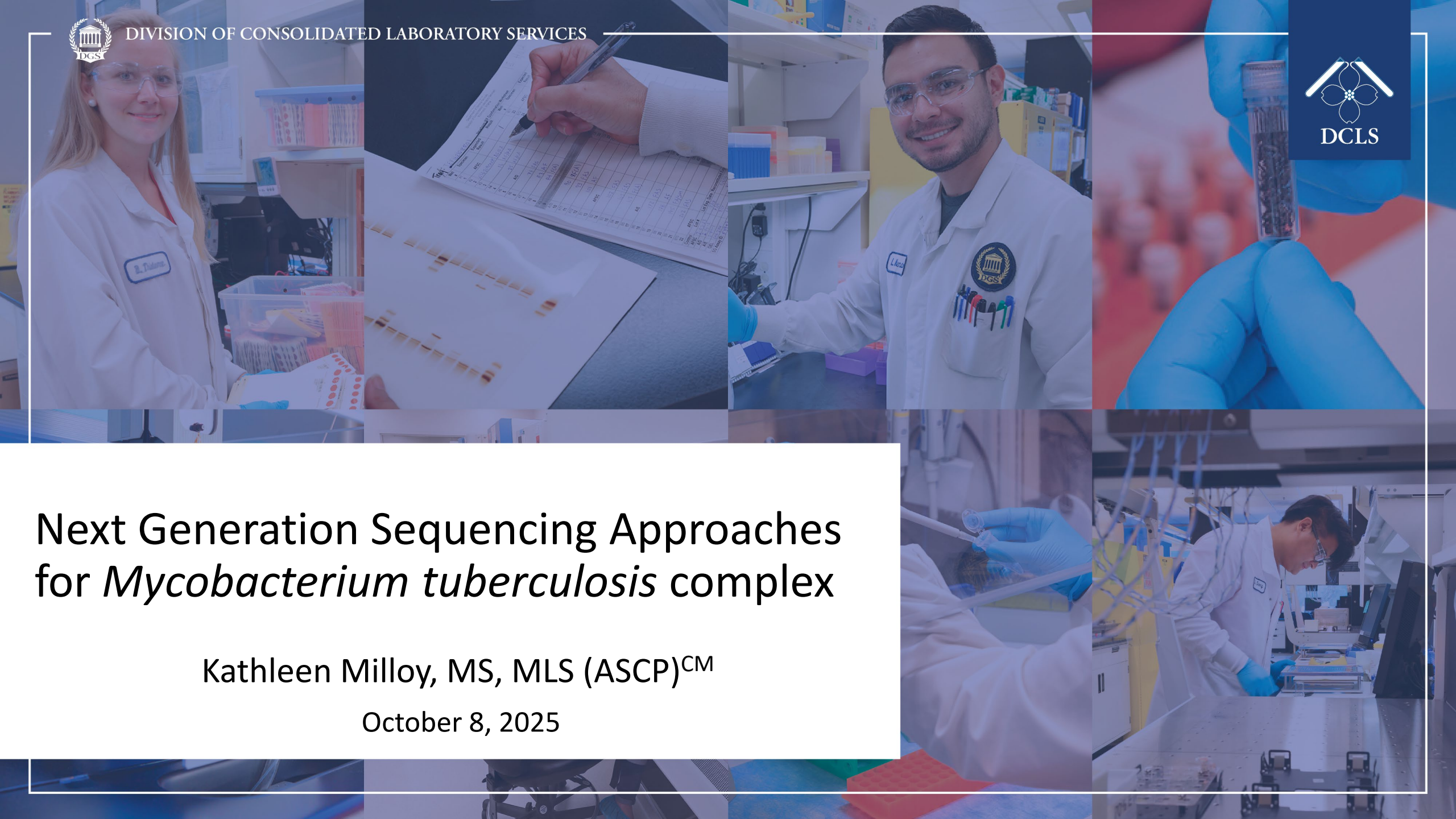

DIVISION OF CONSOLIDATED LABORATORY SERVICES

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Next Generation Sequencing Approaches for *Mycobacterium tuberculosis* complex

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Objectives

- Explain the utility of next generation sequencing (NGS) and the most common methods used for *Mycobacterium tuberculosis* complex (MTBC)
- Describe a NGS workflow for MTBC
- Provide an overview of drug resistant MTBC and susceptibility testing
- Discuss the WHO catalog of mutations
- Describe how targeted next generation sequencing (tNGS) is utilized to detect resistance markers in MTBC
- Provide information for Reference Laboratory susceptibility testing of MTBC
  - CDC MDDR service and Susceptibility Testing Reference Center
  - CA MDL National DST Reference Lab

Next Generation Sequencing (NGS)

- Large-scale, high-throughput DNA sequencing
- Generates a large amount of data that can be used for a variety of purposes

Detection of mutations that predict drug resistance and susceptibility	Identification of strains or species
Compare genotypes	High quality analysis to determine relatedness to other cases

Polling Question 1

What is the definition of a gene?

a) A specific sequence of DNA that contains the information for making a protein

b) An enzyme responsible for making nucleotides

c) A type of cell organelle that contains DNA

d) A random sequence of nucleotides in a chromosome

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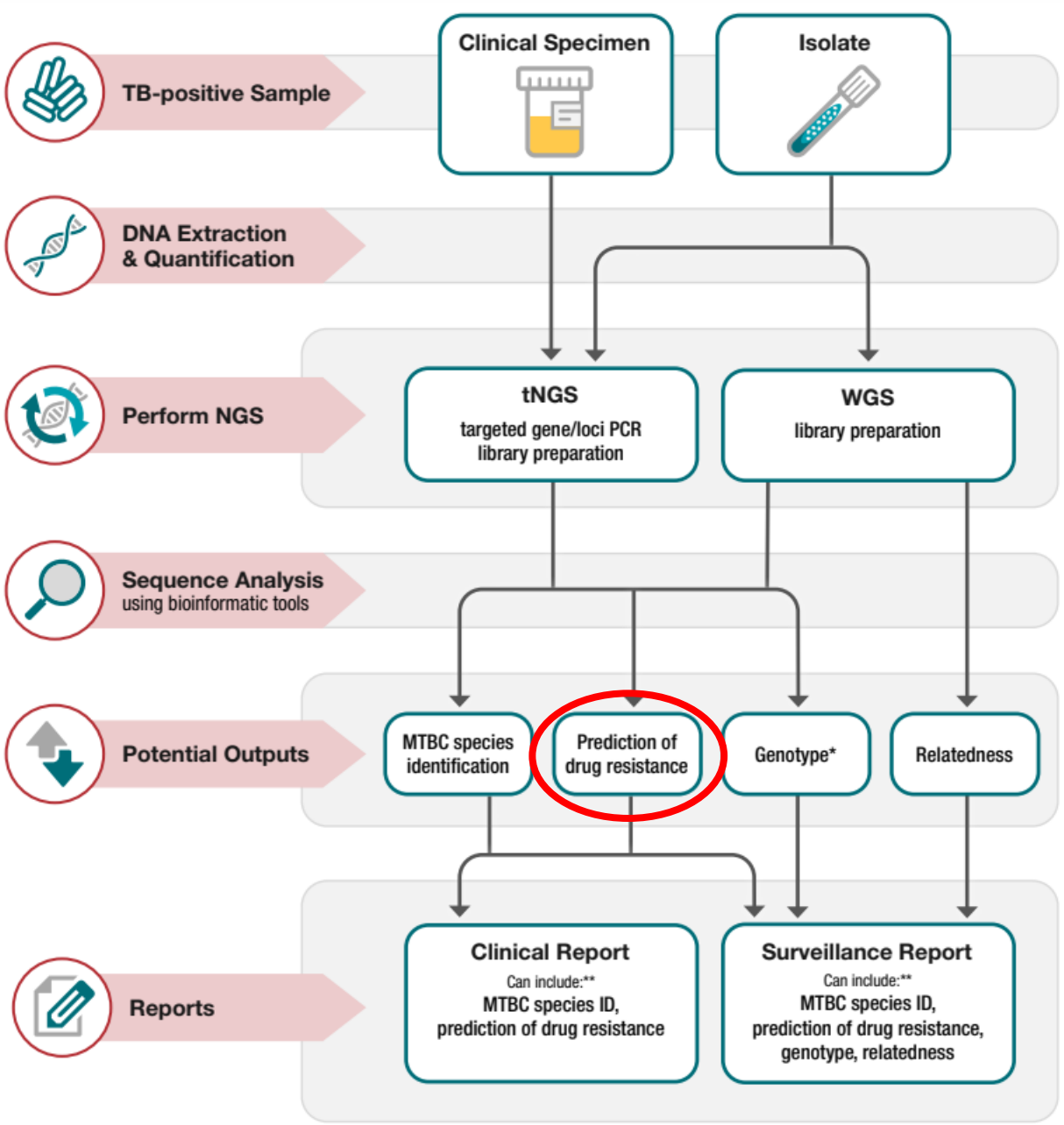
NGS Methods

Targeted NGS (tNGS)	Whole genome sequencing (WGS)
Amplicon-based NGS, to sequence specific parts of the genome (i.e. single gene or group of genes)	Sequence the entire genome of an organism
Useful for diagnostic purposes	Can be used for diagnostic and epidemiological/surveillance purposes
Can be used on primary specimens and isolates	Can be used on isolates

TB NGS Workflow



Reference: APHL TB NGS Fact Sheet

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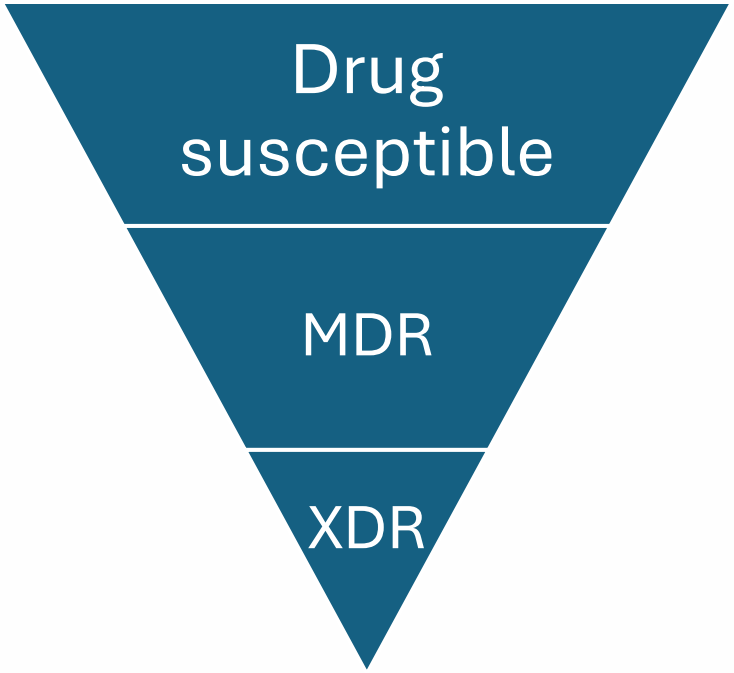
Drug Resistant *Mycobacterium tuberculosis* complex (MTBC)



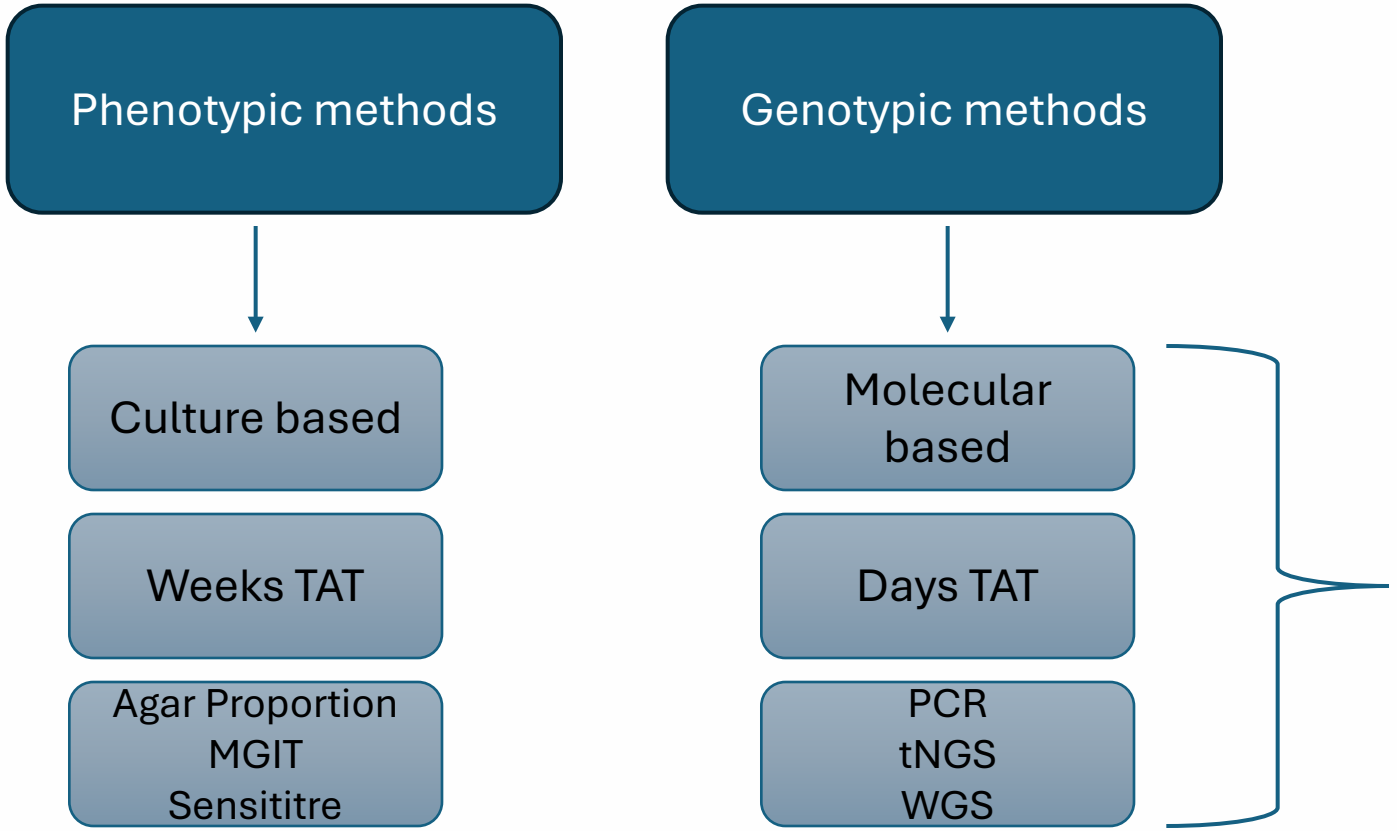
% Drug Resistance: United States (2023 CDC data)

INH Resistant	Multidrug-resistant (MDR)	Pre-extensively drug-resistant (Pre-XDR)
8.5%	1.4%	0.2%

% Drug Resistance Among TB cases: Virginia (2024 VDH data)

First-line Resistant	Multidrug-resistant (MDR)
9.8%	0%

Drug Susceptibility Testing (DST)



Benefits
Faster
More reliable (i.e. PZA)
Primary specimen (tNGS)
Mixed/contaminated cultures
Low-level/hetero-resistance

Genetic Terminology

Variant or Mutation

Alteration in the nucleotide sequence of an organism that may or may not have a phenotypic effect

Silent Mutation

Change to a nucleotide sequence that does not result in a change to the amino acid

Single Nucleotide Polymorphism

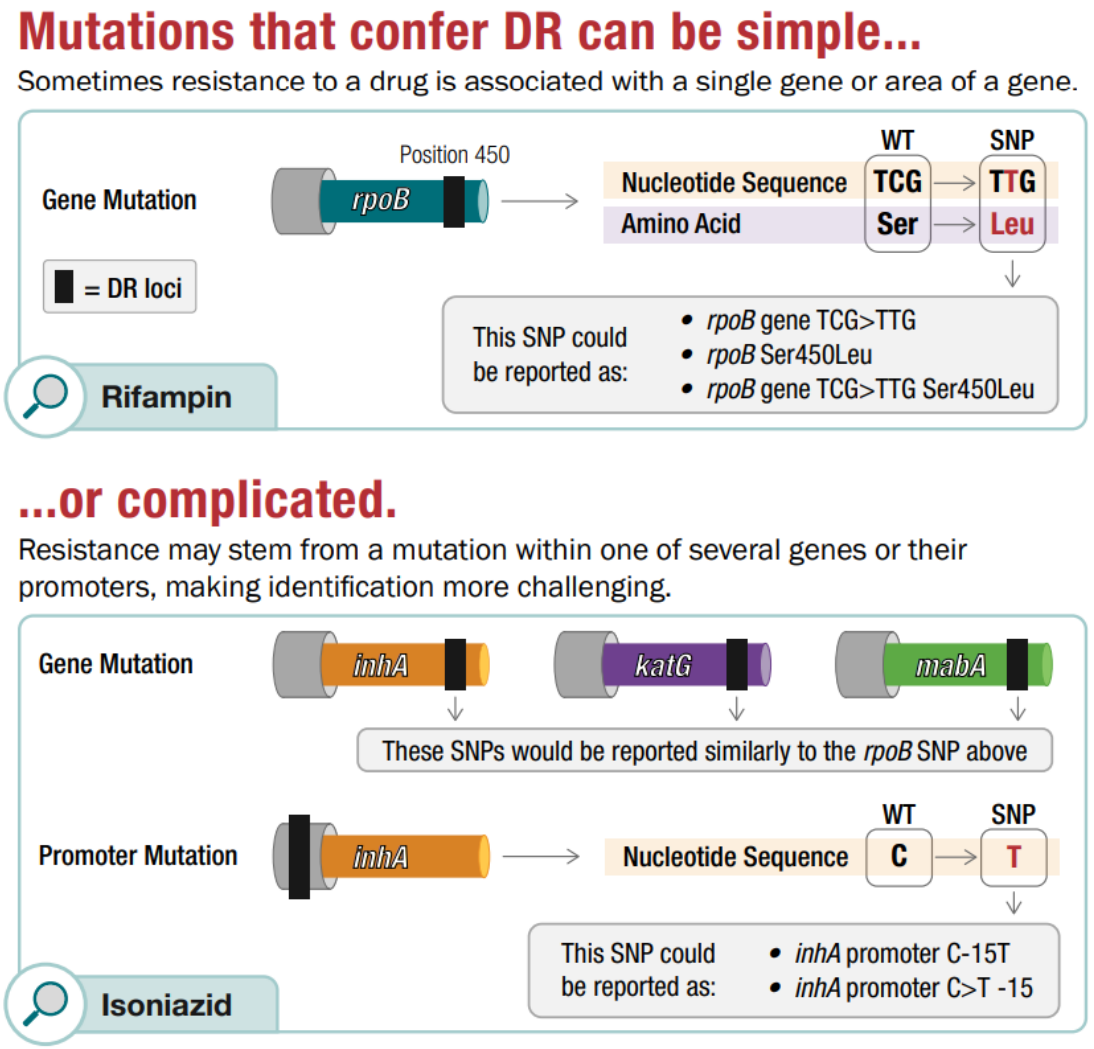
A variation at a single nucleotide position; the most common type of mutation causing drug resistance in MTB

Wild Type (WT)

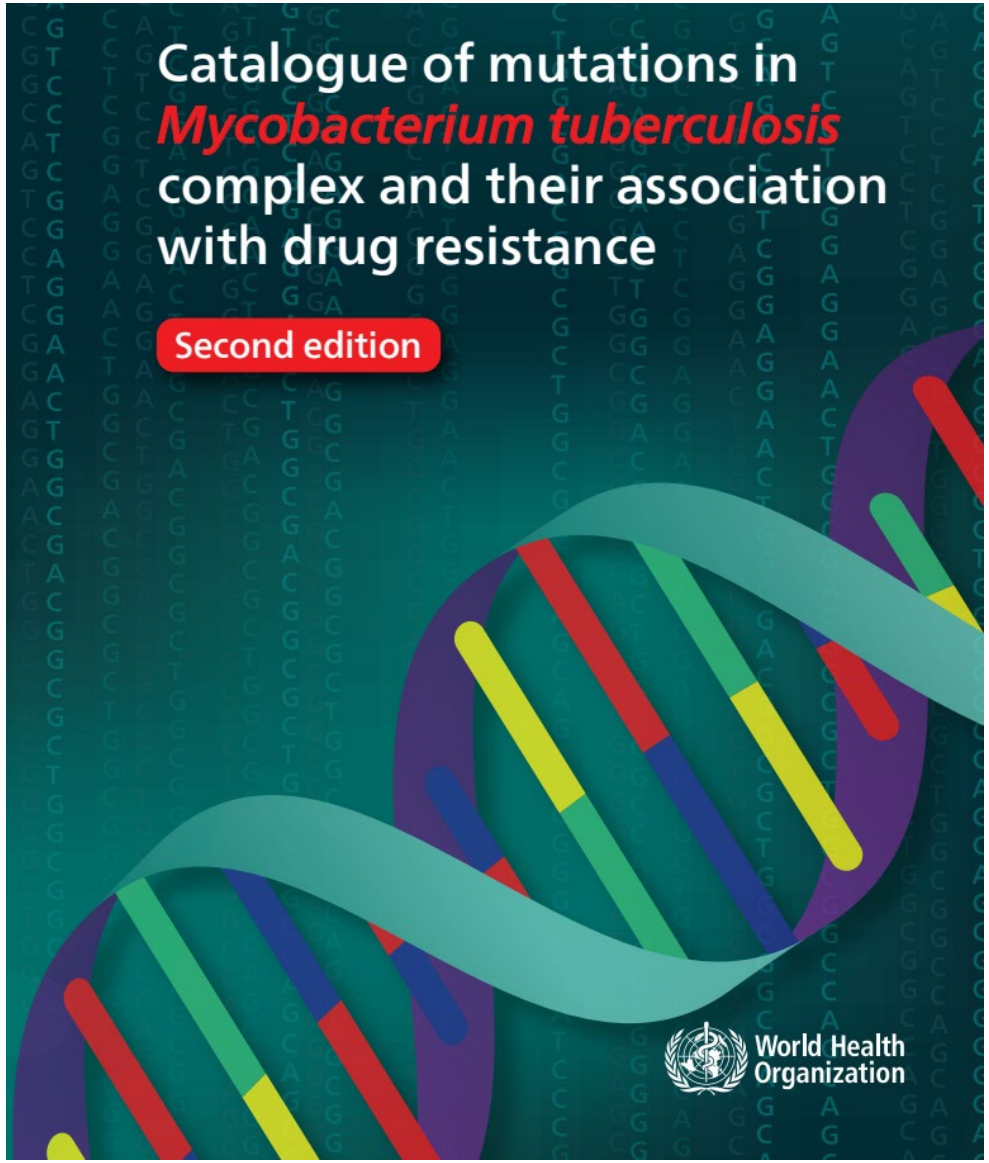
Standard genetic sequence to which mutations are compared (H37Rv commonly used WT (pan-susceptible) strain for MTB)

Molecular DST (mDST) for MTBC

- As NGS capabilities expand, laboratories are using mDST to predict MTBC drug resistance
- Laboratory availability varies based on many factors:
  - Incidence rate
  - Infrastructure
  - Expertise
  - Laboratory developed tests (LDTs)

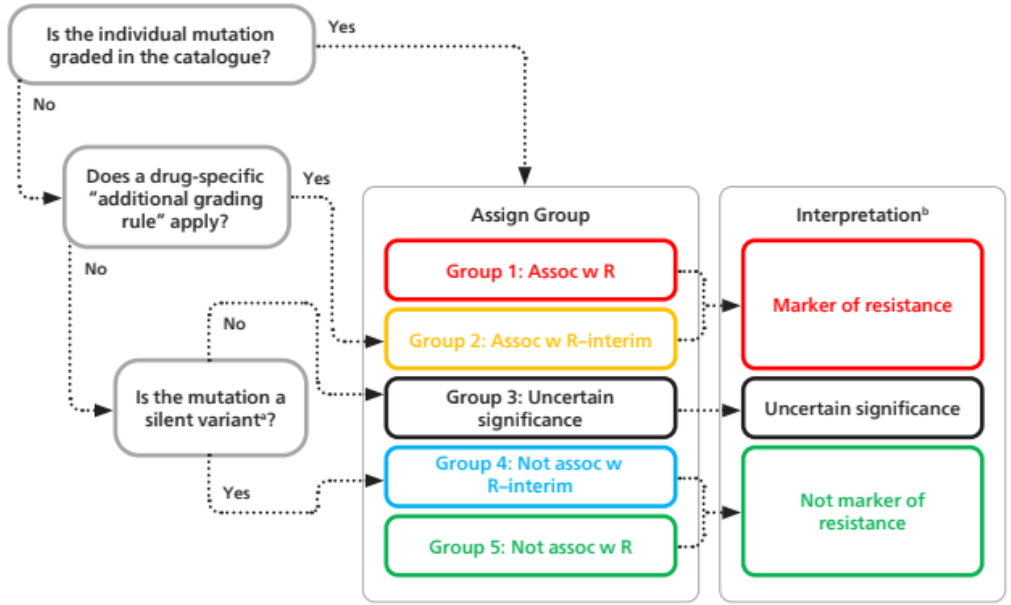


Reference: APHL NGS and Mutations



- Large database of WGS and phenotypic drug susceptibility testing data from analysis of global clinical isolates
- WHO recommends routine testing of all TB patients for rapid detection of resistance to rifampin and isoniazid

Fig. 1. Instructions for using the catalogue



https://www.who.int/publications/i/item/9789240082410

Table 3. Groups 1–5 mutations for the second edition and their performance for predicting phenotypic resistance

		Group 1: Assoc w R	Group 2: Assoc w R-interim	Group 3: Uncertain significance	Group 4: Not assoc w R-interim	Group 5: Not assoc w R
RIF	No. of variants identified	26	110	4484	2 (2568)	52 (32)
	Sens, spec, PPV (% [95% CI])	92.1 (91.7–92.5), 97.1 (96.9–97.3), 94.5 (94.2–94.9)	1.1 (1.0–1.3), 99.8 (99.8–99.9), 76.5 (70.8–81.6)			
	Combined performance	**93.3 (92.9–93.7), 96.9 (96.7–97.1), 94.2 (93.9–94.6)**				
INH	No. of variants identified	7	135	5404	11 (1671)	41 (16)
	Sens, spec, PPV (% [95% CI])	89.6 (89.2–90.0), 98.2 (98.1–98.4), 97.5 (97.2–97.7)	2.0 (1.8–2.2), 99.7 (99.6–99.7), 82.5 (78.9–85.7)			
	Combined performance	**91.6 (91.2–92.0), 97.9 (97.8–98.1), 97.1 (96.8–97.3)**				
EMB	No. of variants identified	13	0	4943	10 (2068)	50 (34)
	Sens, spec, PPV (% [95% CI])	81.1 (80.3–81.9), 91.6 (91.3–91.9), 71.9 (71.0–72.8)	0 (0–0), 100.0 (100.0–100.0), 0 (0–0)			
	Combined performance	**81.1 (80.3–81.9), 91.6 (91.3–91.9), 71.9 (71.0–72.8)**				
PZA	No. of variants identified	139	202	1465	20 (720)	17 (8)
	Sens, spec, PPV (% [95% CI])	63.5 (62.0–64.9), 98.6 (98.5–98.8), 92.4 (91.4–93.4)	14.6 (13.5–15.6), 99.2 (99.1–99.3), 82.9 (80.1–85.5)			
	Combined performance	**78.0 (76.8–79.2), 97.9 (97.6–98.1), 90.5 (89.5–91.4)**				
LFX	No. of variants identified	12	6	2016	2 (983)	19 (9)
	Sens, spec, PPV (% [95% CI])	83.6 (82.6–84.5), 97.3 (97.0–97.5), 89.2 (88.4–90.0)	1.2 (1.0–1.6), 99.6 (99.6–99.7), 48.3 (40.1–56.6)			
	Combined performance	**84.8 (83.9–85.7), 96.9 (96.7–97.1), 88.1 (87.3–89.0)**				

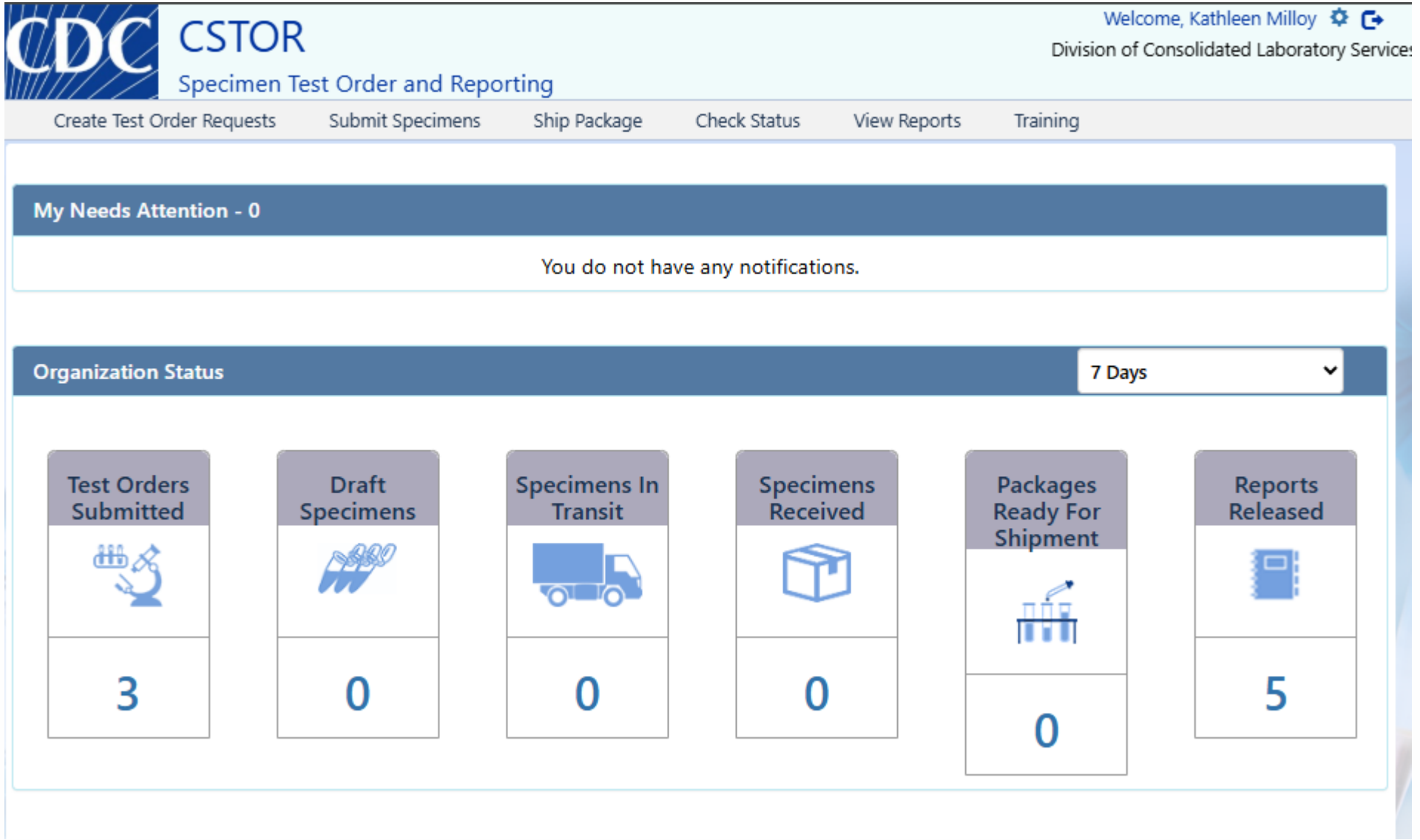
- Resistant
- Likely resistant

- Unknown
- Undetermined

- Susceptible
- Sensitive
- Likely susceptible

CDC Test Offering

CDC TB Laboratory	CDC Pathology
Molecular Detection of Drug-Resistance Service (MDDR)	Infectious disease testing for human tissues (when culture is not available)
Phenotypic DST (agar proportion, first and second-line)	Histopathology, histochemistry, immunohistochemistry, PCR and sequencing
Samples must be routed through state health department/laboratory or authorized facility Pre-approval required for testing	


CDC
CSTOR
Specimen Test Order and Reporting
Welcome, Kathleen Milloy
Division of Consolidated Laboratory Services
Create Test Order Requests
Submit Specimens
Ship Package
Check Status
View Reports
Training
My Needs Attention - 0
You do not have any notifications.
Organization Status
7 Days
Test Orders Submitted
3
Draft Specimens
0
Specimens In Transit
0
Specimens Received
0
Packages Ready For Shipment
0
Reports Released
5

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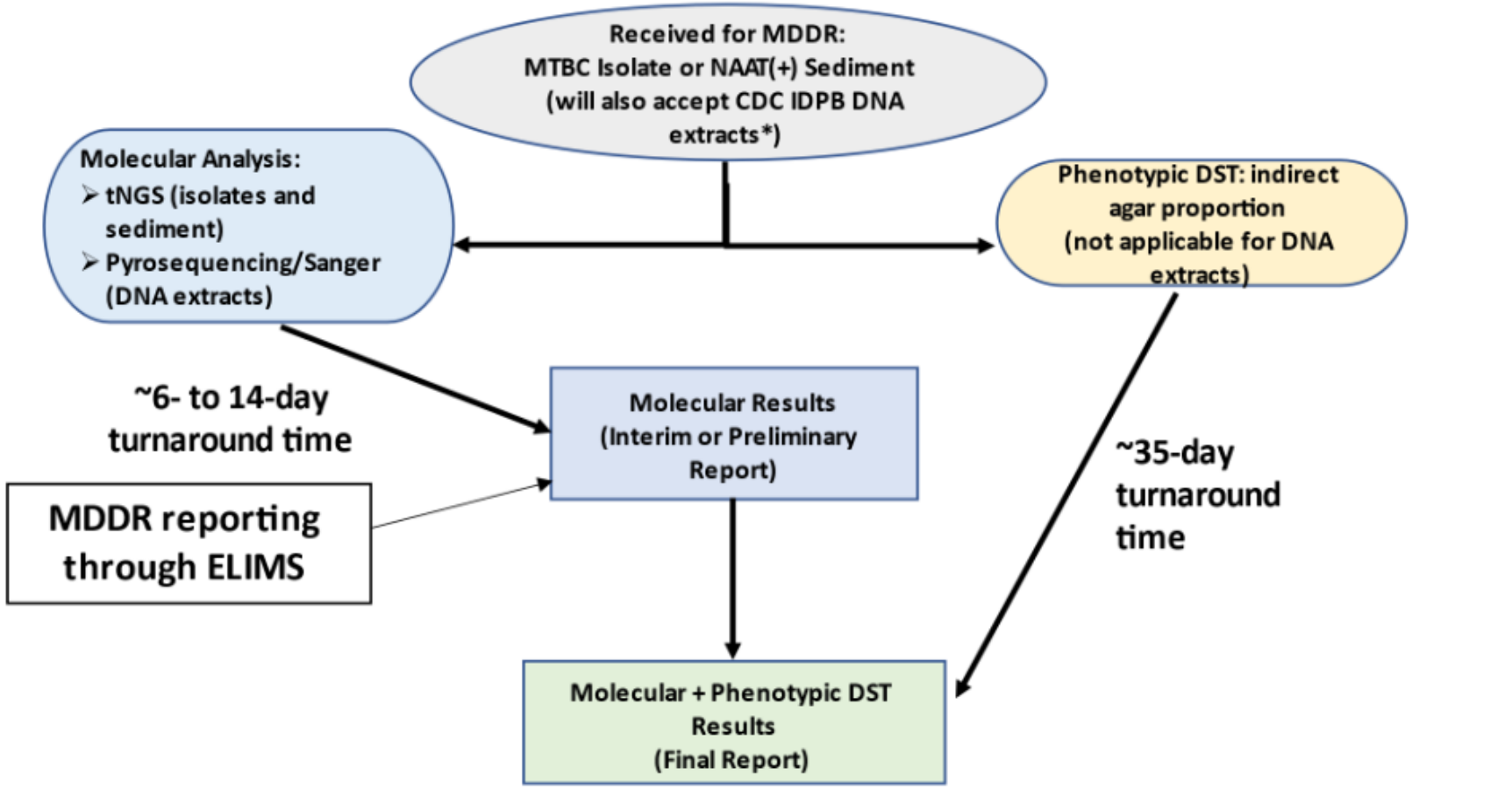
MDDR Testing at CDC

- Utilizes DNA sequencing for detection of mutations most frequently associated with resistance to first and second-line drugs as well as new/repurposed drugs such as bedaquiline, clofazimine, and linezolid
- tNGS assay that is CLIA compliant
  - DNA regions of interest are amplified by PCR
  - Sequencing libraries generated using Illumina Nextera XT Library Prep Kit
  - Libraries sequenced on Illumina MiniSeq platform
- All isolates (including those grown from sediments) will also undergo growth-based DST
- MDDR User Guide

MDDR Algorithm



*DNA extracts only accepted from CDC IDPB and will be tested by conventional sequencing methods (not tNGS)

MTBC *Mycobacterium tuberculosis* complex, IDPB: Infectious Diseases Pathology Branch, ELIMS: enterprise laboratory information management system

Reference: MDDR User Guide

Genetic Loci Examined in MDDR Assay

Drug	Genetic locus
Rifampin	*rpoB*
Isoniazid	*katG*, *fabG1- inhA*
Ethambutol	*embB*
Pyrazinamide	*pncA*
Fluoroquinolones	*gyrA*, *gyrB*
Amikacin Capreomycin Kanamycin	*rrs*
Kanamycin	*eis*
Bedaquiline	*AtpE*, *rv*0678, *pepQ*
Clofazimine	*Rv0678*, *pepQ*
Linezolid	*RplC*, *rrl*

Assistance with MDDR results interpretation:
Laboratorians, TB control personnel, and healthcare providers can contact the CDC by email (TBLab@cdc.gov) or telephone (404-639-2455) for help in interpretation of reports

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Patient Name: [redacted]
Sex: [redacted] Birthdate: [redacted] Age: [redacted] Date of Onset:

Public Health / International Submitter IDs

Patient ID: Alt. Patient ID:
Specimen ID: [redacted] Alt. Specimen ID:

CDC Specimen ID: [redacted] CDC Unique ID: [redacted] CDC Local Aliquot ID: [redacted]

Rifampin (RIF)	Result	Interpretation
RIF interpretation		RIF resistant
rpoB*	Ser450Leu	

Comments and Disclaimers
* DTBE Reference Laboratory has transitioned from the E. coli to the M. tuberculosis numbering system for reporting rpoB gene mutations.

Isoniazid (INH)	Result	Interpretation
INH interpretation		INH resistant
inhA	No mutation	
fabG1	No mutation	
katG	Partial amplification, Ser315Thr	

Ethambutol (EMB)	Result	Interpretation
EMB interpretation		Likely EMB resistant
embB	Met306Ile	

Pyrazinamide (PZA)	Result	Interpretation
PZA interpretation		Likely PZA resistant
pncA	Ile90Thr	

Fluoroquinolones (FQ)	Result	Interpretation
FQ interpretation		Cannot rule out FQ resistance.
gyrA	No mutation	
gyrB	No mutation	

Centers for Disease Control and Prevention
National Tuberculosis Reference Laboratory

Patient Name: [redacted]
Sex: **Female** Birthdate: [redacted] Age: [redacted] Date of Onset:

Public Health / International Submitter IDs

Patient ID: Alt. Patient ID:
Specimen ID: [redacted] Alt. Specimen ID:

CDC Specimen ID: [redacted] CDC Unique ID: [redacted] CDC Local Aliquot ID: [redacted]

MTBC Agar Proportion Susceptibility*	% Resistant	Interpretation
Isoniazid 0.2 µg/mL	100 %	Resistant
Isoniazid 1.0 µg/mL	100 %	Resistant
Isoniazid 5.0 µg/mL	100 %	Resistant
Rifampin 1.0 µg/mL	100 %	Resistant
Ethambutol 5.0 µg/mL	50 %	Resistant
Streptomycin 2.0 µg/mL	100 %	Resistant
Streptomycin 10.0 µg/mL	50 %	Resistant
Rifabutin 2.0 µg/mL	50 %	Resistant
Ciprofloxacin 2.0 µg/mL	0 %	Susceptible
Kanamycin 5.0 µg/mL	0 %	Susceptible
Ethionamide 10.0 µg/mL	100 %	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†	Not Tested

Comments and Disclaimers
† Test not done
This test order is unavailable until further notice.
* Susceptibility testing method: Mycobacteria Growth Indicator Tube (MGIT)

Rifamycins (RIF) & *rpoB* Gene

- Rifamycins include: rifampin (rifampicin), rifabutin and rifapentine.
- ~95% of DR mutations are found in the 81-basepair RIF Resistance Determining Region (RRDR).
- Although less common, mutations outside of the RRDR can confer DR.
- A subset of mutations confer low-level resistance and may not be detected by pDST.

***rpoB* Ser450Leu, His445Tyr, His445Asp, Asp435Val**

Isoniazid (INH) & *katG* Gene

- *katG* mutations are responsible for ~85% of observed INH DR.
- Most frequent *katG* mutation is Ser315Thr, though other *katG* variants also cause resistance.
- Mutations in the promoter and genes for *inhA* and *fabG1(mabA)* may also be associated with INH DR.
- Silent *fabG1(mabA)* mutation Leu203Leu is also know to be associated with INH DR.

***katG* Ser315Thr**

Ethionamide (ETH) & *inhA* and *ethA* Genes

- INH and ETH are structural analogs and cross-resistance is common.
- Mutations in *ethA* are known to be associated with ETH DR.
- *inhA* promoter mutation C-15T is often associated with low-level INH and ETH resistance.
- Mutations in *fabG1(mabA)* are also associated with ETH DR.

***inhA* C-15T**

Pyrazinamide (PZA) & *pncA* Gene/Promoter

~85% of *pncA* genetic variants are associated with phenotypic PZA DR.

All *M. bovis* (including BCG strain) have a *pncA* His57Asp mutation and are resistant to PZA

Ethambutol (EMB) & *embB* Gene

- SNPs in *embA*, *embC* and the embC-embA promoter region have also been associated with EMB DR.
- Not all EMB DR associated mutations are known and discordance between methods can be observed.

***embB* Met306Val**

Fluoroquinolone (FQ) & *gyrA* Gene

- FQs used for MTB treatment include moxifloxacin, levofloxacin and ofloxacin.
- Mutations within the quinolone-resistance-determining region (QRDR) of DNA gyrase subunit *gyrA* are most frequently linked to DR; mutations in the QRDR of *gyrB* can also be linked to DR.
- Heteroresistance is common and can cause discordant results.

***gyrA* Asp94Gly, Ala90Val**

Second Line Injectable Drugs & *rrs, eis* and *tlyA* Genes

Second line injectable drugs include amikacin, kanamycin and capreomycin.

***rrs* A1401G**

New TB Drugs

The following drugs and genes have known DR associations:

- **Bedaquiline** & **Rv0678** and ***atpE***
- **Linezolid** & ***atpE, rplC*** and ***rrl***
- **Delamanid** and **pretomanid** & ***fgd1, ddn, fbiA, fbiB, fbiC*** and ***fbiD***
- **Clofazimine** & ***pepQ, Rv0678, mmpL5*** and ***mmp***

Note that newer drugs have less robust DR data, so continued tracking is essential.

*For more information, see WHO Catalogue of mutations in *Mycobacterium tuberculosis* complex and their association with drug resistance, 2nd edition.

Reference: APHL NGS and Mutations

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Polling Question 2

Which of the following genetic loci is used for determining rifampin resistance?

a) *pncA*

b) *katG*

c) *rpoB*

D) *inhA*

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National Public Health Laboratory DST Reference Center for *Mycobacterium tuberculosis*

- Provides DST for eligible US PHL with low volumes of testing
- Performed at California Department of Public Health's Microbial Diseases Laboratory (MDL)
  - First-line DST using MGIT (rifampin, isoniazid, ethambutol, moxifloxacin)
  - Pyrazinamide DST using MGIT when a mutation with uncertain significance is detected via molecular methods and PZA resistance cannot be ruled out
  - Second-Line DST using MGIT (ethionamide, capreomycin, amikacin, moxifloxacin, kanamycin and rifabutin)
  - Molecular detection of drug resistance performed by WGS on submitted isolates and by tNGS on specimen sediments, mixed and non-viable cultures

MDL WGS and tNGS DST Interpretation Summary

Mutation Interpretation			Individual mutation reporting		Drug interpretation reporting
			Format of individual mutations reported per gene locus	Mutation is listed in clinical report (Yes/No)	Overall value based on the highest severity mutation within all targets associated with given drug
R	R-mutations for all targets, except for *rpoB* (see below)		c.2066C>T (p.Ala689Val)	Yes	Mutation(s) associated with resistance to XXX detected
	R mutations in *rpoB* gene	Low-level RIF R mutations	c.2066C>T (p.Ala689Val)	Yes	Predicted low-level resistance to rifampin. May test susceptible by phenotypic methods
		Other RIF R mutations	c.2066C>T (p.Ala689Val)	Yes	Predicted resistance to rifampin
U			c.2066C>T (p.Ala689Val)	Yes	The detected mutation(s) have uncertain significance. Resistance to XXX cannot be ruled out
S	S-mutations for all targets, except for *rpoB* (see below)		No high confidence mutations detected	No	No mutations associated with resistance to XXX detected
	S mutations in *rpoB* gene	Synonymous mutation present within *rpoB* RRDR (codons 426-452)	c.2066C>T (p.Ala689Ala) [synonymous] [1]	Yes	Predicted susceptibility to rifampin. The detected synonymous mutation(s) do not confer resistance [2] Additionally displayed in comments: The detected in *rpoB* synonymous mutation may result in false-resistance in PCR-based assays targeting the *rpoB* RRDR
		Other RIF S mutations (outside of RRDR)	No high confidence mutations detected	No	Predicted susceptibility to rifampin [2]
WT			No mutations detected	N/A	No mutations associated with resistance to XXX detected
Insufficient Coverage in the gene locus[3] AND Successfully sequenced areas of the same gene OR other genes associated with given drug do NOT contain mutations interpreted as R			No sequence[4]	N/A	Not all targets could be sequenced; resistance to XXX cannot be ruled out[5]

Reference: National PHL DST Reference Center Reporting Language and Bioinformatic Pipeline

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National TB Molecular Surveillance Center

- CDC contracts with Michigan laboratory to provide genotyping services
- Provides support for epidemiologic investigations and surveillance activities
- Performs WGS to distinguish different strains of *M. tuberculosis*
  - Helps distinguish between persons whose TB disease is the result of TB infection that was acquired in the past, compared to recently or newly acquired.
  - Add value to conventional contact investigations
  - Offers a tool to help direct TB prevention and control efforts to preventing transmission
  - Allows monitoring of our progress toward eliminating TB transmission
- State PHL submits index isolate for each patient (1 per year)
- TB Genotyping Information Management System

WGS Analysis

Type of Analysis	Purpose
Whole-genome multilocus sequence typing (wgMLST)	• Analyzes 2,672 gene/loci and assigns a number for each. • Isolates that match at ≥99.7% of loci form a genotype cluster with a wgMLSType name
Whole-genome single nucleotide polymorphism (wgSNP) comparison	• Identify SNPs that distinguish isolates in a genotype-matched cluster. • Number of SNPs that differ between isolates can provide information about recent transmission. • SNPs used to map phylogenetic tree
Detection of possible drug resistance	• Detect mutations associated with drug resistance for surveillance purposes

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Contact

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