Transition of the Molecular Detection of Drug Resistance (MDDR) Service to Use of Next Generation Sequencing

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Advanced TB Topics Webinar: Next-Generation Diagnostics

November 7, 2022

Molecular Detection of Drug Resistance (MDDR) service at CDC since 2009

- **Clinical testing for MDDR**
  - Rapid detection of drug resistant tuberculosis (TB) by DNA sequencing
    - Pyrosequencing for isoniazid (INH) and rifampin (RIF) / Sanger sequencing for 1st and 2nd line drugs
    - Phenotypic drug susceptibility testing (DST) in parallel
  - Available to all 50 states, U.S. territories, and U.S. Affiliated Pacific Islands
  - Testing service is free of charge and shipping costs are covered by FedEx account managed by Association of Public Health Laboratories (APHL)
  - Clinical consultation regarding test results available

https://www.cdc.gov/tb/topic/laboratory/mddrusersguide.pdf

Acceptable sample types and turnaround time

- Confirmed *Mycobacterium tuberculosis* complex (MTBC) isolates or mixed and non-viable MTBC cultures
- MTBC nucleic acid amplification test positive (NAAT+) processed sediments
- Fixed-tissue DNA extracts (through the CDC Infectious Diseases Pathology Branch)
- In 2021: 912 received (21% NAAT+ sediments)
- Mean turnaround time (TAT) from sample receipt: 4 days

Why transition to Next Generation Sequencing (NGS)?

- **NGS / targeted NGS (tNGS) / Whole Genome Sequencing (WGS) ??**

  -NGS = platform, tNGS and WGS = assays that use NGS

- **WGS in future plans, but initial focus on tNGS for testing both sediments and isolates**
- **Reasons for the transition:**
  - Increase the output per run
  - Expand current genetic loci and add new TB drugs (option to expand further)
  - Identification of novel mutations
  - Better detection of heteroresistance
  - Use of bioinformatic pipeline for data analysis (limiting human error)
  - Discontinuation of current methods (96-well PSQ)

tNGS for MDDR service

- **Deep sequencing on the MiniSeq platform (Illumina)* of PCR-amplified drug resistance loci**
- **Suitable for both sediments and isolates**
  - Can accommodate up to 10 clinical patient samples + 2 controls/run
  - Sequences 13,747/ 4,411,709 bp (0.31% of the genome)
  - Loci contained in 16 genes; info about 12+ TB drugs

MiniSeq instrument

Flow cell & reaction cartridge

*Use of trade names and commercial sources is for identification only and does not imply endorsement by the U.S. Department of Health and Human Services

tNGS steps – wet lab (1)

- Heat-kill inactivation of the sample (crude DNA prep)
- 24 PCR reactions/sample = 288 PCR reactions/run (10 samples + 2 controls)
- Amplicon pooling
- Library preparation (purification, normalization, addition of index primers, etc.)
- MiniSeq sample preparation (libraries pooled and loaded into a cartridge)
- MiniSeq instrument run (20h)

tNGS steps – dry lab: bioinformatics pipeline

- ***fastQ* data files retrieved from instrument and fed into the pipeline**
  - Sequences: decontaminated, trimmed, aligned to type strain MTBC H37Rv (ATCC 27294), variants called
  - *vcf* (*variant call format*) files
  - Coverage data

Sample ID	CHROM	POS	REF	ALT	Read Depth	cent Alt al	Annotation	Variant Type	Nt Change	Position w/in CDS	AA change	REF AA	ALT AA	Codon	Gene	Gene ID
2020-XXXX	NC_000962	7362	G	C	2757	100	Non-synonymous	SNP	c.61G>C	61	p.Glu21Gln	Glu	Gln	21	gyrA	Rv0006
2020-XXXX	NC_000962	7585	G	C	3733	100	Non-synonymous	SNP	c.284G>C	284	p.Ser95Thr	Ser	Thr	95	gyrA	Rv0006
2020-XXXX	NC_000962	761155	C	T	4591	99.7	Non-synonymous	SNP	c.1349C>T	1349	p.Ser450Leu	Ser	Leu	450	rpoB	Rv0667
2020-XXXX	NC_000962	2154724	C	A	4850	100	Non-synonymous	SNP	c.1388G>T	1388	p.Arg463Leu	Arg	Leu	463	katG	Rv1908c
2020-XXXX	NC_000962	2155168	C	G	4493	99.7	Non-synonymous	SNP	c.944G>C	944	p.Ser315Thr	Ser	Thr	315	katG	Rv1908c
2020-XXXX	NC_000962	4247730	G	A	3933	99.9	Non-synonymous	SNP	c.1217G>A	1217	p.Gly406Asp	Gly	Asp	406	embB	Rv3795

Annotated *vcf* (example)

tNGS sequencing panel

- **Panel expanded to 24 amplicons,** includes bedaquiline and linezolid loci
- **Isoniazid:** sequencing now the entire *katG* gene
- **Linezolid:** *rplC, rrl*
- **Bedaquiline:** *atpE*, *rv0678* (*mmpR*), *pepQ*
- ***tlyA*** for capreomycin resistance not included in tNGS panel

	SANGER
1	*rpoB*-RRDR
2	*inhA*
3	*katG*
4	*gyrA*
5	*rrs*
6	*pncA*
7	*embB*
8	*eis*
9	*tlyA-1*
10	*tlyA-2*
11	*rpoB-170*
12	*gyrB*
13	*ahpC*
14	*fabG-609*

BEFORE

	tNGS
1	*rpoB*-RRDR
2	*rpoB-170*
3	*katG-1*
4	*katG-2*
5	*katG-3*
6	*katG-4*
7	*inhA*
8	*fabG-609*
9	*pncA*
10	*embB*
11	*gyrA*
12	*gyrB*
13	*rrs*
14	*eis*
15	*rv0678*
16	*atpE*
17	*pepQ-1*
18	*pepQ-2*
19	*pepQ-3*
20	*ahpC*
21	*rplC-1*
22	*rplC-2*
23	*rrl-1*
24	*rrl-2*

AFTER

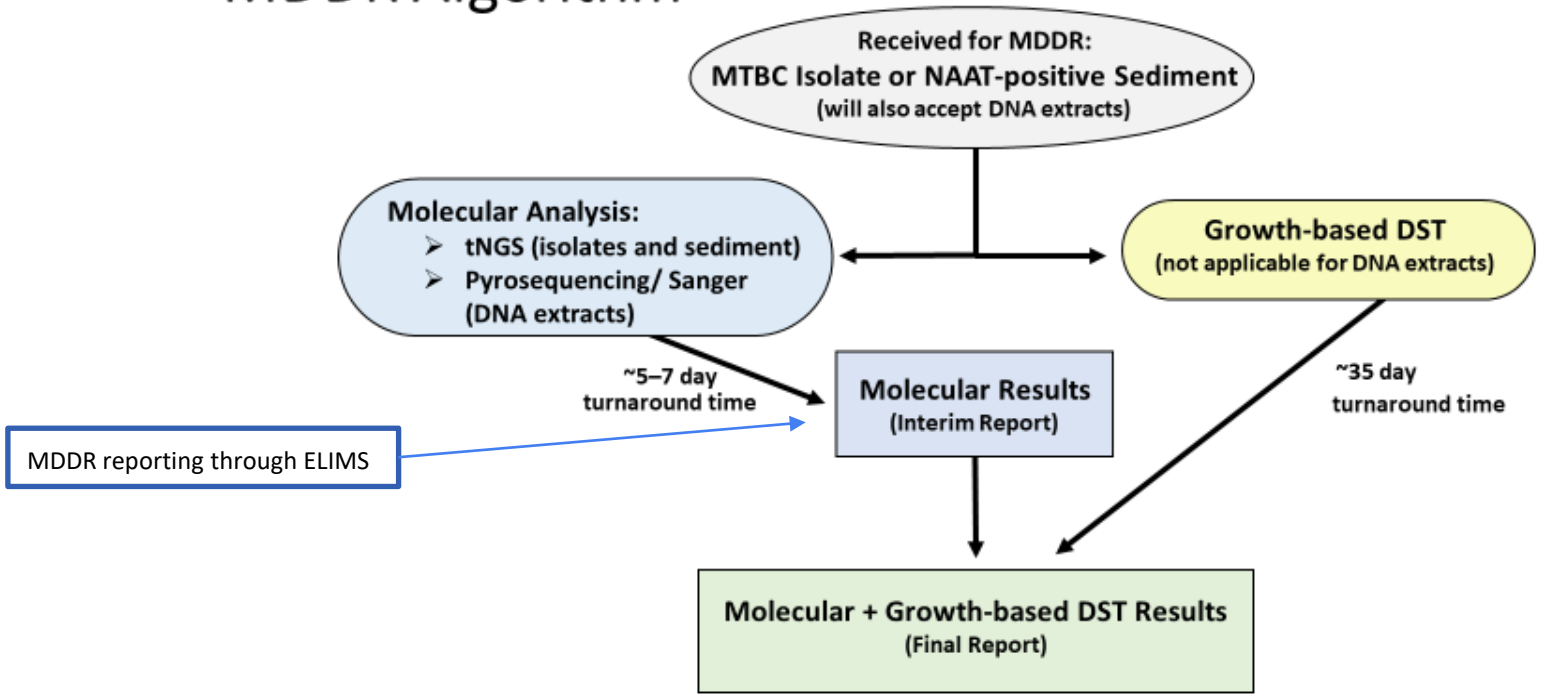
Added
Discontinued

Rubric of reporting rules

- **The minimum reportable alternate allele frequency threshold for the analytic pipeline results is 10%.**

Drug	Genetic locus	Upstream region[1]	Nucleotide position in rRNA[2]	Codons[3]	Rubric for Laboratory Reporting[4]
Rifampin	*rpoB* RRDR			Gly426 to Leu452	All mutations in the RRDR and codons 170 and 491 reported
	rpoB codon 170			Val170	
	rpoB codon 491			Ile491	
Isoniazid	*katG*			Val1 to 741*	All mutations except lineage markers and synonymous mutations at positions other than codon 1
	fabG1-inhA upstream	-140 to -1			All mutations upstream of *fabG1* start codon and mutations at codon 203 only
	fabG1 codon 203			Leu203	
Ethambutol	*embB* (partial)			Thr277 to Thr437	All mutations except lineage markers and synonymous mutations
Pyrazinamide	*pncA*	-40 to -1		Met1 to 187*	All mutations upstream of the *pncA* start codon and all mutations in the open reading frame[5] except synonymous mutations
Fluoroquinolones	*gyrA* QRDR			Gly88 to Asp94	All mutations at codons 88 to 94 except synonymous mutations
	gyrB			Arg446 to Gly537	All mutations except synonymous mutations
Amikacin Capreomycin Kanamycin	*rrs* (partial)		1177 to 1537		Mutations at nucleotides 1401, 1402, and 1484 only
Kanamycin	*eis*_upstream	-127 to -1			All mutations
Bedaquiline Clofazimine	*rv0678*	-84 to -1		Val1 to 166*	All mutations upstream of the *rv0678* start codon and non-synonymous mutations in the open reading frame[5]
	pepQ	-33 to -1		Val1 to 373*	All mutations upstream of the *pepQ* start codon and non-synonymous mutations in the open reading frame[5]
Bedaquiline	*atpE*	-48 to -1		Met1 to 82*	All mutations upstream of the *atpE* start codon and non-synonymous mutations in the open reading frame[5]
Linezolid	*rplC*	-18 to -1		Met1 to 217*	All mutations upstream of the *rplC* start codon and non-synonymous mutations in the open reading frame[5]
	rrl (partial)		2003 to 2367 and 2449 to 3056		All mutations

MDDR Algorithm



*DNA extracts only accepted from CDC IDPB

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The Next Generation Sequencing Quality Initiative

The Next Generation Sequencing (NGS) Quality Initiative is developing an NGS-focused quality management system (QMS) to address the many challenges public health and clinical laboratories encounter when they develop and implement NGS-based tests, by providing ready-to-implement guidance documents, customizable standard operating procedures, and other tools. The project is funded by CDC's Office of Advanced Molecular Detection, and is co-led by the Division of Laboratory Systems, the Office of the Deputy Director for Infectious Diseases, and the Association of Public Health Laboratories.

Get the tools and resources that will support your QMS, learn about the initiative, meet our partners, and find additional NGS materials. Read our feature story about new NGS tools released in 2021.

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Contact Us

For more information about the NGS Quality Initiative, contact us at NGSQuality@cdc.gov.

Page last reviewed: March 1, 2022
Content source: Division of Laboratory Systems (DLS)

https://www.cdc.gov/labquality/ngs-quality-initiative.html

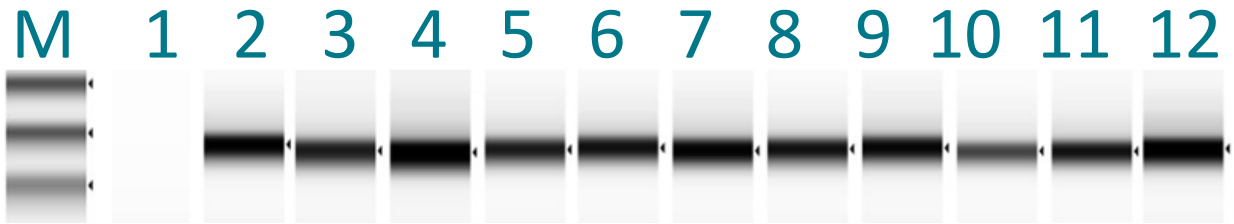
tNGS Validation process for MDDR

- **Objectives: To determine if tNGS….**
  - is specific for MTBC
  - generates comparable results to the current method (Sanger sequencing)
  - can detect all types of MTBC genome mutations (substitutions, insertions, deletions)
  - results are reproducible
  - can detect mutations in the new loci

tNGS specificity

- Nine (9) non-tuberculous mycobacteria (NTM) pure cultures
- +BCG, +MTBC
- All used as templates for the tNGS panel

	TEMPLATES	ATCC #
1	NTC: H_2O	-
2	MTBC H37Rv	ATCC 27294
3	*M. intracellulare*	ATCC 13950
4	BCG Pasteur	ATCC 35734
5	*M. kansasii*	ATCC 35778
6	*M. avium*	ATCC 35713
7	*M. abscessus*	ATCC 35751
8	*M. fortuitum*	ATCC 35754
9	*M. fortuitum*	ATCC 6841
10	*M. gordonae*	ATCC 14470
11	*M. kansasii*	ATCC 12478
12	*M. chelonae*	ATCC 19977



16S rRNA PCRs

tNGS amplicons specificity: results

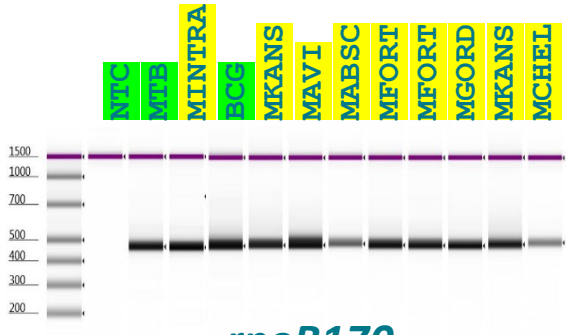
- **New amplicons highly specific to MTBC:**

  katG-1, *katG*-2 (Ser315), *katG*-4, *atpE*

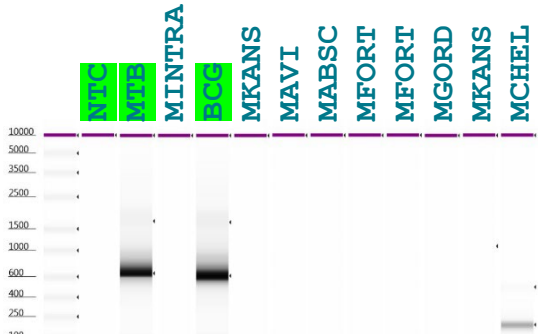
- **Old amplicons highly specific to MTBC:**

  eis, gyrA, gyrB, ahpC

- **Least specific:** *rpoB* Val170
- **NTM sequences in the loci/regions of interest are significantly different**
  - *(large number of mutations compared to MTBC)*
- **Pipeline has a decontamination step**
- **MDDR service does not accept pure NTM cultures**



rpoB170



katG-4

tNGS vs Sanger sequencing (SS)

- **tNGS – SS sample set for comparison of results**
  - 106 samples submitted for MDDR
  - 11 WHO isolates with WGS results
- **Validation sample set**

	Samples (origin, type)	n	%
	Retrospective	72	67.9
	Prospective	34	32.1
	Isolates	84	79.2
	Sediments	22	20.8
	Total	106	100

- **For reportable mutations within the directly comparable regions (12 genetic loci), results matched >99% → tNGS is as good as Sanger sequencing**

MDDR Specificity and Sensitivity

- Performance characteristics of MDDR were updated based on Sanger results 2012 – 2021, using a phenotypic method (agar proportion/MGIT PZA) as a reference (representing ~5,000 samples)

- Based on these results, some of the MDDR interpretation comments have also been updated

- This table will be updated regularly based on tNGS results

Drug	Locus or loci examined	Sensitivity (%)	Specificity (%)
Rifampin	*rpoB* RRDR, codons 170 and 491	99.8	91.8*
Isoniazid	*fabG1-inhA*_upstream, *katG* codon 315, *fabG1* codon 203	93.6	99.2
Ethambutol	*embB* (partial)	80.6	94.2
Pyrazinamide	*pncA*	69.8#	95.7
Fluoroquinolones	*gyrA* QRDR	86.4	99.3
Kanamycin	*rrs* (partial) *eis*_upstream	93.9	99.3
Amikacin	*rrs* (partial)	95.8	99.9
Capreomycin	*rrs* (partial) *tlyA*	98.3	95.3

*RIF specificity likely impacted by challenges of detection of phenotypic resistance in isolates with low-level RIF-R mutations (“disputed”)

#PZA sensitivity likely impacted by Clade 1 isolates (PZA-R without pncA mutations), as well as poor reproducibility of MGIT PZA test.

Summary

- **tNGS**
  - Reproducible and robust
  - Will successfully replace traditional sequencing methods of MDDR service early 2023
  - Earlier detection (sediments) of heteroresistance possible
  - May be customized to include resistance loci to other drugs
  - Reports will be issued through ELIMS

Future Enhancements

Broth Microdilution (BMD) – Minimum Inhibitory Concentration

SENSITITRE CUSTOM PLATE FORMAT

2011140914

Plate Code: CML1FCMY Date: 10-Jun-19

	1	2	3	4	5	6	7	8	9	10	11	12
A	BDQ 0.008	BDQ 0.015	BDQ 0.03	BDQ 0.06	BDQ 0.12	BDQ 0.25	BDQ 0.5	BDQ 1	BDQ 2	BDQ 4	RIF 0.06	RIF 0.12
B	RIF 0.25	RIF 0.5	RIF 1	RIF 2	RIF 4	INH 0.03	INH 0.06	INH 0.12	INH 0.25	INH 0.5	INH 1	INH 2
C	INH 4	INH 8	INH 16	OFL 0.12	OFL 0.25	OFL 0.5	OFL 1	OFL 2	OFL 4	OFL 8	LEVO 0.12	LEVO 0.25
D	LEVO 0.5	LEVO 1	LEVO 2	LEVO 4	MXF 0.06	MXF 0.12	MXF 0.25	MXF 0.5	MXF 1	MXF 2	MXF 4	KAN 0.12
E	KAN 0.25	KAN 0.5	KAN 1	KAN 2	KAN 4	KAN 8	KAN 16	AMI 0.12	AMI 0.25	AMI 0.5	AMI 1	AMI 2
F	AMI 4	AMI 8	AMI 16	CAP 0.12	CAP 0.25	CAP 0.5	CAP 1	CAP 2	CAP 4	CAP 8	CAP 16	LZD 0.12
G	LZD 0.25	LZD 0.5	LZD 1	LZD 2	LZD 4	LZD 8	CFZ 0.015	CFZ 0.03	CFZ 0.06	CFZ 0.12	CFZ 0.25	POS
H	CFZ 0.5	CFZ 1	CFZ 2	CFZ 4	EMB 0.25	EMB 0.5	EMB 1	EMB 2	EMB 4	EMB 8	EMB 16	NEG

ANTIMICROBICS	
BDQ	Bedaquiline
RIF	Rifampin
INH	Isoniazid
LEVO	Levofloxacin
KAN	Kanamycin
AMI	Amikacin
LZD	Linezolid
CFZ	Clofazimine
OFL	Ofloxacin
CAP	Capreomycin
MXF	Moxifloxacin
EMB	Ethambutol
POS	Positive Control
NEG	Negative Control

Acknowledgements

- **Applied Research Team**
  - **Jamie Posey**, **Scott Burns**, **Lauren Cowan**, **Matthew Ezewudo**
- **Reference Laboratory Team**
  - Kate Klein, **Scott Chancey**, David Sikes, Richard Ebai
- **Former TB Laboratory Branch employees and fellows who contributed**
  - Beverly Metchock (retired), **Calin Chiribau**, Connie Lam,
  - Melissa Willby, Frank Ambrosio, Eric Ransom
- **Office of Branch Chief and Systems Group**
  - Angela Starks, Tracy Dalton, Lois Diem, Wan Moon

Thank You!

For more information, contact CDC
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