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Perspectives from a Public Health Laboratory

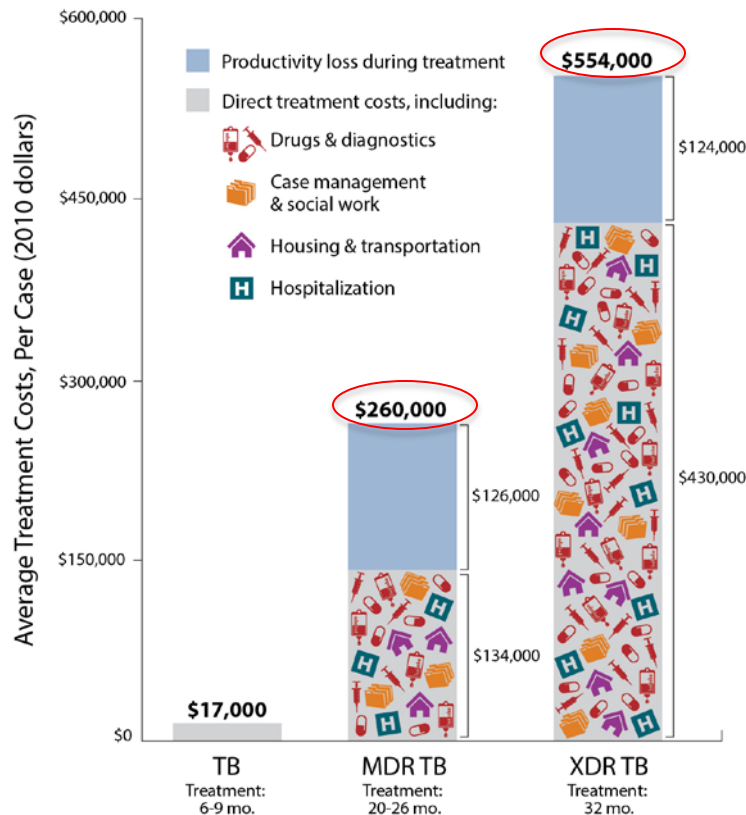Kimberlee Musser, PhD
Chief, Bacterial Diseases
Wadsworth Center

*I have no disclosures.

Drug Resistant Tuberculosis is a Global Health Concern

- **Multi-drug resistant TB (MDR):** resistant to at least rifampin and isoniazid

- **Extensively drug resistant TB (XDR):** resistant to rifampin and isoniazid plus any fluoroquinolone and at least one of three injectable second-line drugs (i.e., amikacin, kanamycin, or capreomycin).

The Outsized Financial Toll of MDR and XDR TB
Cost increases with greater resistance:

Average Treatment Costs, Per Case (2010 dollars)

Legend:
- Productivity loss during treatment
- Direct treatment costs, including:
  - Drugs & diagnostics
  - Case management & social work
  - Housing & transportation
  - H Hospitalization

$600,000
$450,000
$300,000
$150,000
$0

- **TB** Treatment: 6-9 mo. — $17,000
- **MDR TB** Treatment: 20-26 mo. — $260,000 ($126,000 / $134,000)
- **XDR TB** Treatment: 32 mo. — $554,000 ($124,000 / $430,000)

http://www.cdc.gov/nchhstp/newsroom/2014/WorldTBDay-graphics.html

Preventing and Controlling MDR and XDR TB in the U.S. Requires:

BETTER TREATMENT OPTIONS	**RAPID DIAGNOSIS**	**EXPERT TREATMENT OF EVERY TB CASE**	**IMPROVING GLOBAL TB DIAGNOSIS AND TREATMENT**

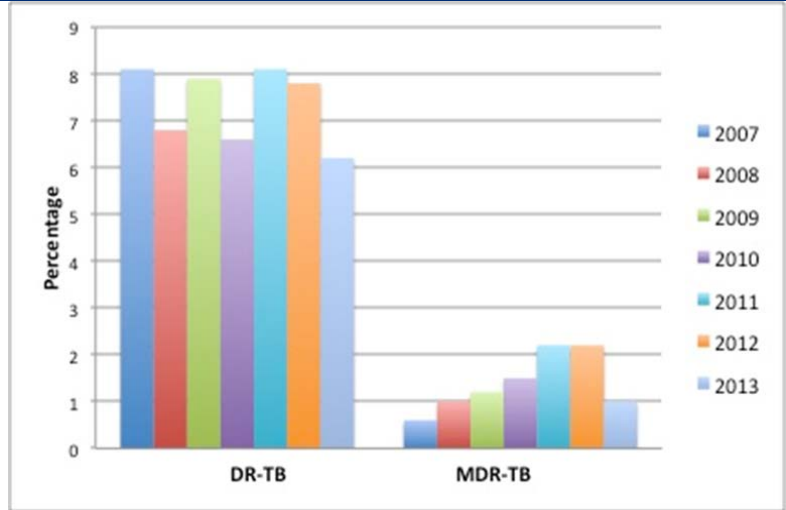http://www.cdc.gov/nchhstp/newsroom/2014/WorldTBDay-graphics.html

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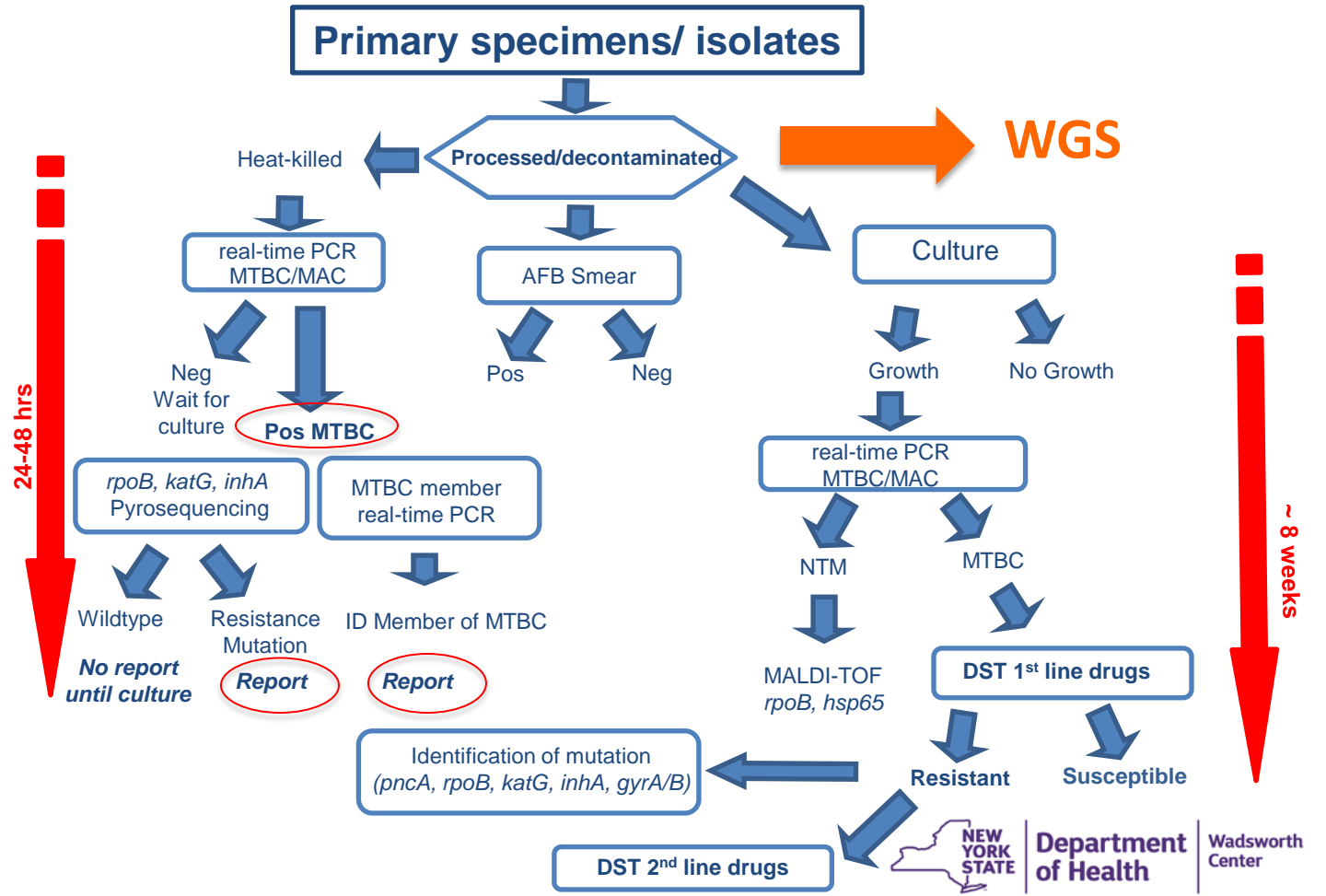# Why perform WGS on *Mycobacterium tuberculosis*?

- **Faster turn-around time**
- **More comprehensive results**
    - **Detect mixed infections**
    - **Many predictors of drug resistance**
    - **Emerging resistance**
- **Cost effective**
    - **Replace existing assays (real-time PCR, pyrosequencing, spoligotyping)**
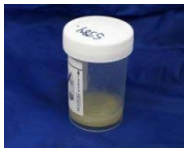   - **Staff time savings**

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	2007	2008	2009	2010	2011	2012	2013	2014
TB Cases	1175	1200	1007	954	910	864	872	786

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Primary specimens/ isolates

Processed/decontaminated → **WGS**

Heat-killed

real-time PCR MTBC/MAC

Neg Wait for culture

Pos MTBC

rpoB, katG, inhA Pyrosequencing

MTBC member real-time PCR

Wildtype

Resistance Mutation

ID Member of MTBC

No report until culture

Report

Report

AFB Smear

Pos

Neg

Culture

Growth

No Growth

real-time PCR MTBC/MAC

NTM

MTBC

MALDI-TOF *rpoB, hsp65*

DST 1st line drugs

Resistant

Susceptible

Identification of mutation *(pncA, rpoB, katG, inhA, gyrA/B)*

DST 2nd line drugs

24-48 hrs

~ 8 weeks

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Whole Genome Sequencing

Next Generation Technologies
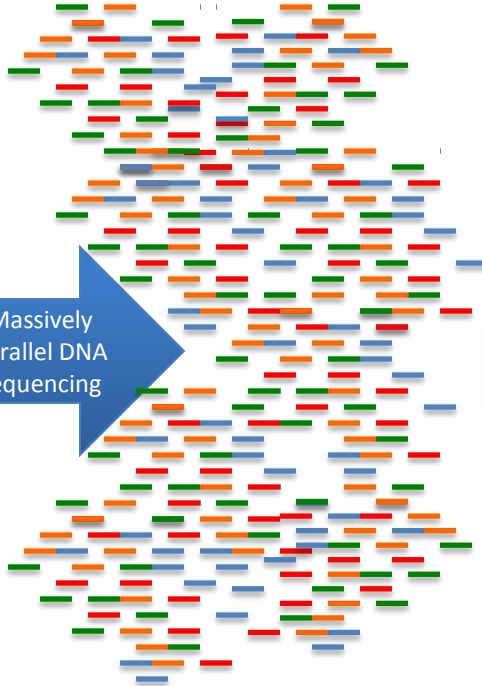
Validating a WGS assay for TB
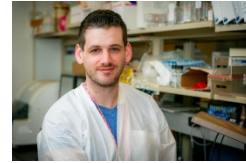
Bacteriology/Myco bacteriology Laboratory

Selecting validation approach, culture, optimization of DNA preparation

Core Facilities

Library preparation and Miseq sequencing, optimizing, planning overall decisions for _balancing runs_ (3 Illumina Miseqs)

Bioinformatics

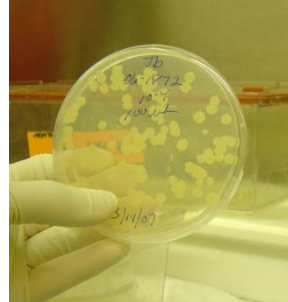Development, testing and continual improvements to pipeline, data storage

What to validate first?

- Isolates
  - Solid
  - MGITs
- Primary specimens
  - sputum
  - other

Need to keep in mind available testing volumes, what is needed for other tests, archiving, etc...

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How Can We Mimic a Clinical Isolate?

- **Grow 2 different strains in MGIT tube**
  - *M. tuberculosis* : **ATCC strain (ATCC)**
  - *M. bovis BCG* : **patient strain (BCG)**
- **Aliquots made and heat-killed before leaving BSL-3**

MGIT

10^5 to 10^6 colony-forming units
per milliliter (CFU/mL)

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Preparing TB DNA for WGS

- Assess methods used in lab
- Research TB WGS methods
- Assess worse case scenario
  - 1- 2 ml MGIT
  - early MGIT positive (Day 0-3 flagged positive)
- Ease of use, cost
- DNA concentration
- Ultimately- WGS 40X depth and close to 100% coverage

Breaking TB Open is Critical for DNA Extraction

Important TB Characteristics

- **~24 hour doubling time**
- **TB clumps together**
- **Unique cell wall**
  - **Rich in lipids (>60%)**
  - **Mycolic acids**

CDC

Initial Methods Tested

- **Typical bacterial extraction**
- **Zymo Research Kit**
  - **Meant for tough to lyse fungi/ bacteria**
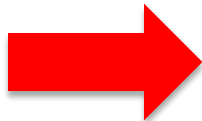- **CTAB method**
  - **Ideal for plant cell nucleic acid extraction/ MTB**

DNA yield too low, labor intensive, WGS variable results

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# InstaGene Matrix and Tissue Homogenizer

- **InstaGene matrix (Chelex resin)**
  - The Chelex matrix binds to PCR inhibitors rather than DNA, preventing DNA loss due to irreversible DNA binding.

- **Fastprep tissue homogenizer**
  - **Good enough yield to provide reliable WGS data even with 0 day MGIT**

Success!

Successful WGS

8X Depth

```
ATTGC
ATTGC
 TTGCATAAAATTC
ATTGCAT
   TGCATAAATTC
ATTGCAT
ATTG
   TGCATAAAT
```

- **Depth**: **Essentially the number of times the base was read; measure of confidence in correct call**
  - **Can be given as a genome average**
  - **We are aiming for 40X**

- **Coverage**: **A percentage that describes how much of the genome was sequenced**
  - **Best 100%**

Not sequenced

67% Coverage

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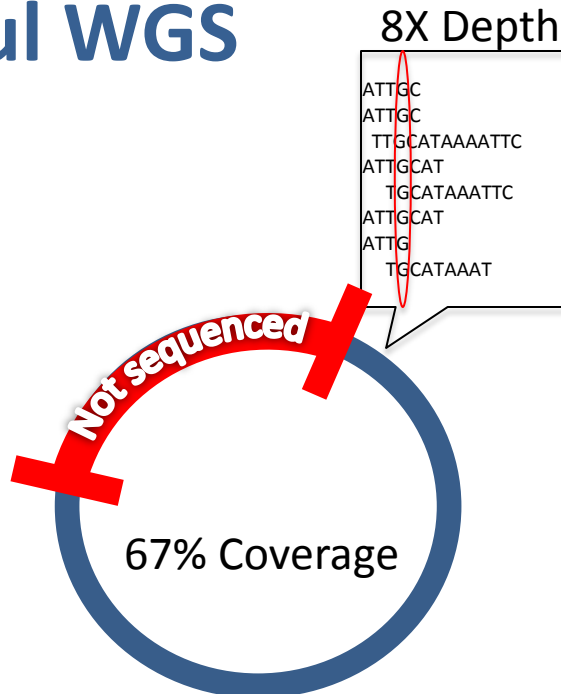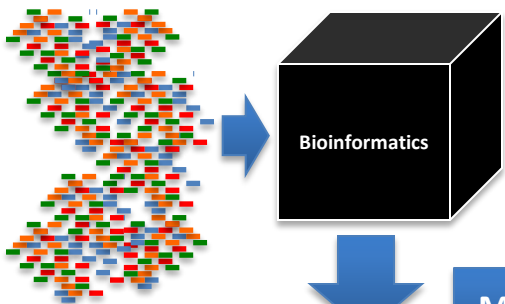
Library Preparation is Another Key Factor

- **Votintseva *et al.* suggested using 15 cycle library preparation**
  - **2015 paper about WGS of early positive MGIT**

Sample	Method	stock ng/ul	12 cycle library prep Nextera XT DNA Library Preparation Kit		15 cycle library prep Nextera XT DNA Library Preparation Kit	
			Avg depth	coverage %	Avg depth	coverage %
M. bovis BCG (0day)	InstaGene	0.268	FAIL	FAIL	27.66	97.23
	InstaGene	0.344	FAIL	FAIL	19.4	97.07
	InstaGene	0.346	FAIL	FAIL	14.22	96.78

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Bioinformatics Pipeline

Pascal Lapierre, PhD
Michael Palumbo, PhD

TB Bioinformatics Pipeline
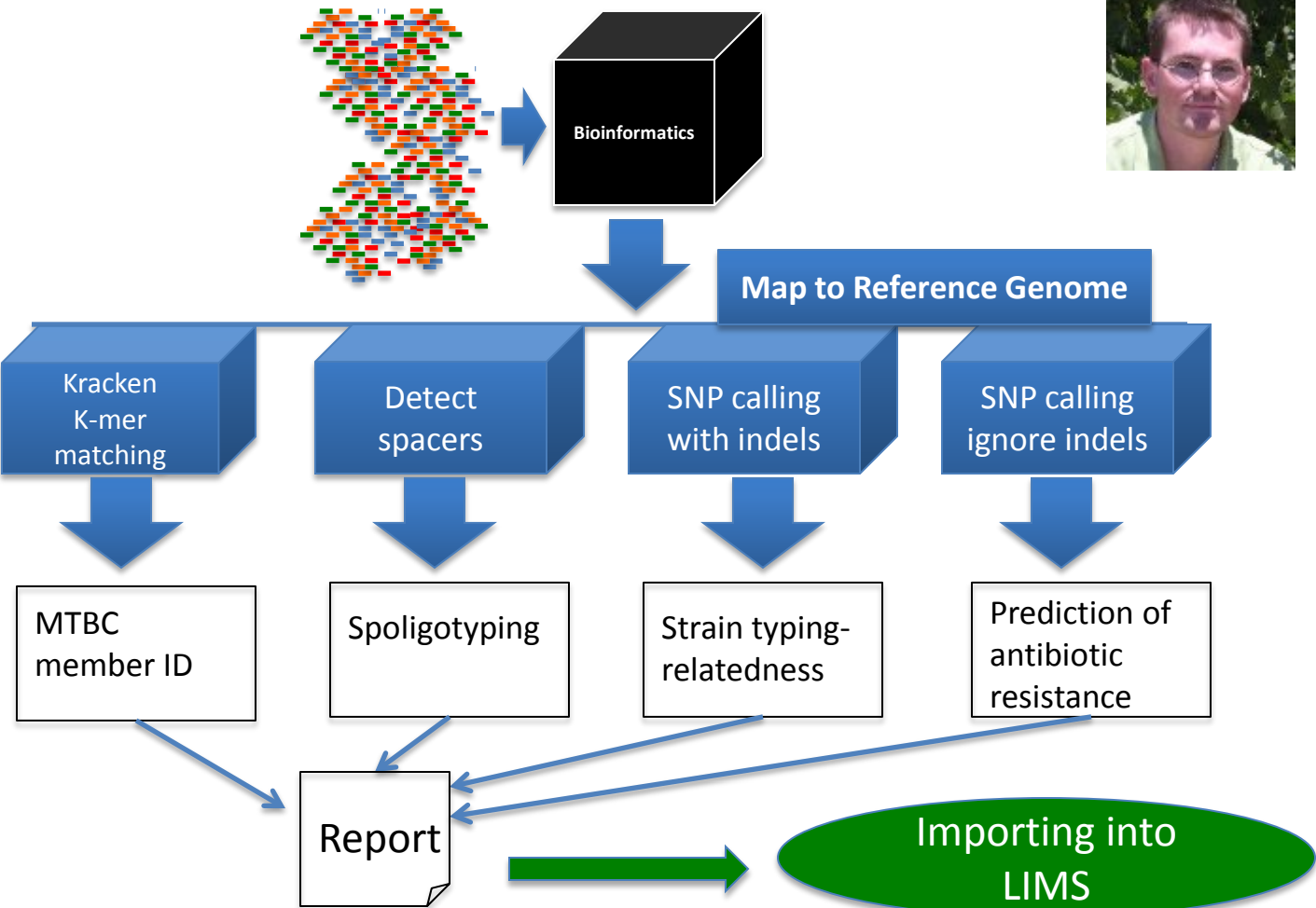
Example report excerpt

```
All Mutation in screened loci (except silent mutations):

7362      21      GAG -> CAG      Glu -> Gln      Rv0006  gyrA    Fluoroquinolones
7584      95      AGC -> ACC      Ser -> Thr      Rv0006  gyrA    Fluoroquinolones
9303      668     GGC -> GAC      Gly -> Asp      Rv0006  gyrA    Fluoroquinolones
9555      752     ACG -> ACC      Thr -> Thr      Rv0006  gyrA    Fluoroquinolones (Silent)
761154    450     TCG -> TTG      Ser -> Leu      Rv0667  rpoB    Rifampicin
764816    483     GTG -> GCG      Val -> Ala      Rv0668  rpoC    Rifampicin compensatory
765149    594     GGG -> GAG      Gly -> Glu      Rv0668  rpoC    Rifampicin compensatory
775639    948     ATT -> GTT      Ile -> Val      Rv0676c mmpL5   Clofazimine/Bedaquiline
1416234   372     TGT -> GGC      Cys -> Gly      Rv1267  embR    Ethambutol
1416222   376     TTC -> CTC      Phe -> Leu      Rv1267  embR    Ethambutol
1674046   203     CTG -> CTA      Leu -> Leu      Rv1483  mabA    Isoniazid (Silent)
1917970   11      CTA -> CTG      Leu -> Leu      Rv1694  tlyA    Aminoglycosides (Silent)
2155169   315     AGC -> ACC      Ser -> Thr      Rv1908c katG    Isoniazid
2288896   116     CTG -> CGG      Leu -> Arg      Rv2043C pncA    Pyrazinamide
3336825   365     ACA -> GCA      Thr -> Ala      Rv2981c ddlA    D-Cycloserine
4242641   927     CGC -> CGT      Arg -> Arg      Rc3793  embC    Ethambutol (Silent)
4242803   981     GTG -> CTG      Val -> Leu      Rc3793  embC    Ethambutol
4243221   -12     C -> T  intergenic      embC-embA       Ethambutol
4247429   306     ATG -> ATA      Met -> Ile      Rv3795  embB    Ethambutol
4407968   79      TTG -> TCG      Leu -> Ser      RV3919c gidB    Streptomycin



High confidence mutations detected :

embB      306     ATG -> ATA      Met -> Ile      Ethambutol
katG      315     AGC -> ACC      Ser -> Thr      Isoniazid
mabA      203     CTG -> CTA      Leu -> Leu      Isoniazid
pncA      116     CTG -> CGG      Leu -> Arg      Pyrazinamide
rpoB      450     TCG -> TTG      Ser -> Leu      Rifampicin


-----------------------------------------------
Resistance Report:

Ethambutol        PASS    Resistant
Fluoroquinolones          PASS    Sensitive
Isoniazid         PASS    Resistant
Pyrazinamide      PASS    Resistant
Rifampicin        PASS    Resistant
Streptomycin      PASS    Sensitive
```

Validation of TB WGS

- SOP, reports, interpretation, QC, assay controls, metrics
- Specificity, intra-assay and inter-assay reproducibility
- Retrospective testing
- Prospective testing
- Evaluate each drug

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TB WGS Reports

Whole genome sequencing

Concentrated Smear(Ziehl - Neelsen/1,000 X)
(03/13/14):   Numerous (>9 acid-fast bacilli per field)

Direct Molecular Detection - Real-time PCR
Mycobacterium tuberculosis complex DNA by real-time PCR: DETECTED
Mycobacterium avium complex DNA by real-time PCR: Not Detected

Molecular Identification - Real-time PCR
Mycobacterium tuberculosis complex species DNA identified: Mycobacterium tuberculosis

Culture
(03/25/14):   acid-fast bacillus was isolated

Direct Molecular Drug Susceptibility Detection- Pyrosequencing
Rifampin (rpoB):   Mutation present (Ser531Leu) suggests Rifampin resistance. Result must be confirmed by culture based susceptibility testing.
Isoniazid (katG):   Mutation absent. Culture must be performed for final susceptibility result.
Isoniazid (inhA):   Mutation absent. Culture must be performed for final susceptibility result.

Identification
(03/26/14):   Mycobacterium tuberculosis was identified by culture and molecular analysis.

Susceptibility Testing for M. tuberculosis complex (MGIT)
Streptomycin [1.0 ug/ml]:   Susceptible
Isoniazid [0.1 ug/ml]:   Susceptible
Rifampin [1.0 ug/ml]:   RESISTANT
Ethambutol [5.0 ug/ml]:   Susceptible
Pyrazinamide [100 ug/ml]:   Susceptible

Whole Genome Sequencing of TB:
A "One Stop Shop"

WGS

Single assay

Species identification

Genotyping (<u>more accurate</u>)

Drug resistance mutations

(<u>more comprehensive</u>)

COST

Estimated around $100 per sample

TURNAROUND TIME

DNA preparation (1 days)

WGS result (4-5 days)

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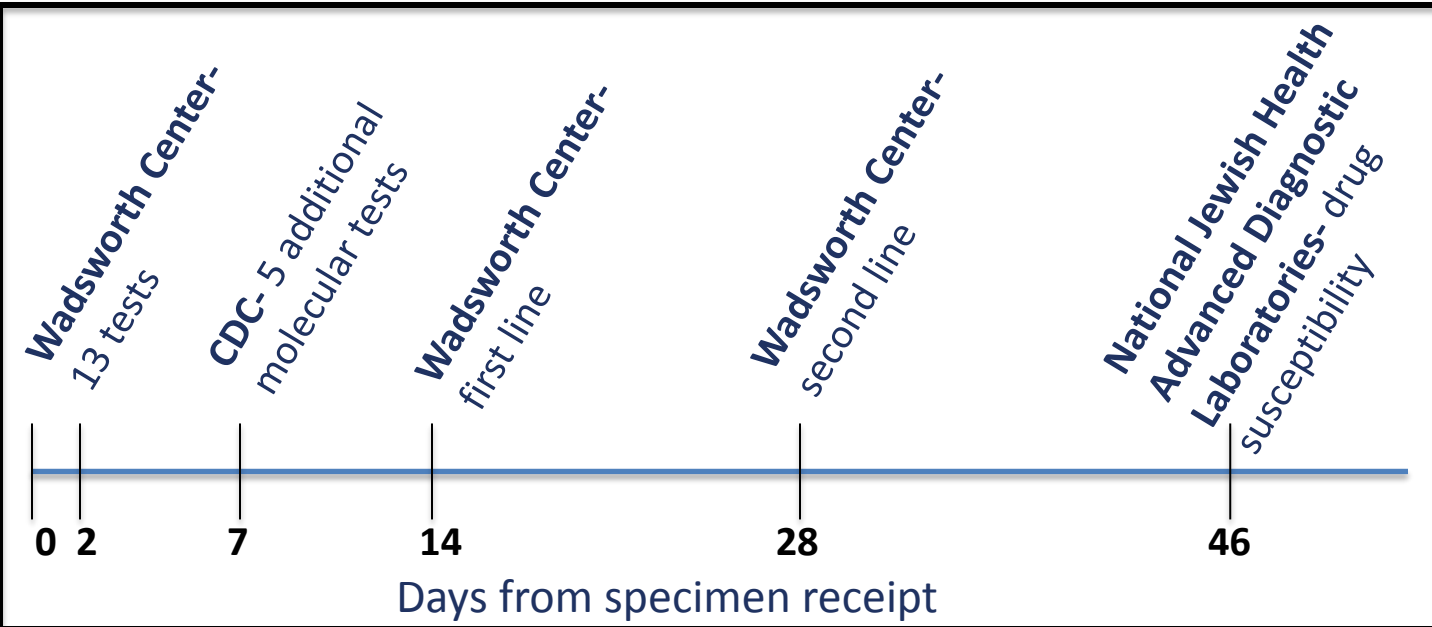# WGS prediction spoligotypes and genotyping with increased resolution
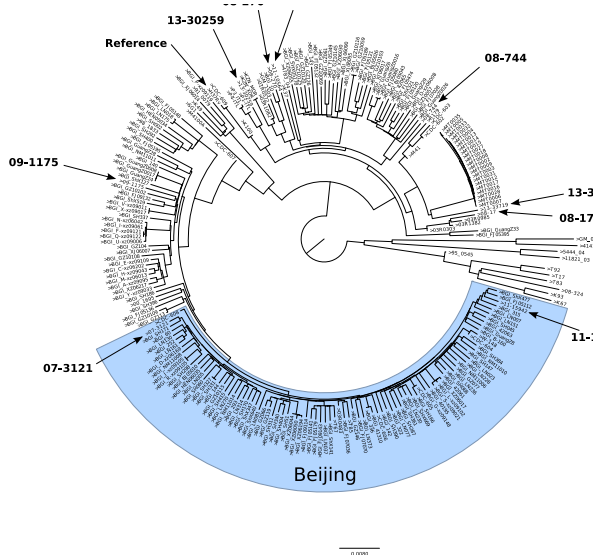
Sample #	Extraction	AccId	Phenotype (DST)	Resistance Associated mutations (WGS)	Other mutations noted (WGS)	WGS Spoligotype	CLIMS spoligo. NYS	Do results correlate?
21	CTAB	14-7386	SM (low level in 2nd line), INH, RIF, RBT	rpoB aa 450 (RIF) rpsL aa 88 (SM) katG aa 315 (INH)		S00034	Scheduled 12/15/14	Yes
22	CTAB	14-7387	INH (low level), RIF, RBT	rpoB aa 445 (RIF) intergenic hyp-fabG1 aa. -15 (INH)		S00197	S00197	Yes
23	CTAB	14-7388	SM, INH, PZA, EMB (2nd line only), KM, AN,	rpoB aa 450 (RIF) rpsL aa 43 (SM) katG aa 315 (INH) embB aa 306 (EMB/INH)	rrs (AN/SM) pncA aa 119 (PZA)	S00034	S00034	Yes
24	CTAB	14-7391	SM, EMB, CAP, KM, AN	rrs (AN/SM)	embC aa 981 (EMB) gid aa 97 (SM)	S00241	S00241	Yes
25	CTAB	14-7392	SM, RIF, EMB, PZA, KM, RBT, FLQ	gyrA aa 94 (FLQ) rpoB aa 450 (RIF) rpoC aa 452 (RIF) embB aa 306 (EMB/INH) embB aa 497 (EMB/INH)	gid aa 92 (SM) rrs (AN/SM) rpsL aa 43 (SM) pncA TG-TGG (PZA) eis promoter (AN/KM)	S00034	S00034	Yes
26	CTAB	14-7393	INH, RIF, PZA, EMB 5.0 (2nd line only), ETA, RBT,	rpoB aa 450 (RIF) intergenic hyp-fabG1 aa. -15 (INH/ETA) embB aa 306 (EMB/INH)		S00002	S00002	Yes
27	CTAB	14-7395	SM, INH, RIF, EMB, PZA, RBT	rpoB aa 450 (RIF) rpsL aa 43 (SM) katG aa 315 (INH) pncA aa 12 (PZA) embB aa 306 (EMB/INH)		S00034	S00034	Yes
28	CTAB	14-7396	SM, INH, RIF, PZA, EMB (2nd line only), RBT	rpoB aa 450 (RIF) rpsL aa 43 (SM) katG aa 315 (INH) embB aa 306 (EMB/INH)	pncA aa 151 (PZA)	S00034	S00034	Yes

A Glimpse of the One Stop Shop in Action

Sample	# Day Pos	Qubit stock []	Clims				Whole Genome Sequencing							spoligo / speciation match?	DR match?
			species	DST	Pyro/Sanger results	spoligotype	Depth	%Coverage	spoligotype	HC mutations	large deletions	other notable mut's			
15-5031	1	1.16	*M. africanum?*	NOT DONE	NOT DONE	S01519 (700740007774671)	173.30	97.80%	S01519 6(7)00740007774671	none	~13Kb deletion at RD12 region		YES: SNP in #3 & *africanum* on tree	
14-26616	0	0.378	*M. tuberculosis*	SM, INH, ETA	inhA C-15T (INH)	S00034	125.593	98.69	S00034	rpsL 88 (SM);mabA -15 (INH/ETA)	In-frame deletion iniB (INH)		YES	YES
15-6146	1	1.1	*M. tuberculosis*	SM, INH, FLQ(OFL)	katG Ser315Thr (INH), gyrA Asp94Gly (FLQ)	S00034	79.9	98.4	S00034	gyrA 94 Asp-Gly (FLQ), katG 315 (INH); rpsL 88 (SM)	None		YES	YES
14-7137	1	0.552	*M. tuberculosis*	SM, INH, RIF(MGIT only), ETA	rpoB Asp516Val (RIF), katG Ser315Leu (INH)	not done	168.6	99	S00035	katG 315 (INH); rpoB Asp-Val (RIF); rpsL 43 (SM)	None	ethA 329, ethA 403 (ETA)	YES	No for ETA

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XDR Case (November 2014)

Wadsworth Center- 13 tests · 0, 2

CDC- 5 additional molecular tests · 7

Wadsworth Center- first line · 14

Wadsworth Center- second line · 28

National Jewish Health Advanced Diagnostic Laboratories- drug susceptibility · 46

Days from specimen receipt

**Can we develop <u>one</u> assay
capable of generating
the same results...and more?
Can we do it in <1 week?**

XDR Case (November 2014)

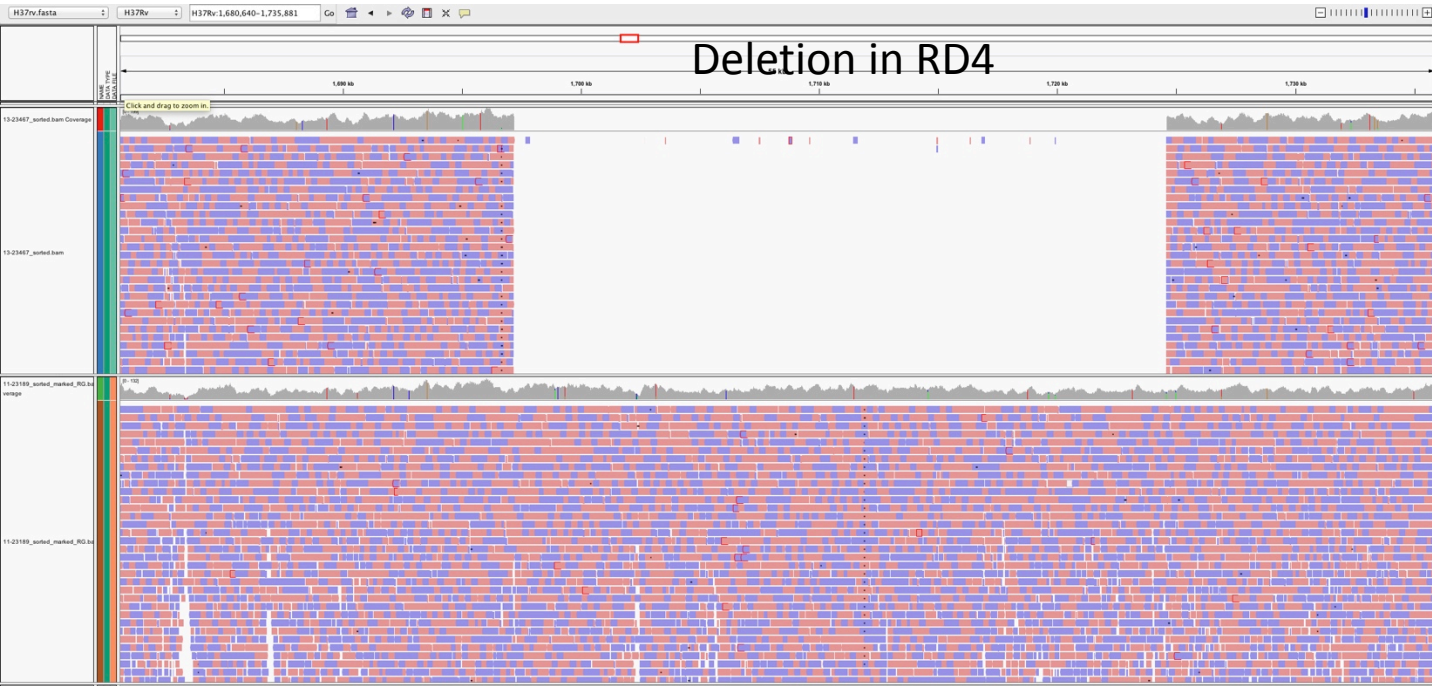**Spoligotype: S00062 (777740777760771)**

Lineage Euro-American
M. tuberculosis X1 family

Drug Resistant phenotype:
FLQ (OFL,LVX, MX)
RIF
INH
SM
EMB
PZA
RBT
KAN
AMI
CAP (11%)

Gene	Genome position	Gene Position		SNP	Res. associated		Codon AA change	Known mutation?
rrs	1473246	1400	A -> G	AMI/SM				Putative mutation*
gyrA	7362	61	G -> C	FLQ	21	Glu/Gln	No	GAG -> CAG
gyrA	7582	281	A -> G	FLQ	94	Asp/Gly	HC mutation	GAC -> GGC
gyrA	7585	284	G -> C	FLQ	95	Ser/Thr	No	AGC -> ACC
gyrA	9304	2003	G -> A	FLQ	668	Gly/Asp	No	GGC -> GAC
rpoB	761155	1349	C -> T	RIF	450	Ser/Leu	HC mutation	TCG -> TTG
rpoC	764948	1579	T -> G	RIF	527	Leu/Val	No	TTG -> GTG
rpoC	765150	1781	G -> A	RIF	594	Gly/Glu	No	GGG -> GAG
tlyA	1917972	33	A -> G	AMI	11	Leu/Leu	No	Silent CTA -> CTG
katG	2154678	1434	G -> C	INH	478	Ala/Ala	No	Silent GCG -> GCC
katG	2155168	944	G -> C	INH	315	Ser/Thr	HC mutation	AGC -> ACC
pncA	2289049	193	T -> TA	PZA	Insertion	Frameshift	No	
ahpC	2726409	217	G -> C	INH	73	Asp/His	No	GAC -> CAC
embC	4242643	2781	C -> T	EMB	927	Arg/Arg	No	Silent CGC -> CGT
embC	4242803	2941	G -> C	EMB	981	Val/Leu	No	GTG -> CTG
embB	4247730	1217	G -> C	EMB/INH	406	Gly/Ala	HC mutation	GGC -> GCC
embB	4249408	2895	G -> A	EMB/INH	965	Pro/Pro	No	Silent CCG -> CCA
embB	4249678	3165	C -> A	EMB/INH	1055	Arg/Arg	No	Silent CGC -> CGA
ethA	4326718	756	CCGCG -> CCGCGCG	ETH	Insertion	Frameshift	No	
gid	4407934	269	T -> G	SM	90	Leu/Arg	No	CTC -> CGC

Evolving Pipeline

Deletion in RD4

- **Bedaquiline mutations detected**

Future Directions WGS TB

- Finalize validation and implement WGS for TB MGIT testing
- Refining pipeline and data interpretation
- TB Primary specimens
- LIMS importing
- NCBI
- Data Storage

MYCOBACTERIOLOGY LAB

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Matt Schudt
Patrick VanRoey
Pascal Lapierre
Mike Palumbo

Questions?

http://biocomicals.blogspot.com/2011_05_01_archive.html

TB MGIT

200 ul

Centrifuge

56°C 15 min

15 sec 2X

Centrifuge

Proteins, inhibitors, etc...

WGS