



# **Laboratory Diagnosis of Tuberculosis**

Benjamin Alpers, BA  
July 16, 2024

TB Intensive  
July 16 – 18, 2024  
San Antonio, Texas

# **Benjamin Alpers, BA** has the following disclosures to make:

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- No conflict of interests
- No relevant financial relationships with any commercial companies pertaining to this educational activity





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**Texas Department of State  
Health Services**

# Laboratory Diagnosis of Tuberculosis

or

Desperately Seeking Tuberculosis

Benjamin Alpers

Applications Scientist/TB Reference Team Lead DSHS Austin Laboratory

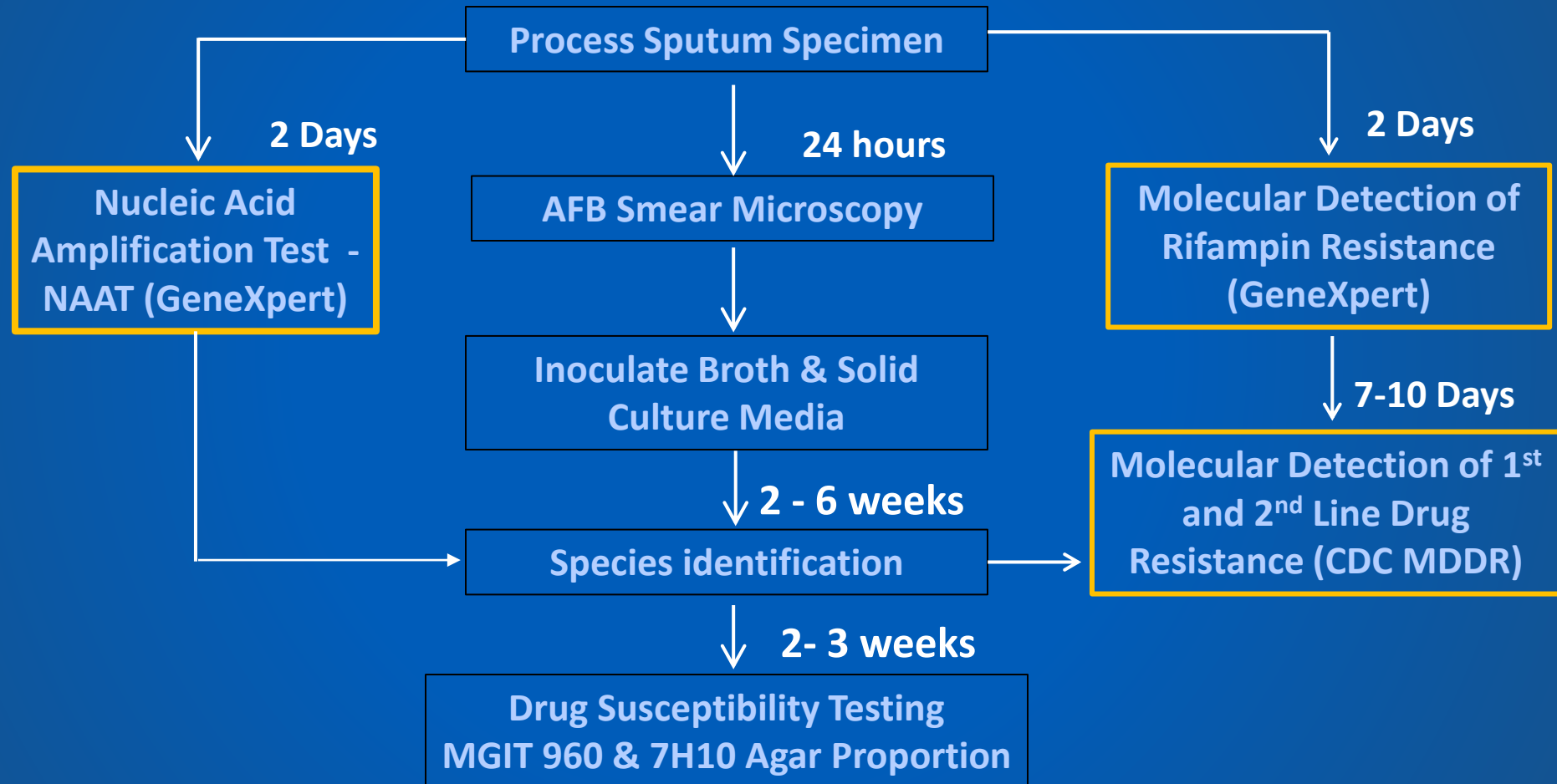
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# TB Laboratory Testing Algorithm

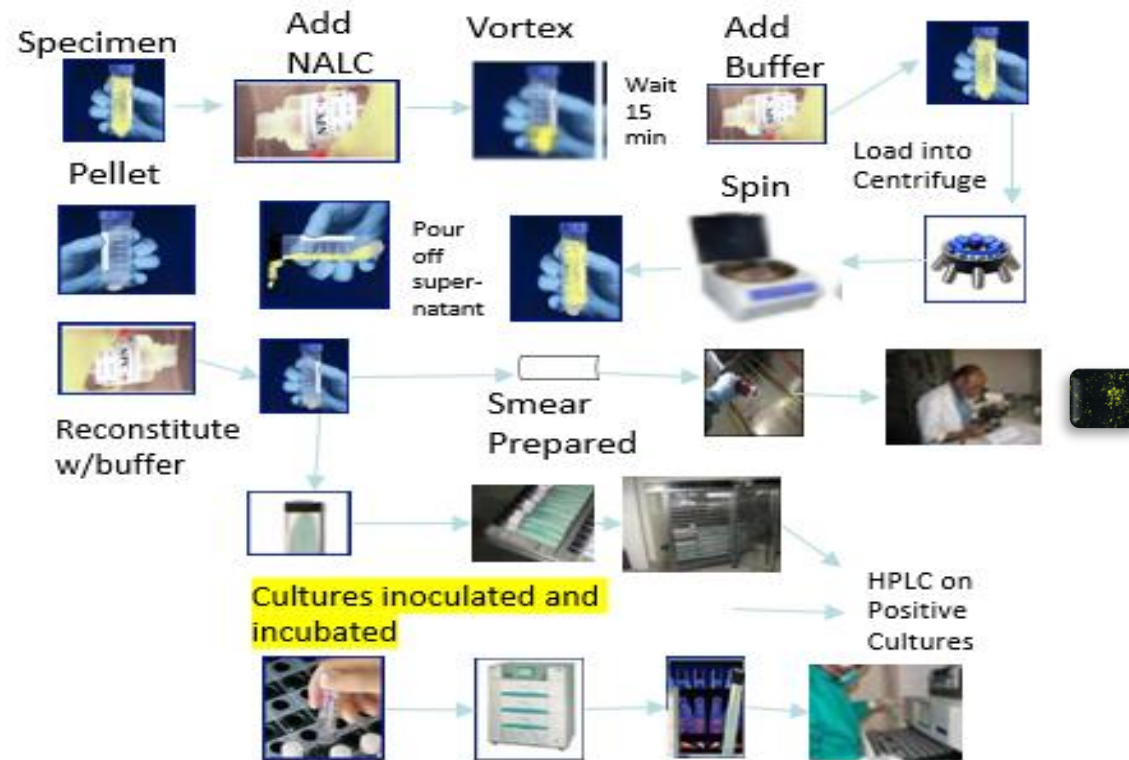


# Specimen Quality

- Accurate laboratory results are directly related to the quality of the specimen
- Sputum
  - *Recently* discharged material from the bronchial tree, with minimal amounts of upper respiratory tract secretions
    - Well coached patient, collect at least 3ml
    - Label tube, form, and indicate test:
      - initial Dx: Smear, NAAT, & Culture
      - Follow-up: Smear and Culture
      - Release from respiratory isolation? Order Smear only
- Transport to lab cool and quickly



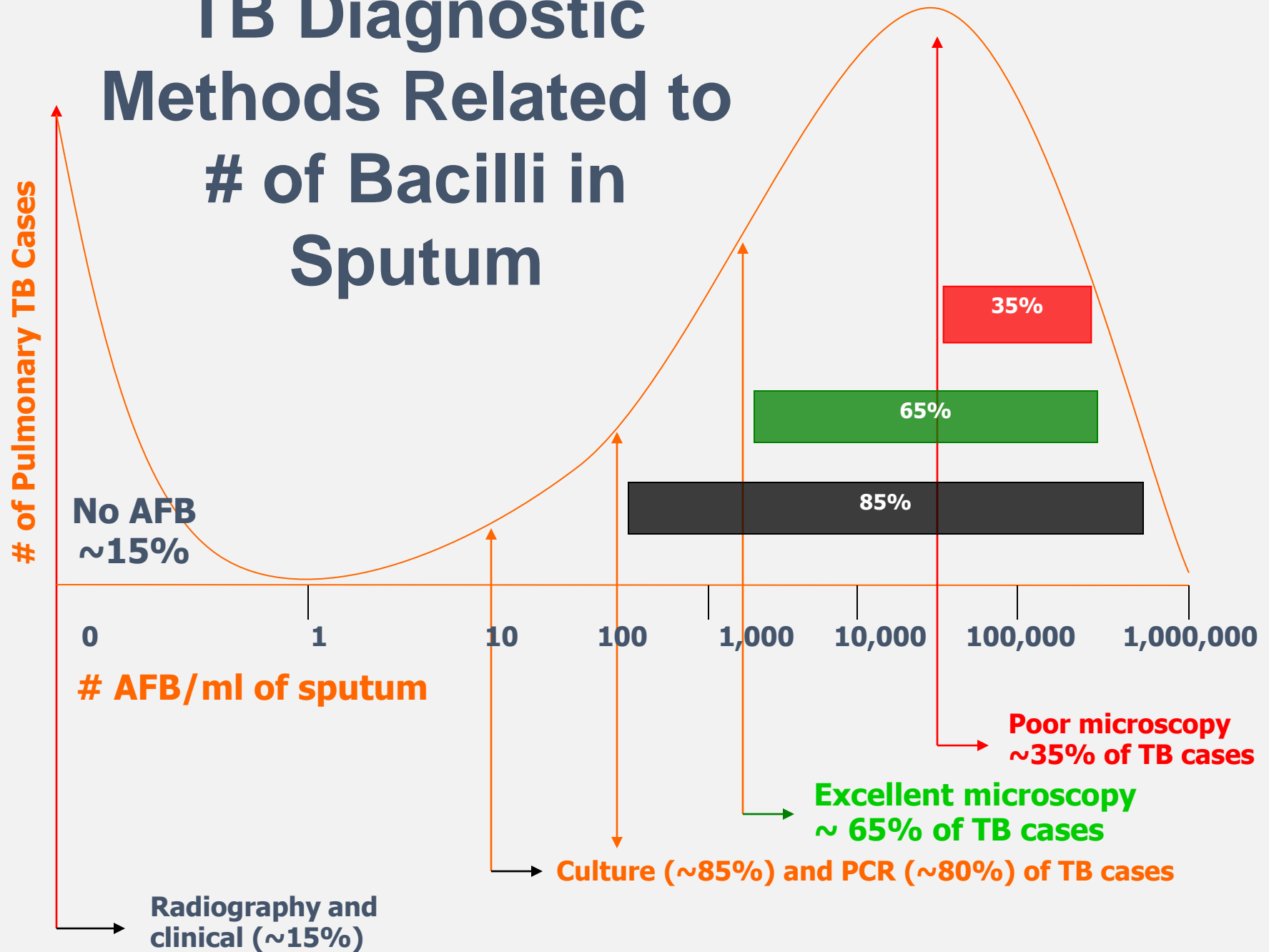
# AFB Specimen Processing Series Of Many Manual Steps!



Adapted from slide by Frances Tyrrell, CDC (retired)



# TB Diagnostic Methods Related to # of Bacilli in Sputum



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# AFB Culture

- More sensitive than smear
  - 5,000 to 10,000 AFB/ml for smear
  - ~10 viable AFB/ml for culture
- Positive for only ~85% of Pulmonary TB
  - Requires a quality specimen
  - May be invalid due to contamination
- Used to monitor patient response to treatment (like smear)
- Required for conventional drug susceptibilities & genotype
- Lengthy
  - 1-6 weeks by liquid media
  - 2-8 weeks by solid media



# Becton Dickinson BACTEC™ MGIT™ 960

- A fluorescent compound is embedded in silicone on the bottom of tubes.
- Tube initially contains dissolved oxygen which quenches emissions from the compound and little fluorescence can be detected.
- Later, actively respiring microorganisms consume the oxygen and allow the fluorescence to be observed (instrument takes a reading once an hour).



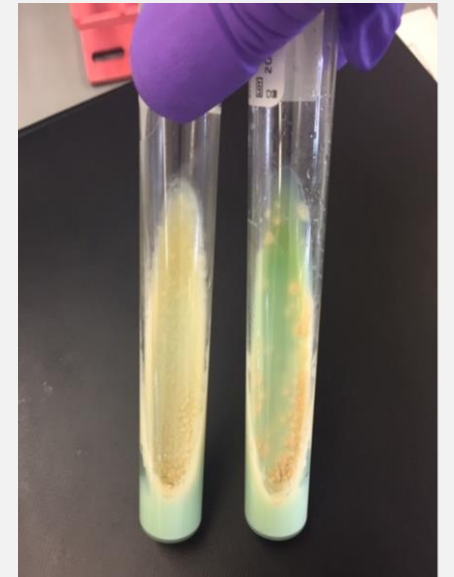
# Becton Dickinson BACTEC™ MGIT™ 960, cont.

- Fluorescence can also be manually observed using a transilluminator.
- Particulates are often observed at bottom of tube.
- Modified 7H9 broth that requires supplement for each process (growth and antibiotic).
- The same MGIT™ tube used for IIRE drug susceptibility testing (tube with different pH used for PZA).



# Solid Media for MTBC Detection

- Middlebrook 7H11 agar
  - Primary media
  - Morphology can be viewed microscopically
- Middlebrook 7H10 agar
  - Used as secondary media (less selective)
- Lowenstein Jensen slant
  - Long life span for storage



# Methods for Diagnosis Used in Conjunction with Culture

- Acid Fast Bacilli Microscopy (AFB Smear)
- Nucleic Acid Amplification Test (NAAT)
  - Cepheid GeneXpert<sup>®</sup>
- High Performance Liquid Chromatography (HPLC)
- MALDI-TOF Mass Spectrometry
- Real-time Polymerase Chain Reaction (PCR)
  - Used in *M. tb* cx. speciation



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# Acid Fast Bacilli Microscopy (AFB Smear)

- **Has many qualities of an ideal diagnostic test**
  - Rapid & universally available
  - Detects the most infectious cases
  - Used to support diagnosis and identify need to isolate
  - Helps monitor response to therapy
  - Identifies priority cases for nucleic acid amplification (NAA)
- **Problems**
  - Not sensitive - misses ~50% of TB
  - Not specific in low TB prevalence areas (e.g. Texas)
    - Positive smear may be NTM (~20% at DSHS-Austin)
- **Highly specific where TB is highly prevalent**



# Nucleic Acid Amplification Tests (NAAT)

- Real time reverse transcription polymerase chain reaction (qRT-PCR or qPCR)
- Tiny amounts of DNA/RNA amplified (copied) until a significant signal compared to the background
- GeneXpert examines DNA for:
  - Identification
  - Detection of Rifampin Resistance
- Test turnaround time measured in hours



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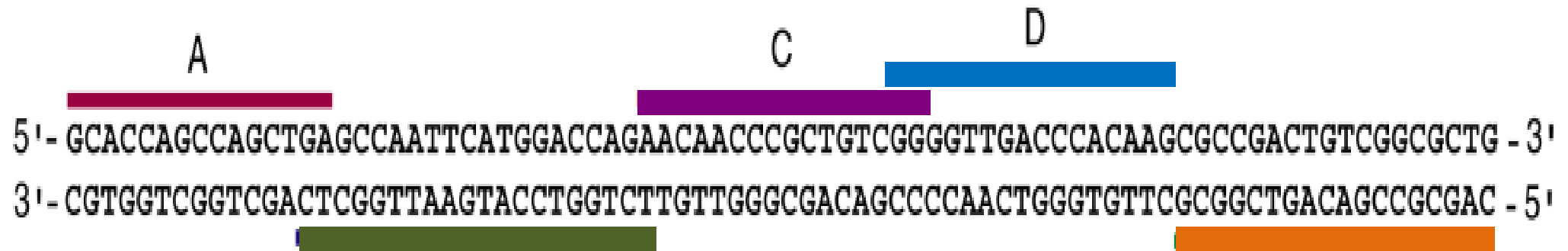


# Nucleic Acid Amplification Tests (NAAT)

- Detects *M. tuberculosis* complex nucleic acids; does not distinguish between live and dead bacilli
  - For initial diagnostic specimens only
  - Not suitable for follow-up specimen or monitoring; cured patients may be NAAT + for years!
- Xpert sensitivity compared to TB culture
  - >95% for AFB smear-positive
  - Only 55-75% for AFB smear-negative
- Does not replace culture for bacteriological diagnosis
  - (Yet)



# Cepheid GeneXpert® Target Region



**The MTB assay target is the 81 bp rifampin resistance determination region of the rpoB gene.**

Approx. 10% of rifampin resistant predictions are false  
(ex. Phe433Phe silent mutation)

**GX Rifampin resistant results must be confirmed**



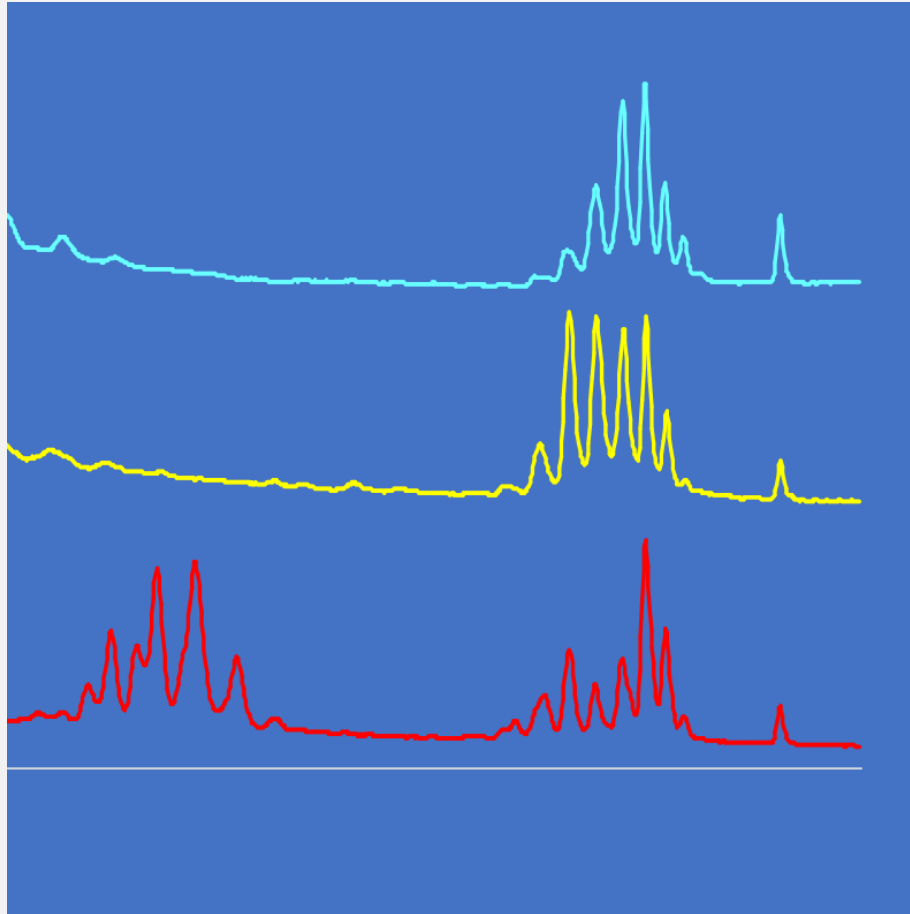
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# High-Performance Liquid Chromatography (HPLC)

- Identifies mycobacteria by analysis of mycolic acids in the thick cell wall.
- Mycolic acids are chemically cleaved, separated, and extracted from cell wall.
- Mycolic acid esters are then separated and detected with reverse-phase HPLC with fluorescent detection (FL-HPLC) to produce a chromatogenic pattern with diagnostic peaks.



# Fluorescence-HPLC Patterns



- M. tuberculosis cx.
- M. kansasii
- M. avium cx.

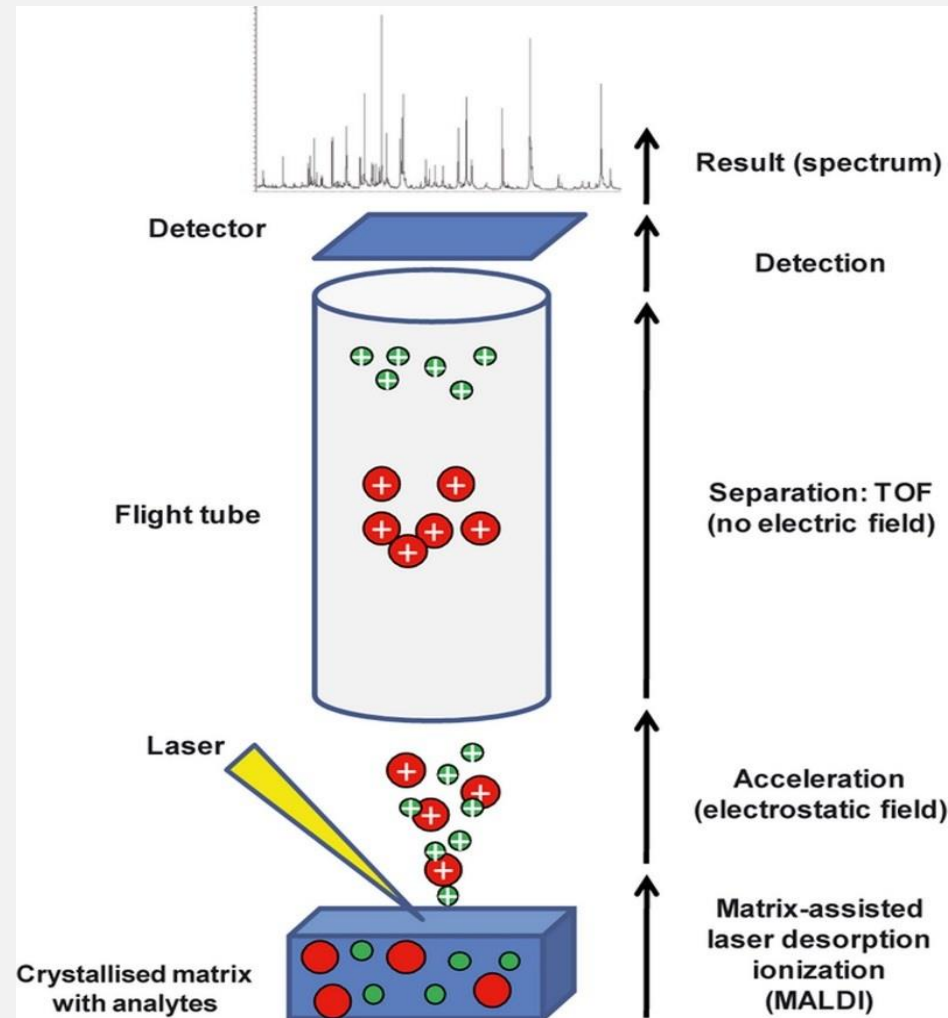
# MALDI-TOF Mass Spectrometry

## Matrix-Assisted Laser Desorption/Ionization Time Of Flight

- Prepared organism is applied to sample plate and overlaid with a chemical matrix.
- When a laser is applied, the matrix provides proteins with a charge in the vacuum.
- Proteins move toward the detector via an electric field.
- Sample spectra is compared to a library database.



# MALDI Diagram Principle



# MALDI vs. HPLC

- MALDI able to identify many more species than HPLC, also break some groups and complexes up into distinct organisms
- MALDI is less sensitive than HPLC, especially for MTB. Liquid media may need to incubate an additional couple days for valid ID.
- MALDI is currently validated filamentous mold and Candida yeast; working on AFB validation



# M. tb cx. Speciation through Real-Time Polymerase Chain Reaction (PCR)

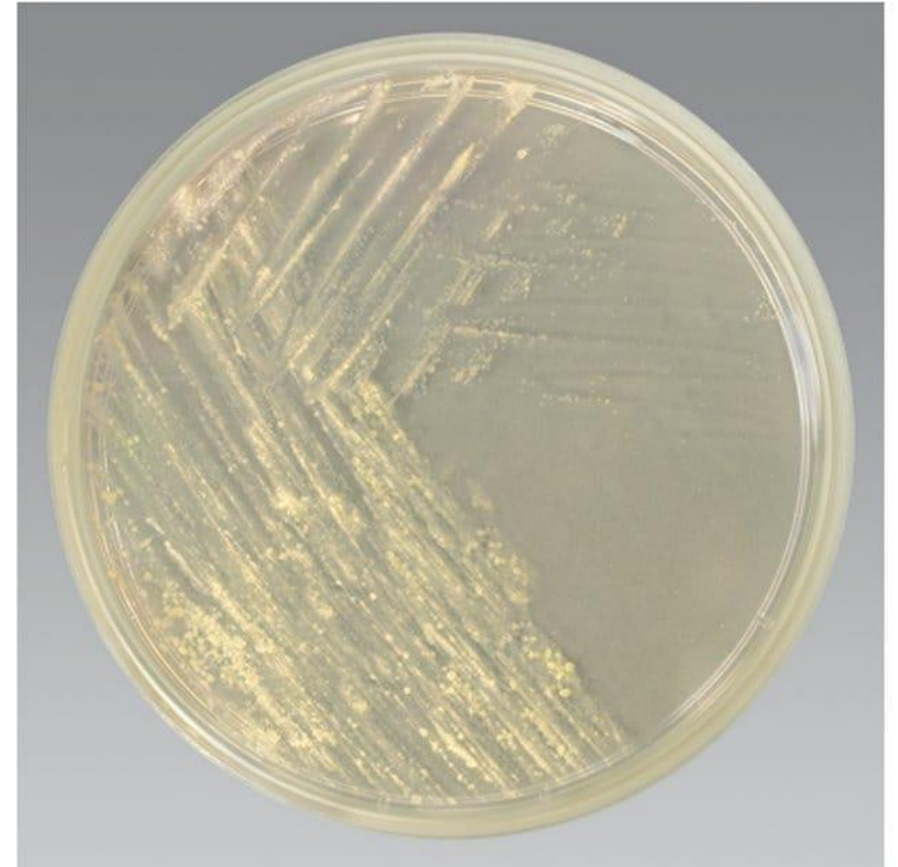
- New laboratory developed test
- Currently send to CDC when bovis or bovis BCG suspected with an average of 4 week turnaround
- Once implemented should produce a result in <4 days
- Can differentiate between species within the complex using 5 sets of probes and primers targeting known regions of difference (RD)





# *M. tuberculosis* complex

- *M. tuberculosis*
  - *M. bovis*
  - *M. bovis* BCG
- } inherently  
PZA resistant
- and
- *M. africanum*
  - *M. microti*
  - *M. canettii*
  - *M. caprae*
- ...and others





Species	RD 1	RD 4	RD 9	RD 12	Ext-RD9
<i>M. tuberculosis</i>	+	+	+	+	+
<i>M. bovis</i>	+	-	-	-	+
<i>M. bovis</i> BCG	-	-	-	-	+
<i>M. africanum</i>	+	+	-	+	+
<i>M. microti</i>	-	+	-	+	+
<i>M. canettii</i>	+	+	+	-	+
NTM	-	-	-	-	-

# Drug Susceptibility Testing (DST)

- Conventional growth-based method performed at DSHS
- Molecular Detection of Drug Resistance (MDDR) performed at the CDC



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# Conventional Drug Susceptibility Testing (DST) of *M. tuberculosis* Complex

## Current Laboratory Protocol

- Initial isolate should be tested against first-line drugs (FLD)
  - Isoniazid, rifampin, ethambutol, pyrazinamide plus a fluoroquinolone (ofloxacin)
  - Repeat test if patient cult+ after 3 mo. Rx
- For isolates resistant to Rifampin or to any 2 FLDs: **test second-line drug panel!**
  - Rifabutin, ethionamide, & injectable (capreomycin, kanamycin)



# Important Considerations

- A rapid report of “INH & RMP susceptible” is the single most common & important DST report issued by the TB lab.
  - ~90% of U.S. cases are susceptible to the primary drugs
  - “INH & RMP susceptible” predicts primary drug panel efficacy
  - “INH & RMP susceptible” allows discontinuation of relatively toxic antibiotics in treatment continuation phase



# Additional Considerations

- CDC recommends RMP DST results be reported within 17 days after M. tuberculosis culture identification
- Most U.S. laboratories use a rapid commercial system for DST (MGIT 960)
- Commercial DST methods miss some clinically significant RMP resistance that can be detected by agar proportion
- Agar Proportion (AP) is the “gold standard” method for conventional DST...however AP is not a rapid method; conventional AP method takes 21 days (3 weeks) for full results
- DSHS is able to regularly report INH & RMP susceptibility within 17 days of culture identification



# Agar Proportion DST Method Principle

- A standardized suspension of *M. tuberculosis* is inoculated to quadrant plates of drug-containing Middlebrook 7H10 agar and a drug-free control.
- If growth of *M. tuberculosis* on the drug quadrant is 1% or greater than the growth on the control, the drug can no longer be counted on as being effective for treatment.

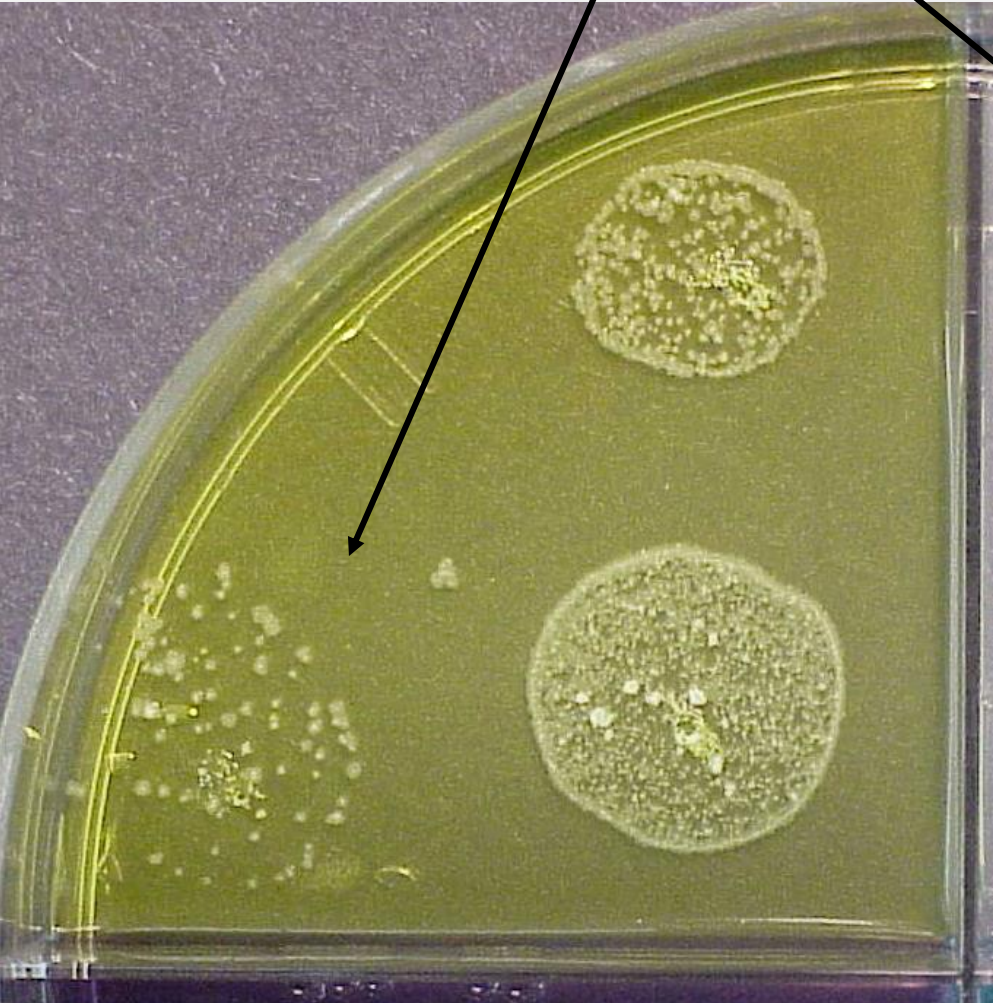




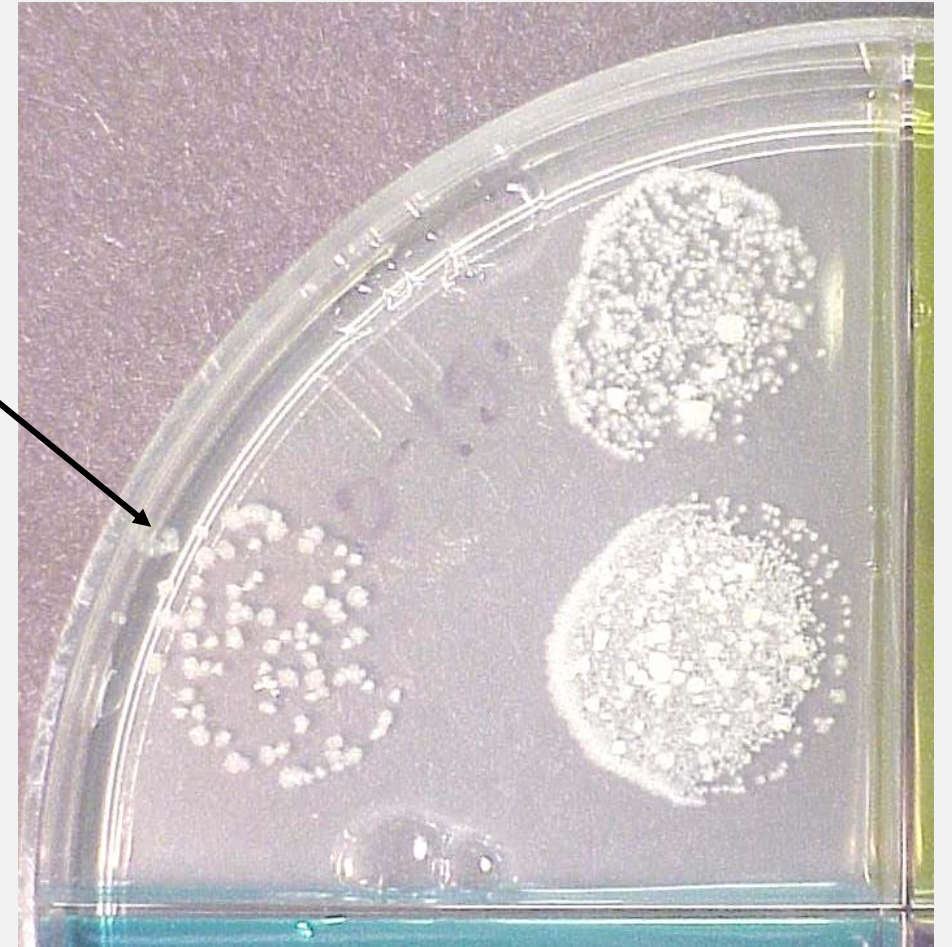




**100cfu/100cfu = 100% Resistance**

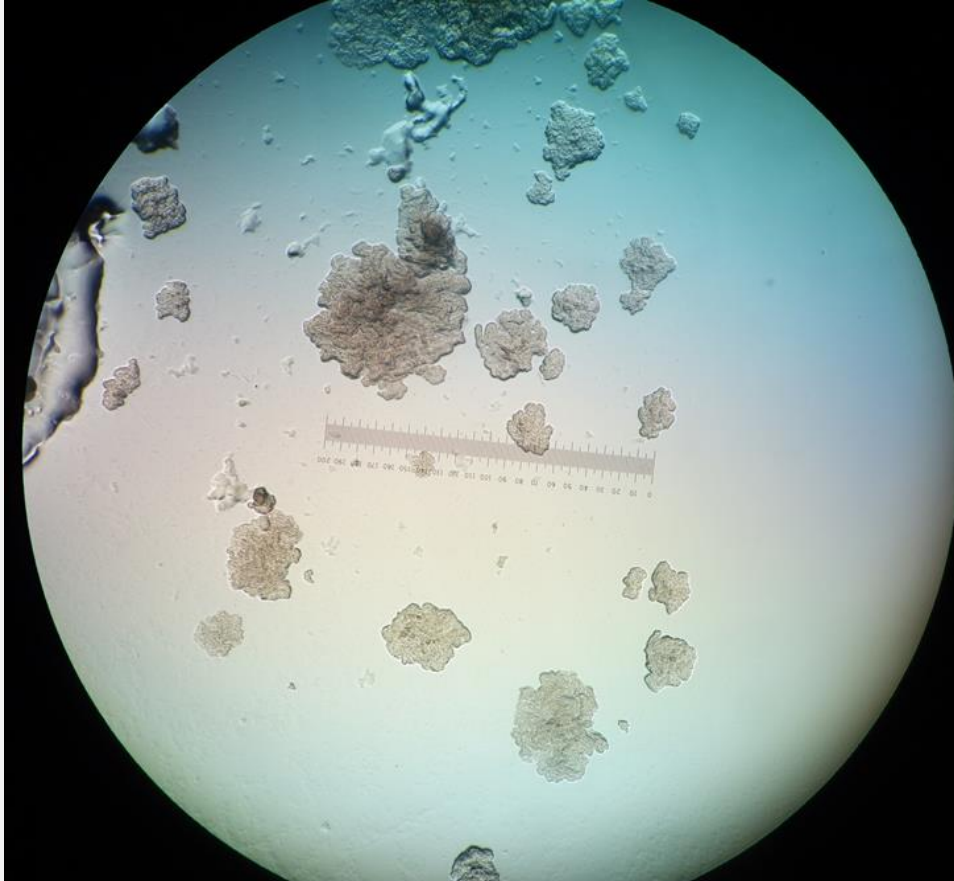


Isoniazid, 1.0 mcg/ml

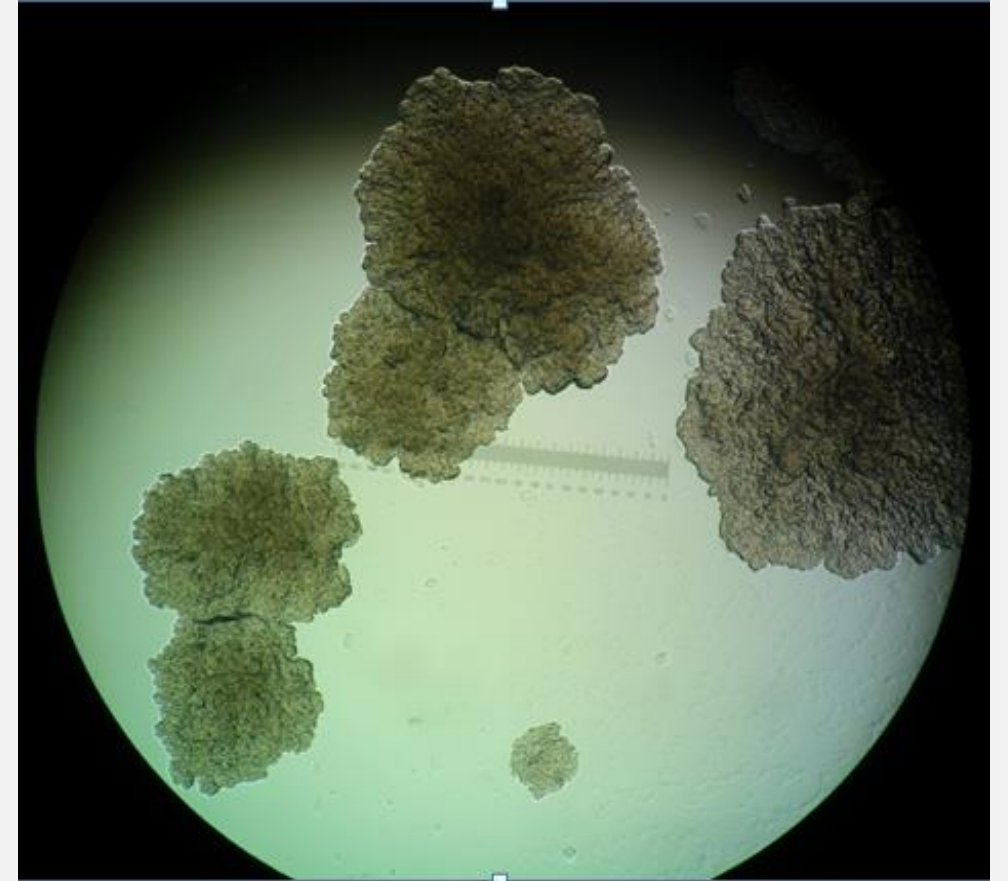


Drug-Free Control

# Typical MTBC 7H10 Agar Proportion Growth at 50X



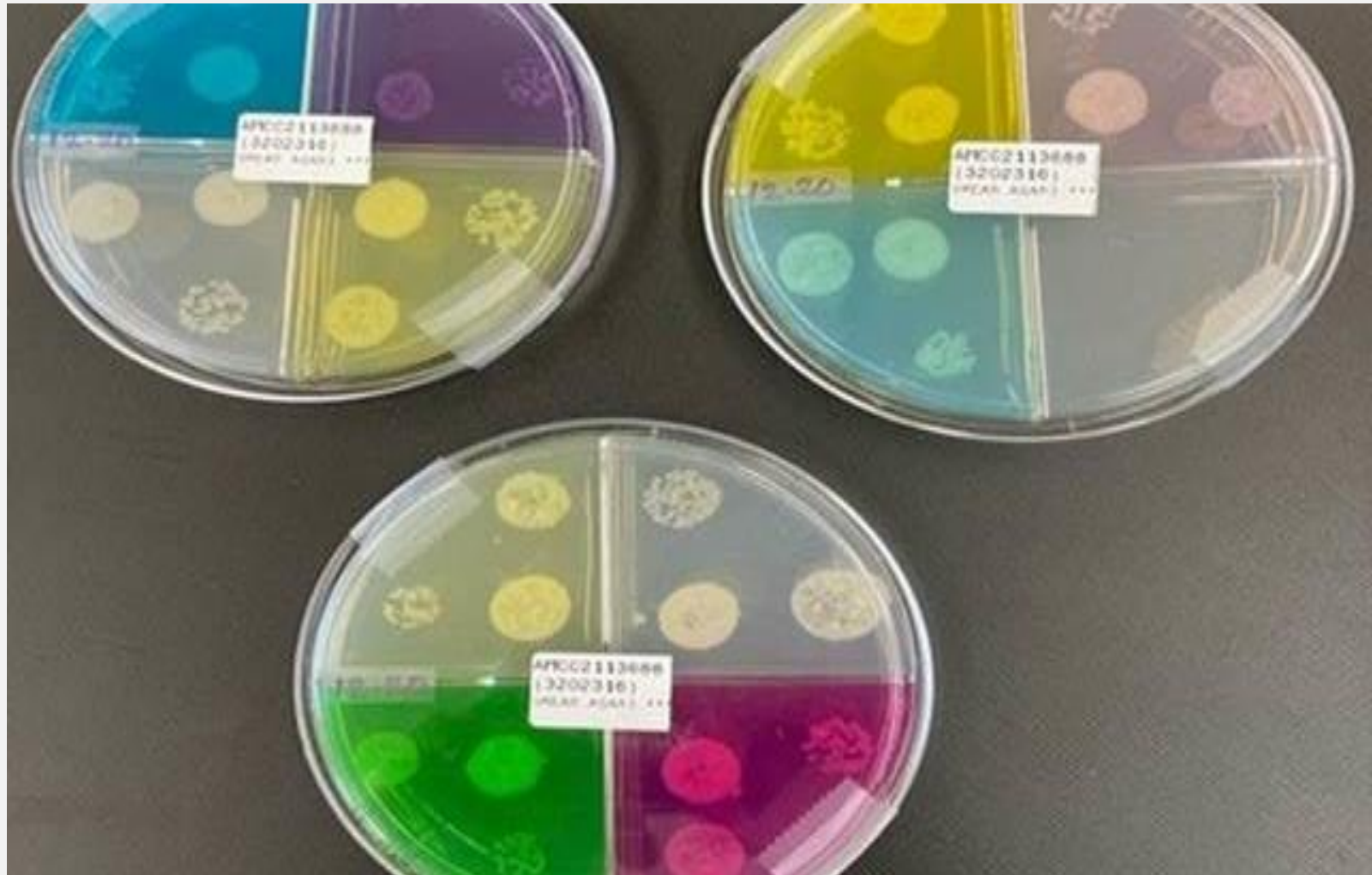
Two Weeks Incubation



Three Weeks Incubation



# Example of XDR by Agar Proportion



# Molecular Detection of Drug Resistance (MDDR)

- Can provide rapid detection of drug resistance
- Both NAAT positive and culture positive specimens are candidates
- Particularly useful for high-risk patients, RMP positive Xpert sediment, contaminated specimens, or those specimens that do not grow well or are non-viable in standard TB media
- Examines 24 amplicons across 16 genes providing information on more than 12 antituberculosis drugs



# Genetic Loci Sequenced through MDDR

## Genetic Locus

RRDR within the *rpoB* gene with the addition of two codons outside of the RRDR

*inhA, katG, fabG1*

*embB*

*pncA*

*gyrA, gyrB*

*rrs*

*eis*

## Associated Drug

Rifampin (RMP)

Isoniazid (INH)

Ethambutol (EMB)

Pyrazinamide (PZA)

Fluoroquinolones

Amikacin Capreomycin

Kanamycin

Kanamycin



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# CDC's MDDR Assay

- New sequencing method using Targeted Next Generation Sequencing (tNGS)
- More regions sequenced including genes associated with bedaquiline, clofazimine, and linezolid
- Improved limit of detection for heteroresistance
- More TB DNA needed for successful amplification than pyrosequencing (1-10/field smear result or greater)
- Turnaround time now 7-10 days



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# Additional Genetic Loci Sequenced through tNGS

## Genetic Locus

Expanded *katG*  
(sensitivity increased to >93%)

*atpE*  
*rv0678*  
*pepQ*

*rv0678*  
*pepQ*

*rplC*  
*rrl* (partial)

## Associated Drug

INH

Bedaquiline

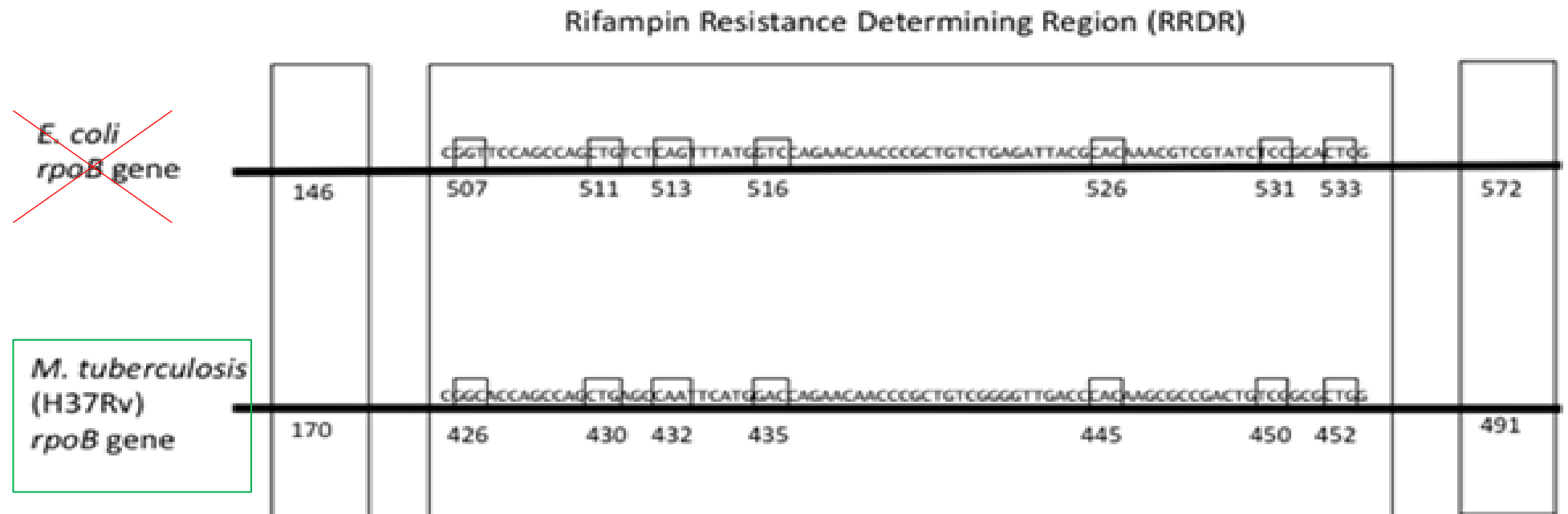
Clofazimine

Linezolid



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# *M. tuberculosis* numbering in *rpoB*



The *M. tuberculosis* numbering is minus 81 codons from the *E. coli* numbering except for the 146/170 codon.

Figure is adapted from Andre, 2017 et al and kindly provided by the Association of Public Health Laboratories



# CDC Molecular Detection of Drug Resistance (MDDR) vs. Agar Proportion (AP) Method

- MDDR provides 7-10 day DNA sequence analysis for drug resistance prediction, AP 11-22 day growth-based susceptibility
  - 1<sup>st</sup> and 2<sup>nd</sup> line drugs tested
- MDDR complements conventional DST
  - Used alone, MDDR and conventional DST are imperfect
  - Used together, accuracy of drug resistance or susceptibility detection can be improved.
- Conventional DST results are still needed, or at least desirable, to confirm susceptibility to individual drugs.



# Whole Genome Sequencing (WGS)

- Current genotyping method
- CDC began performing this in 2012, regularly in 2014, and exclusively in 2022
- Primarily used to determine relatedness between strains infecting individuals for epidemiological purposes
- Can be helpful in false-positive investigations
- Potential to establish reactivation vs. reinfection
- Reflexively alerts to any significant *rpoB* mutation



# WGS (con't)

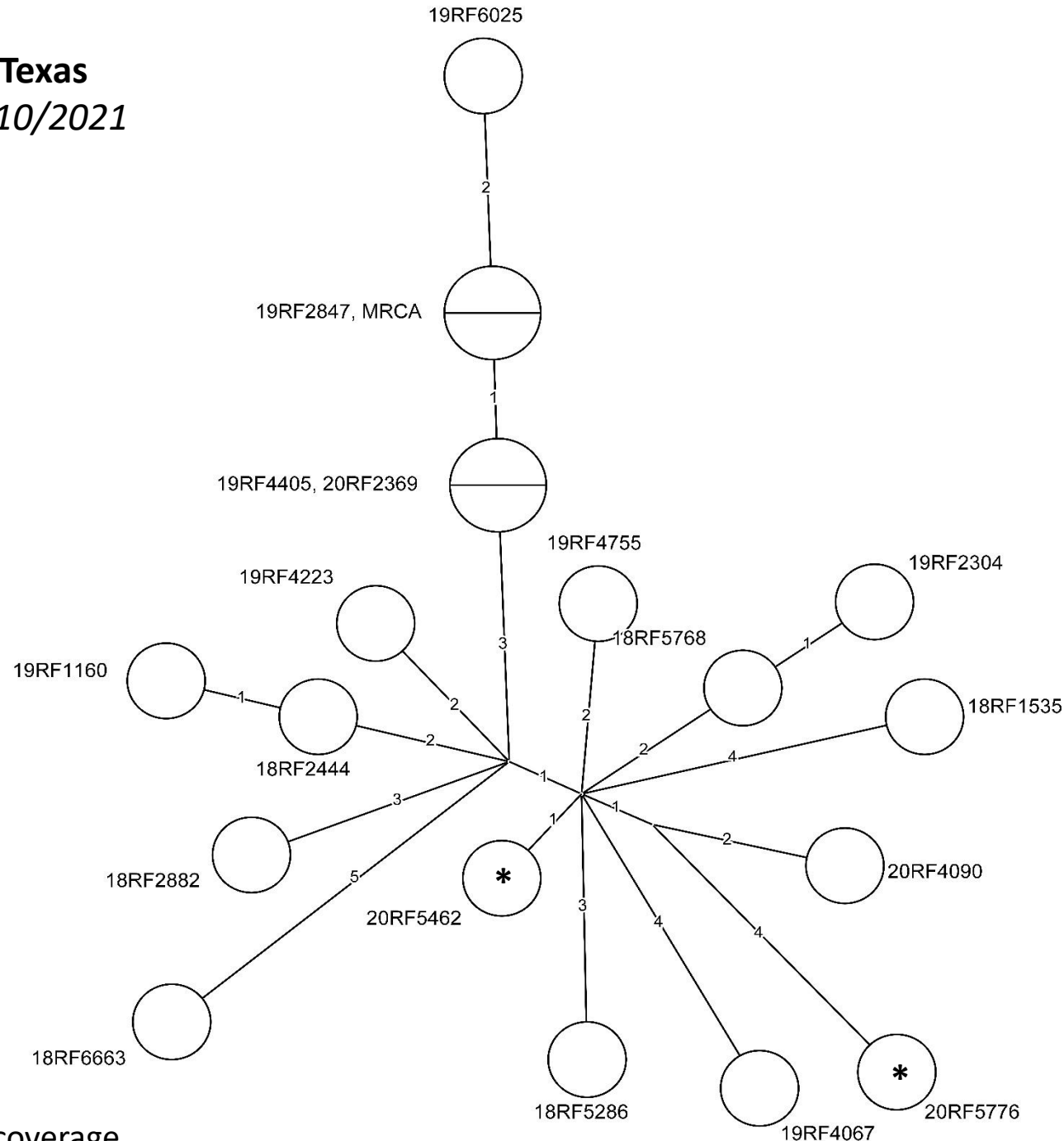
- 2,690 genetic loci examined and compared
- Those that are 99.7% similar clustered by wgMLSType
- This translates as <8 SNPs difference to at least one isolate in cluster
- Phylogenetic trees can be created within clusters
- **Not** indicative of drug resistance pattern!



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**MTBC000025 in Texas**  
*Results received 2/10/2021*

*Analysis updated with  
20RF5776 and 20RF5462  
(isolates from the same patient)*



**Excluded isolates:**

- 19RF3857 – contaminated
- 18RF6976 – contaminated
- 18RF6084 – contaminated
- 18RF4713 – contaminated
- 18RF3636 – low sequence coverage

*\*Isolates denoted with an asterisk are from the same patient. Isolate 20RF5462 was collected from a sputum specimen and 20RF5776 was collected from a urine sample.*

# WGS (con't)

- DSHS has incorporated this method into our algorithm for predictive DST
- Can only be performed on isolate
- One isolate per patient per year in most instances
- Will report mutations detected from genes associated with primary drug resistance (INH, RMP, EMB, FQN, and PZA)
- MDDR will not be replaced by this; used as an alternative unless MDDR indicated initially or reflexively



# Diagnostics for an MDR Patient

A Case Study for the Way Things Should Work



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- Collection from a binational patient 3/13/23



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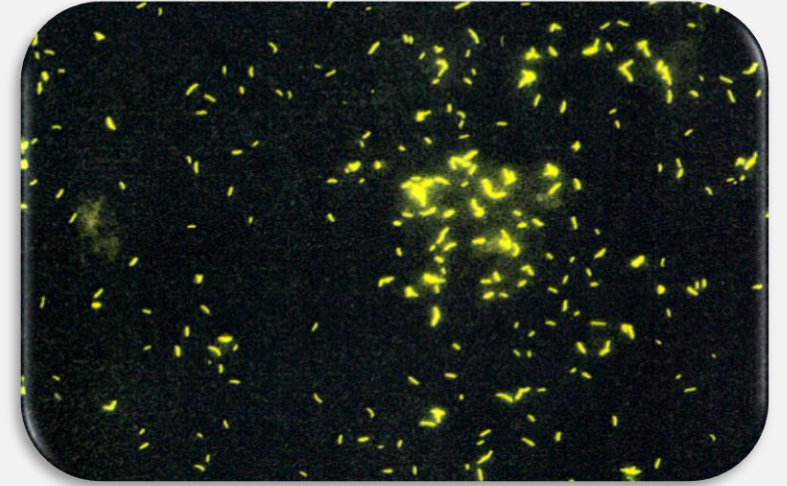
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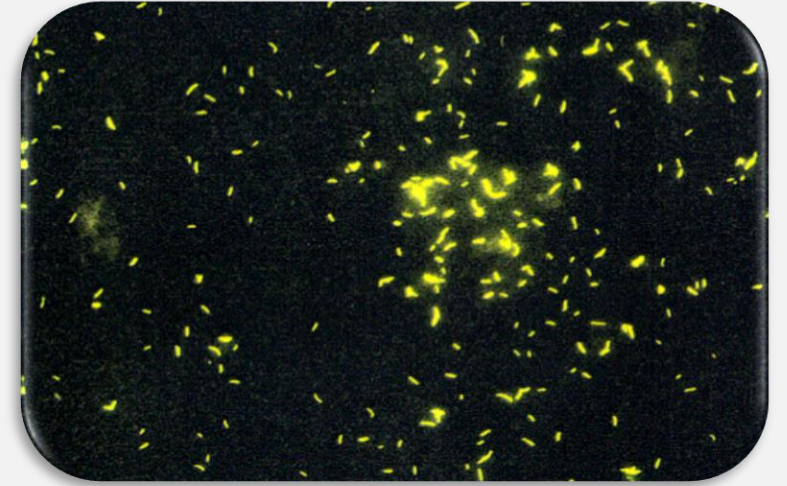


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- Received at lab 3/15/23
- Smear reported 3/15/23
- **>10/field**



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- Collection from a binational patient 3/13/23
- Received at lab 3/15/23
- Smear reported 3/15/23
- **>10/field**
- Xpert reported 3/16/23
- **Positive, Rifampin resistance detected**



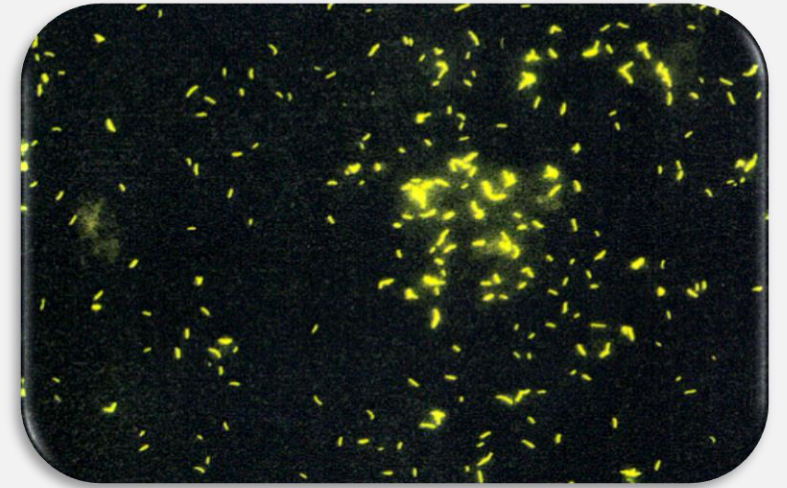
Test	Result
MTB Direct Detection, NAA	POSITIVE: M.tuberculosis complex DNA detected
Rifampin by Direct NAA	Rifampin resistance mutation detected; likely rifampin Resistant; confirmatory testing in progress.

Note: This real-time PCR assay was developed and its performance characteristics determined by the Texas Department of State Health Services Laboratory. It has not been cleared or approved by the US Food and Drug Administration . A result of "Not Detected" is the reference range. Results from this assay should be interpreted in conjunction with other laboratory data and clinical findings.

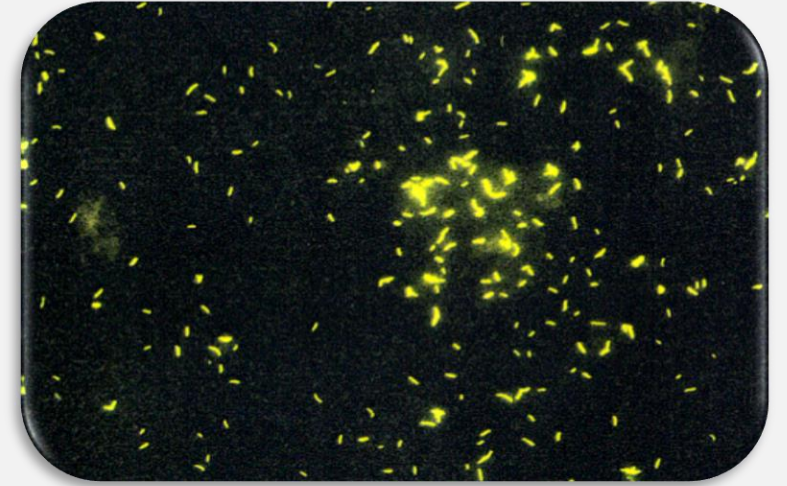


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- Sent for MDDR 3/16/23

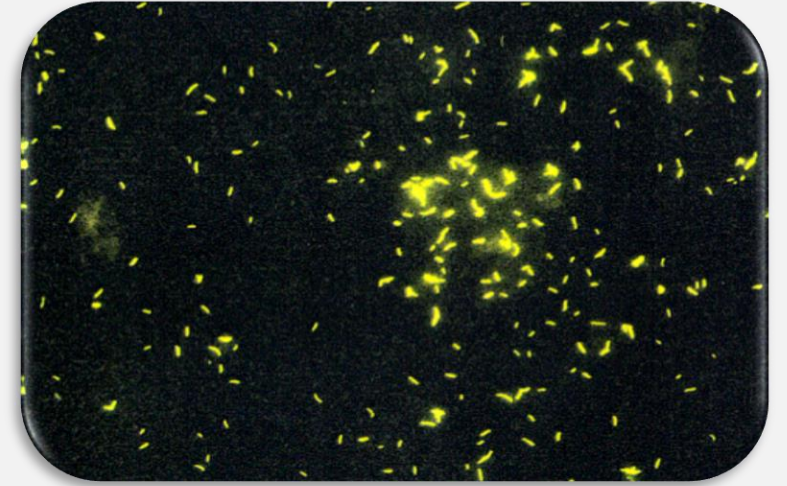


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- Culture positive 3/21/23





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- Culture positive 3/21/23
- MDDR report 3/31/23
- **Mutations in *rpoB*, *katG*, and *embB***





Specimen ID: **AMCC2303733**

Alt. Specimen ID:

CDC Specimen ID: **3003783519** CDC Unique ID: **N8KHJYAT** CDC Local Aliquot ID: **23-2244**

<b>Rifampin (RIF)</b>	<b><u>Result</u></b>	<b><u>Interpretation</u></b>
RIF interpretation		RIF resistant
rpoB*	His445Asp	
<b>Comments and Disclaimers</b>		
* DTBE Reference Laboratory has transitioned from the E. coli to the M. tuberculosis numbering system for reporting rpoB gene mutations.		

<b>Isoniazid (INH)</b>	<b><u>Result</u></b>	<b><u>Interpretation</u></b>
INH interpretation		INH resistant
inhA	No mutation	
fabG1	No mutation	
katG	Ser315Thr	

<b>Ethambutol (EMB)</b>	<b><u>Result</u></b>	<b><u>Interpretation</u></b>
EMB interpretation		EMB resistant
embB	Met306Val	

<b>Pyrazinamide (PZA)</b>	<b><u>Result</u></b>	<b><u>Interpretation</u></b>
PZA interpretation		Cannot rule out PZA resistance.
pncA	No mutation	

<b>Fluoroquinolones (FQ)</b>	<b><u>Result</u></b>	<b><u>Interpretation</u></b>
FQ interpretation		Cannot rule out FQ resistance.
gyrA	No mutation	
gyrB	No mutation	

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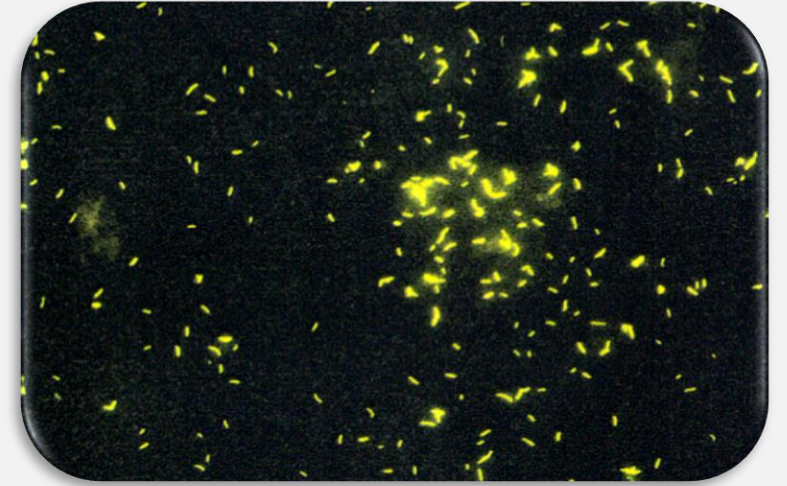
<b>Amikacin, Capreomycin, and Kanamycin (AMK, CAP, and KAN)</b>	<b><u>Result</u></b>	<b><u>Interpretation</u></b>
AMK CAP and KAN interpretation		Cannot rule out resistance to AMK, CAP, and KAN.
rrs	No mutation	
eis	No mutation	

<b>Bedaquiline (BDQ)</b>	<b><u>Result</u></b>	<b><u>Interpretation</u></b>
BDQ interpretation		Cannot rule out BDQ resistance.
atpE	No mutation	
rv0678	No mutation	
pepQ	No mutation	

<b>Clofazimine (CFZ)</b>	<b><u>Result</u></b>	<b><u>Interpretation</u></b>
CFZ interpretation		Cannot rule out CFZ resistance.
pepQ	No mutation	
rv0678	No mutation	

<b>Linezolid (LZD)</b>	<b><u>Result</u></b>	<b><u>Interpretation</u></b>
LZD interpretation		Cannot rule out LZD resistance.
rplC	No mutation	
rri	No mutation	

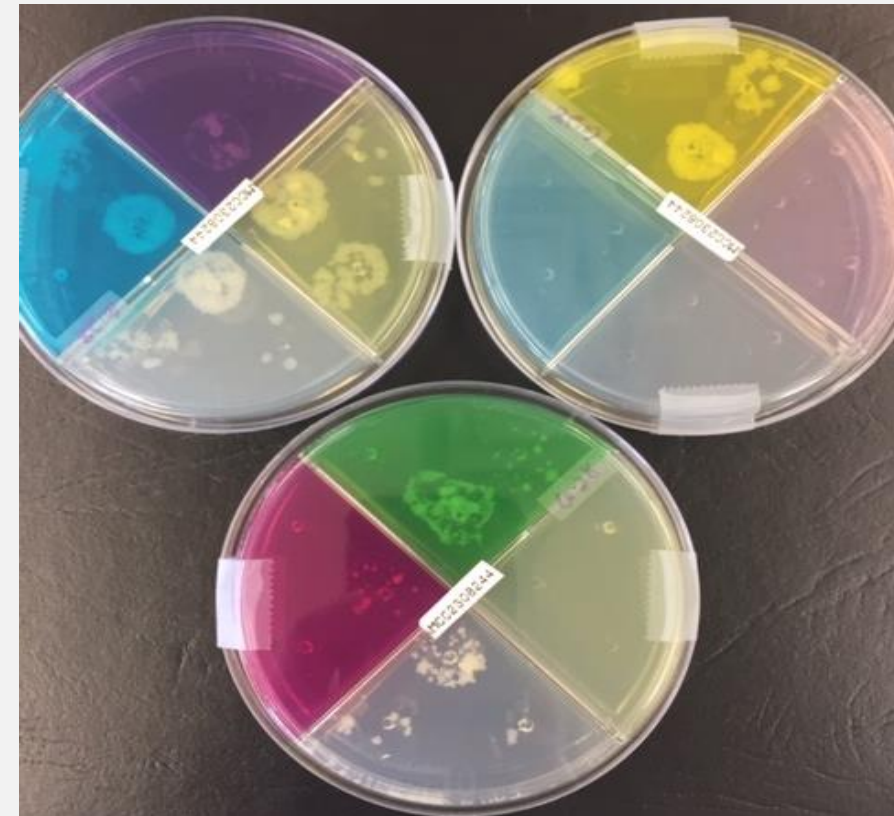
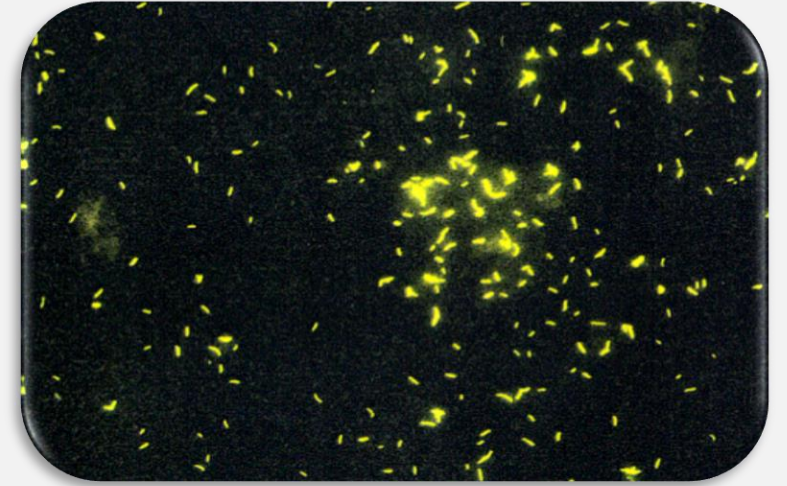
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- MDDR report 3/31/23
- **Mutations in *rpoB*, *katG*, and *embB***
- Preliminary conventional DST reported
- **Resistance to RMP, INH 1.0µg/mL, EMB, ETO, RBT**



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- Final report 4/12/23
- **Resistance to SM**



Isoniazid 0.2 mcg/ml by Agar Proportion

**Resistant**

Note: **MEDICAL EMERGENCY:** This patient is resistant to one or more drugs. Notify responsible physician and Infection Control prior to filing this report.

Rifampin 1.0 mcg/ml by Agar Proportion

**Resistant**

Pyrazinamide 100 mcg/ml by MGIT 960

Susceptible

Ethambutol 5.0 mcg/ml by Agar Proportion

**Resistant**

Isoniazid 1.0 mcg/ml by Agar Proportion

**Resistant**

Ethionamide 5.0 mcg/ml by Agar Proportion

**Resistant**

Streptomycin 2.0 mcg/ml by Agar Proportion

**Resistant**

Ofloxacin 2.0 mcg/ml by Agar Proportion

Susceptible

Rifabutin 2.0 mcg/ml by Agar Proportion

**Resistant**

Kanamycin 5.0 mcg/ml by Agar Proportion

Susceptible

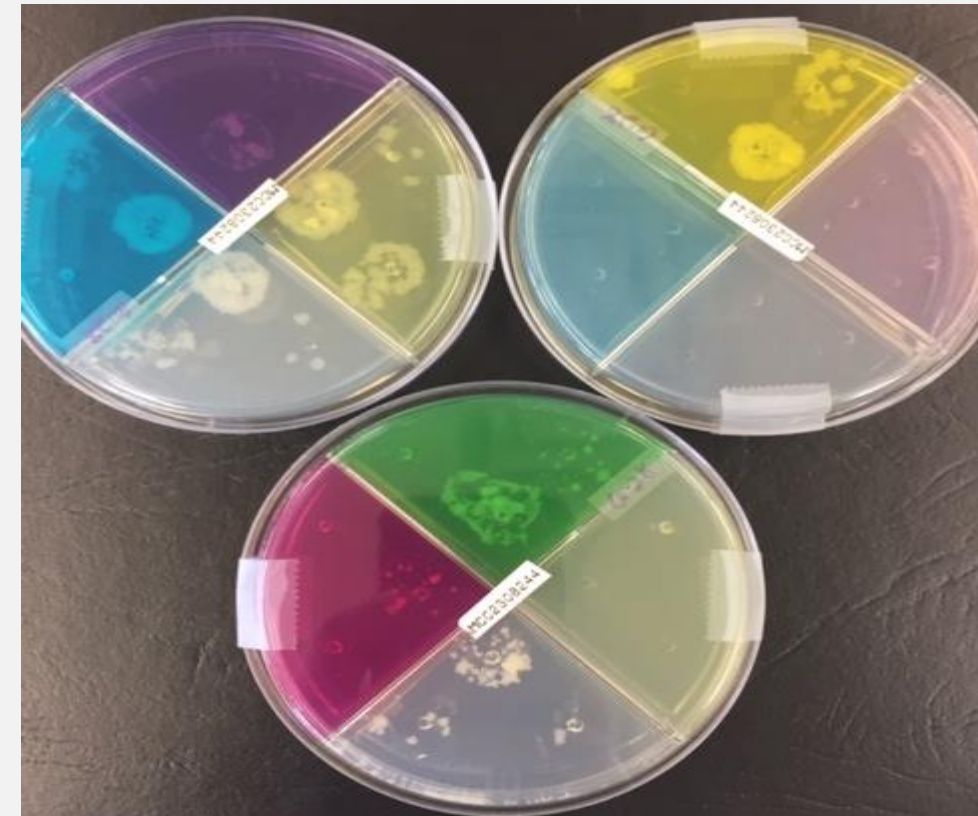
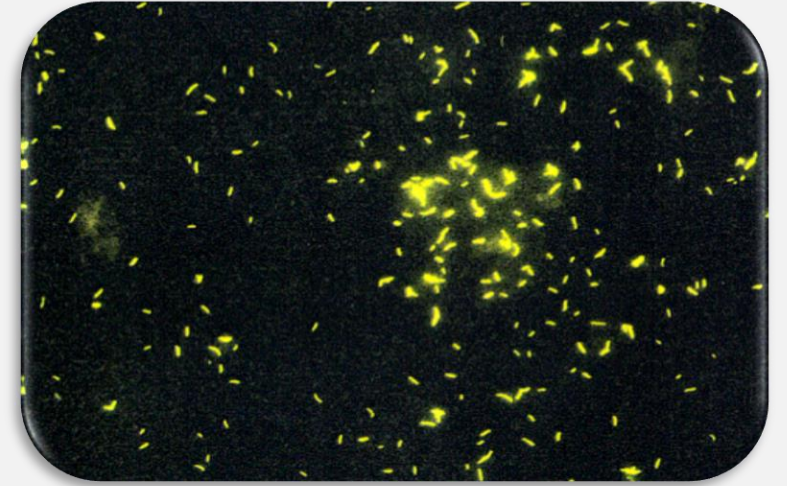
Capreomycin 10.0 mcg/ml by Agar Proportion

Susceptible



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- CDC final 4/27/23





Specimen ID: **AMCC2303733**

Alt. Specimen ID:

CDC Specimen ID: **3003783519** CDC Unique ID: **N8KHJYAT** CDC Local Aliquot ID: **23-2244**

<b>MTBC Agar Proportion Susceptibility*</b>	<b>% Resistant</b>	<b><u>Interpretation</u></b>
Isoniazid 0.2 µg/mL	100 %	Resistant
Isoniazid 1.0 µg/mL	100 %	Resistant
Isoniazid 5.0 µg/mL	0 %	Susceptible
Rifampin 1.0 µg/mL	100 %	Resistant
Ethambutol 5.0 µg/mL	33 %	Resistant
Streptomycin 2.0 µg/mL	67 %	Resistant
Streptomycin 10.0 µg/mL	0 %	Susceptible
Rifabutin 2.0 µg/mL	67 %	Resistant
Ciprofloxacin 2.0 µg/mL	0 %	Susceptible
Kanamycin 5.0 µg/mL	0 %	Susceptible
Ethionamide 10.0 µg/mL	33 %	Resistant
Capreomycin 10.0 µg/mL	0 %	Susceptible
PAS 2.0 µg/mL	0 %	Susceptible
Ofloxacin 2.0 µg/mL	0 %	Susceptible
Amikacin 4.0 µg/mL	0 %	Susceptible

**Comments and Disclaimers**

\* Susceptibility testing method: Indirect agar proportion, 7H10 medium. Resistance is defined as >1% (growth on drug-containing medium compared to drug-free medium).

This test has not been cleared or approved by the FDA. The performance characteristics have been established by the DTBE Reference Laboratory.

**MTBC Pyrazinamide Susceptibility\*****Result**

Pyrazinamide 100 µg/mL

Susceptible

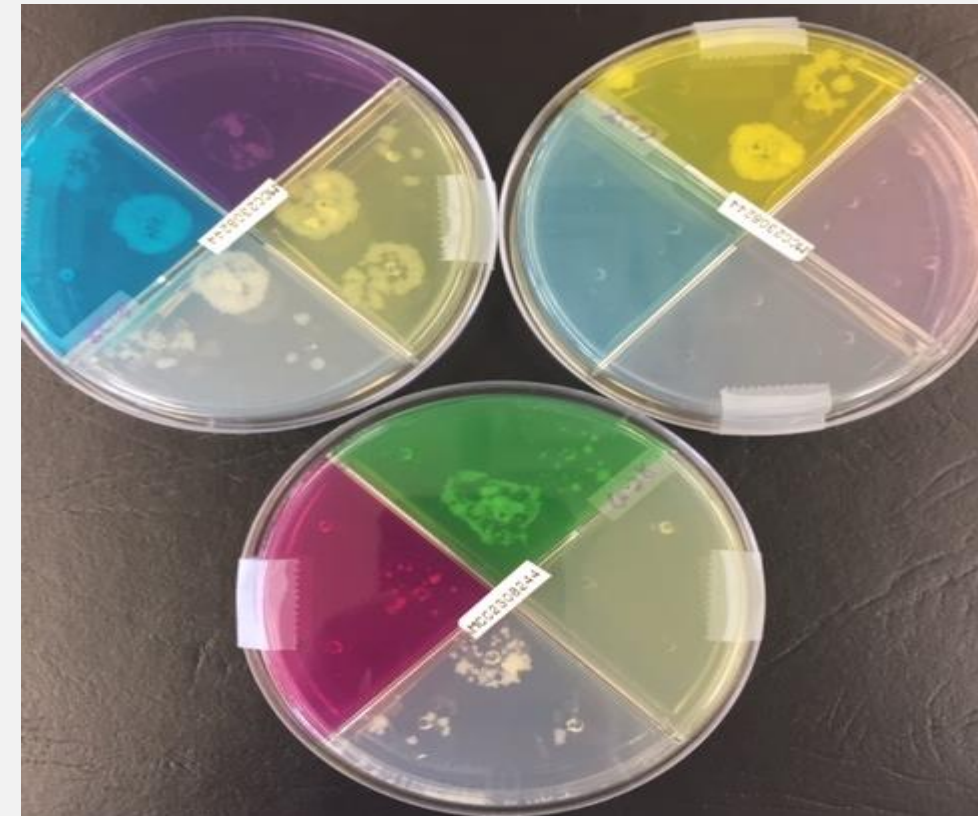
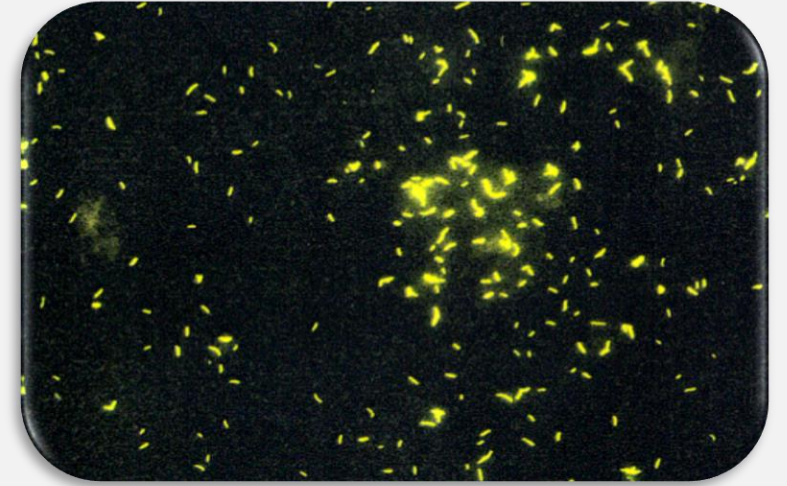
**Comments and Disclaimers**

\* Susceptibility testing method: Mycobacteria Growth Indicator Tube (MGIT)

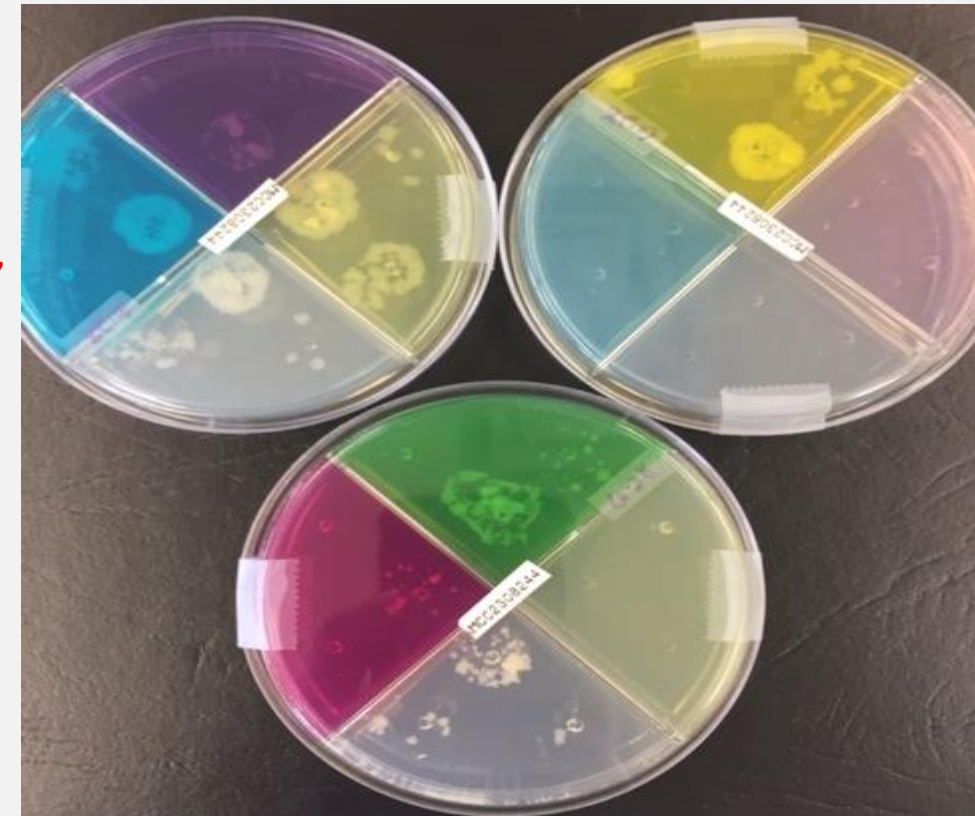
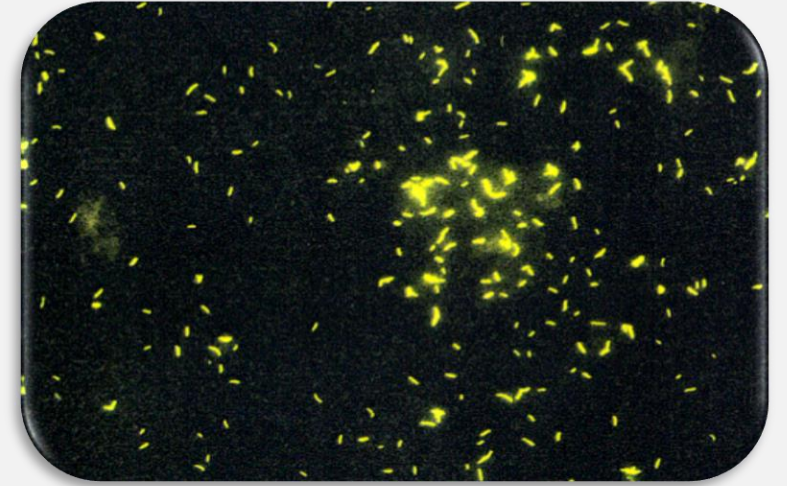


Texas Department of State  
Health Services

- Collection from a binational patient 3/13/23
- Received at lab 3/15/23
- Smear reported 3/15/23
- **>10/field**
- Xpert reported 3/16/23
- **Positive, Rifampin resistance detected**
- Sent for MDDR 3/16/23
- Culture positive 3/21/23
- MDDR report 3/31/23
- **Mutations in *rpoB*, *katG*, and *embB***
- Preliminary conventional DST reported
- **Resistance to RMP, INH 1.0µg/mL, EMB, ETO, RBT**
- Final report 4/12/23
- **Resistance to SM**
- CDC final 4/27/23
- **Agrees with DSHS**

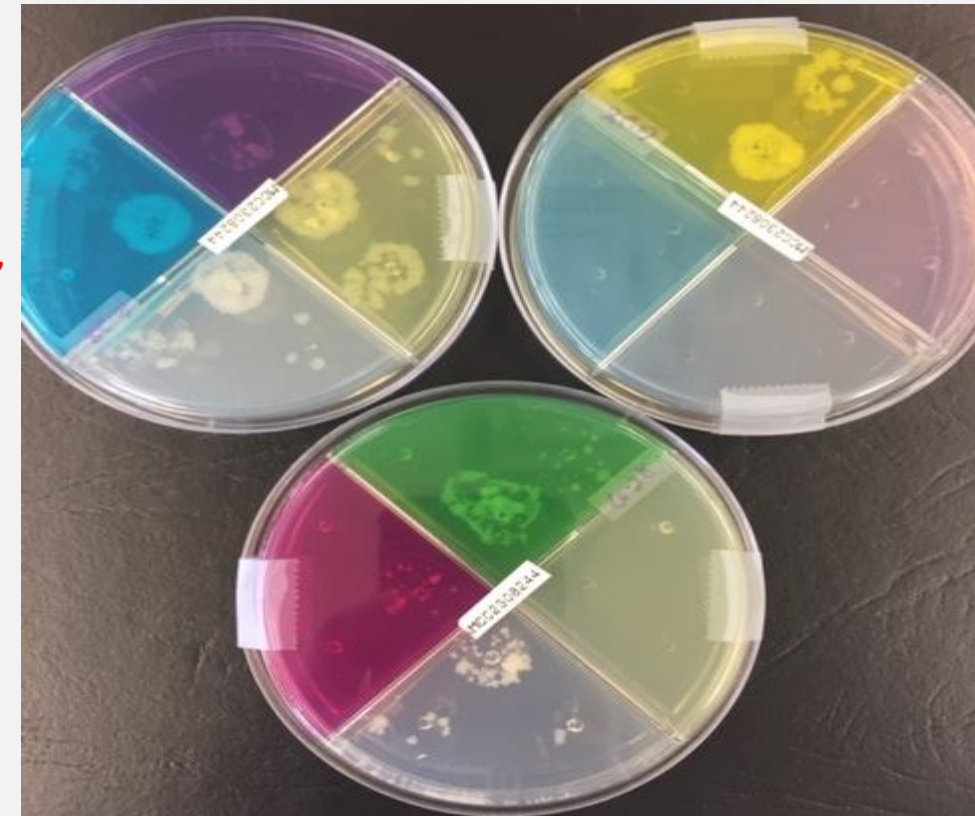
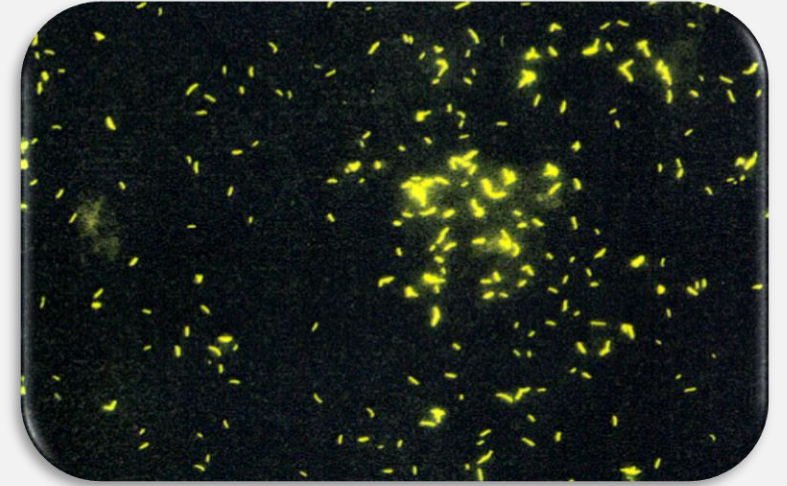


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- Repeat susceptibilities after 3 months of culture positive 6/27/23
- **No acquired additional resistance**



# Genotype Investigation

DST	Relation	Originating Lab	Date of Collection	Submitter Number	Date Received	wgMLSType	GENType	SpoligoType	MIRU	MIRU2
MDR	(current pt)	GRUPO SIN FRONTERAS BINATIONAL PROJ	03/13/2023	AMCC2303733	03/29/2023	MTBC002441				



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MDR	(current pt)	GRUPO SIN FRONTERAS BINATIONAL PROJ	03/13/2023	AMCC2303733	03/29/2023	MTBC002441				
MDR	(father)	SOUTH TEXAS LABORATORY	09/24/2012	AMRC1202584	10/31/2012		G11225	777776777760771	224325153314	333334213338
MDR	(brother-in-law)	GRUPO SIN FRONTERAS BINATIONAL PROJ	08/06/2018	AMRC1802226	10/03/2018	MTBC002441	G40790	777776777760771	224325133314	333334213338
pre-XDR	(b-in-law's sister)	GRUPO SIN FRONTERAS BINATIONAL PROJ	07/09/2022	AMCC2209108	08/04/2022	MTBC002441				

# Thank you!

## Laboratory Diagnosis of Tuberculosis

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