing

AKA: genotypic testing, DNA-based

Principle: Use DNA amplification and detection methods to identify specific gene mutations that are known to confer resistance to antituberculosis drugs.



Molecular Drug Susceptibility Testing

Advantages:

- Rapid turnaround time—result in 1-2 days vs.
 2-3 weeks
- Test can be performed on mixed or non-viable cultures
- Characterization of new mutations

Disadvantages:

Interpretation of uncommon or unknown mutations



Examples of Molecular DST

	Method			
	Cepheid	Sanger	Pyrosequencing	
	GeneXpert® MTB/RIF	Sequencing		
Genetic loci	<i>rpoB</i> (for RMP)		Varies but can include <i>rpoB</i> , <i>inhA, katG, aphC,</i>	
		<i>embB</i> (EMB), <i>pncA</i> (PZA), <i>gyrA</i> (FQ), and <i>rrs</i> (injectables)	<i>gyrA</i> , and <i>rrs</i>	
Format	Semi-automated real-time PCR	DNA sequencing	DNA sequencing	
FDA approved	Yes	N/A (laboratory developed test)	N/A (laboratory developed test)	
Expected turnaround time from receipt in laboratory	1-2 working days	· •	1-2 working days (depends on how often performed in lab)	



MDDR Testing at WSLH

- WSLH performs GeneXpert MTB/RIF assay on all new TB patients identified in WI (sputum sediment, BAL sediment, or broth culture—MGIT)
 - Any other specimen type is sent to CDC or Milwaukee City Public Health Department for testing
- Used as a rapid method to detect Rifresistance/potential MDR TB



GeneXpert MTB/RIF

Workflow: Self contained cartridge - just add sample

Pour Sample Reagent into sample tube.Pipette diluted sample into cartridge.Insert cartridge and start assay.Incubate for 15 minutes at room
temperature.
(Acceptable sample types: unprocessed
sputum or sediment from concentrated
specimen.)Pipette diluted sample into cartridge.Insert cartridge and start assay.Image: Acceptable sample types: unprocessed
sputum or sediment from concentrated
specimen.)Image: Acceptable sample types: unprocessed
specimen.)Image: Acceptable sample types: unprocessed
sputum or sediment from concentrated
specimen.)Image: Acceptable sample types: unprocessed
specimen.)Image: Acceptable sample type: Acceptable sample type:

TOTAL HANDS-ON TIME = 2 MINUTES



GeneXpert Result Interpretation

Result	Interpretation
MTB DETECTED; Rif Resistance DETECTED	Likely Resistant to Rifampin
MTB DETECTED; Rif Resistance NOT DETECTED	Likely Susceptible to Rifampin

If RIF resistance mutation is detected, specimen is sent to CDC for full MDDR panel and 2nd line drug agar proportion testing

CDC MDDR Result Interpretation



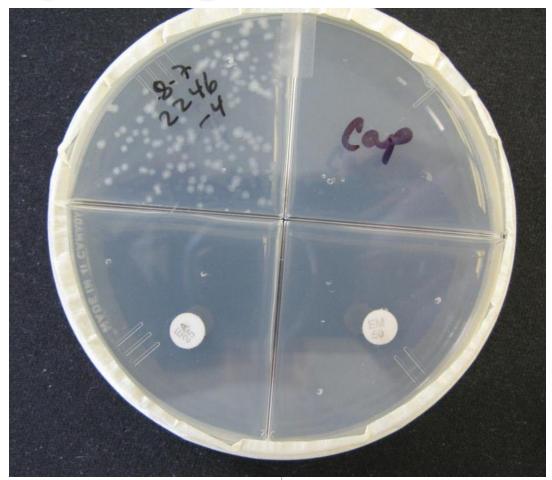
Results for Molecular Detection of Drug Resistance (Sanger Sequencing, complete panel); Conventional Drug Susceptibility Test in progress.

Locus (region) examined*	Result	Interpretation (based on in-house evaluation of 550 clinical isolates) Rifampin resistant. (100% of isolates in our in-house evaluation of 550 clinical isolates with this mutation are RMP-R.)		
rpoB (RRDR)	Mutation: TCG>TGG; Ser531Trp			
inhA (promoter)	No mutation	Cannot rule out INH resistance. (66% of INH-R isolates in our in-house evaluation of		
katG (Ser315 codon)	No mutation	550 clinical isolates have a mutation at one or both of these loci.)		
embB (Met306,Gly406)	No mutation	Cannot rule out ethambutol resistance, (79% of EMB-R isolates in our in-house evaluation of 550 clinical isolates have a mutation at this locus.)		
pncA (promoter. coding region)	No mutation	Cannot rule out PZA resistance. (86% of PZA-R isolates in our in-house evaluation of 550 clinical isolates have a mutation at this locus.)		
gyrA (QRDR)	No mutation	Cannot rule out fluoroquinolone resistance. (80% of FQ-R isolates in our in-hous evaluation of 550 clinical isolates have a mutation at this locus.)		
rrs (1400 region)	No mutation	Cannot rule out resistance to injectable drugs (kanamycin, capreomycin, amikacin). (In our in-house evaluation of 550 clinical isolates:		
eis (promoter)	No mutation	 91% of AMK-R isolates have a mutation in the rrs locus; 		
tlyA (entire ORF)	No mutation	 87% of KAN-R isolates have a mutation in either the rrs locus or the eis locus; 55% of CAP-R isolates have a mutation in either the rrs locus or the tlyA locus.) 		

*A negative result (e.g., no mutation) does not rule out contributory mutations present elsewhere in the genome.



Agar Proportion Method



*3 weeks incubation at 35-37C



AP Result Interpretation

Susceptibility Testing Method: Indirect agar proportion, 7H10 medium; Susceptibility is defined as < 1% resistance compared to colonies that develop on drug-free media

RESULTS:

3	Percent Resistance	Interpretation		Percent Resistance	Interpretation
Isoniazid 0.2 ug/ml	0%	S	Kanamycin 5.0 ug/ml	0%	S
Isoniazid 1.0 ug/ml	0%	S	Ethionamide 10.0 ug/ml	0%	S
Isoniazid 5.0 ug/ml	0%	S	Capreomycin 10.0 ug/ml	0%	S
Rifampin 1.0 ug/ml	100%	R	PAS 2.0 ug/ml	0%	S
Ethambutol 5.0 ug/ml	0%	S	Ofloxacin 2.0 ug/ml	0%	S
Streptomycin 2.0 ug/ml	0%	S	Amikacin 4.0 ug/ml	0%	S
Streptomycin 10.0 ug/ml	0%	S			
Rifabutin 2.0 ug/ml	50%	R			
Ciprofloxacin 2.0 ug/ml	0%	S			

Susceptibility Testing Method: MGIT 960

Pyrazinamide 100 ug/ml : Sus

: Susceptible

Comments: Molecular Detection of Drug Resistance (MDDR) report was issued 6/27/2017.

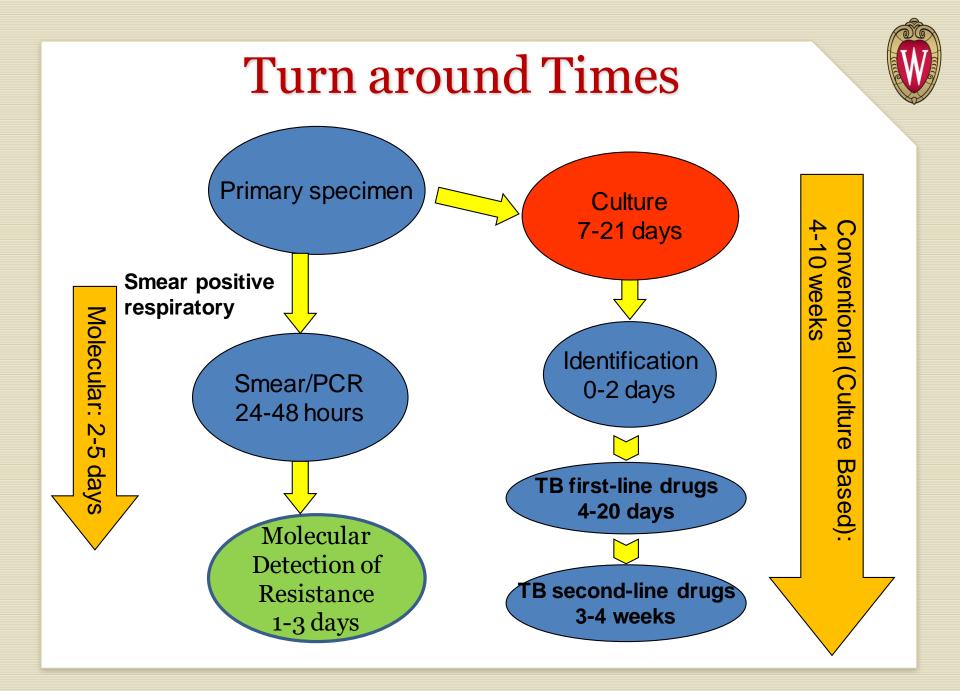
These conventional agar proportion results agree with the MDDR results.



Agar Proportion Limitations

Slow---3 week incubation

- Compared to 4-12 days with broth method
- Media preparation—cannot purchase commercially
- CDC goal
 - Report RIF DST result within 17 days of organism ID (impossible to meet!)





Acknowledgements

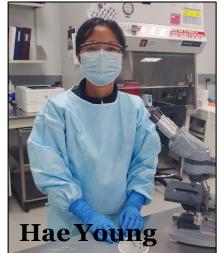
Dave Warshauer, PhD Julie Tans-Kersten Laura Louison, MLS(ASCP) The WSLH TB Laboratory Staff

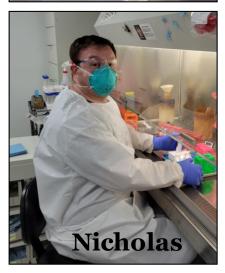


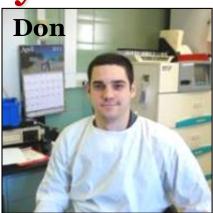
WSLH TB Laboratory Team













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Questions?

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