LABORATORY TESTS FOR TB: IMPLICATIONS FOR CASE MANAGEMENT

OBJECTIVES

Upon completion of this session, participants will be able to:

1. Describe three laboratory methods used in the diagnosis and control of TB resulting in a better understanding of laboratory results and improved communication between the clinician, the laboratory, and the patient

INDEX OF MATERIALS

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1. Laboratory test for TB: Implications for case management – slide outline
   Presented by: Nicole Green, PhD, D(ABMM), MASCP

SUPPLEMENTAL READING MATERIALS

None
### ADDITIONAL REFERENCES


Barnes, P., Cave, D. Molecular epidemiology of tuberculosis. NEJM. 2003;349(12):1149-1155. Review article.


Laboratory Diagnostics for Mycobacterium tuberculosis

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Disclosures/Disclaimers

• No potential conflicts of interest

• Financial disclosures – none

• Speaker conclusions do not necessarily represent the views of the Los Angeles County Public Health Department

• Use of brand names does not imply endorsement by the speaker or the Los Angeles County Public Health Department
Objectives

• Learn about the role of public health laboratory in TB diagnosis
• Discuss core TB functions for public health laboratories
• Review TB diagnostics
  – Historical aspects
  – Conventional methods for mycobacterial identification/DST
  – Molecular methods for mycobacterial identification
  – Molecular drug susceptibility testing
  – Molecular epidemiology typing methods
• Provide case study example
Role of public health labs and core functions related to TB services
Role of the local PHL

- Rapid response to community needs and generate data
  - Make decisions and policies
  - Evaluate programs
  - Provide testing at site of patient care
- Support mission of the local health department
- Serve as conduits for disease screening or control programs
- Serve as surge capacity for state PHLs

TB services are an important part of public health laboratory work

WHO core elements of TB lab services

- Lab infrastructure, appropriate biosafety measures and maintenance
- Equipment validation and maintenance
- Specimen transport and referral mechanisms
- Management of laboratory commodities and supplies
- Lab information and data management systems
- Lab quality management systems
- Appropriate, adequate strategies and funding for laboratory HR development
- Coordination of technical assistance
- Integration of diagnostic algorithms in lab strengthening plans
• Defines core TB services that public health laboratories must provide or assure access (in-house and reference)
• Provide basic set of services locally to improve turnaround times and expertise for leadership and consultation

Public health laboratories should have the ability to provide accurate and precise analytical results in a timely manner. Serve as a “first line of defense” in the rapid recognition and prevention of the spread of communicable diseases. Serve as a center of expertise for the detection and identification of biologic agents of importance in human disease.
Core TB lab services for PHLs

- DISEASE PREVENTION, CONTROL, AND SURVEILLANCE
- INTEGRATED DATA MANAGEMENT
- REFERENCE AND SPECIALIZED TESTING
- LABORATORY IMPROVEMENT AND REGULATION
- POLICY DEVELOPMENT
- PUBLIC HEALTH RELATED RESEARCH
- TRAINING AND EDUCATION
- PARTNERSHIP AND COMMUNICATION
TB diagnostics
TB Complex

- *M. tuberculosis*
- *M. bovis* (M. bovis subsp. bovis, M. bovis subsp. caprae, M. bovis BCG)
- *M. africanum*
- *M. cannetti*
- *M. microti*
- *M. pinnipedii*

Stain of *M. tuberculosis* from liquid culture showing cording
From my numerous observations, I conclude that these tubercle bacilli occur in all tuberculous disorders, and that they are distinguishable from all other microorganisms.
Conventional algorithm for ID

Identification of Mycobacteria takes A LONG TIME!!!!

1-2 hours
4 hours
1-8 weeks*
1-2 days to 4 weeks**
6-8 weeks**

Process specimen
AFB smear microscopy
Growth on solid and liquid media
Species ID
DST

* Species dependent; **method dependent

Modified from D. Warshauer, WI SPHL
Modern algorithm for ID

1 hour

- Molecular based tests
  - Processed or raw specimen
    - AFB smear microscopy
      - Growth on solid and liquid media
        - Species ID
          - DST

1-3 days*

*method dependent

Modified from D. Warshauer, WI SPHL
### Acceptable specimen types

<table>
<thead>
<tr>
<th>Pulmonary</th>
<th>Extra-pulmonary</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sputum (expectorated, induced)</td>
<td>Tissue</td>
</tr>
<tr>
<td>Bronchoalveolar lavage (BAL)</td>
<td>Body fluids</td>
</tr>
<tr>
<td>Bronchial wash/brush</td>
<td>Blood</td>
</tr>
<tr>
<td>Transtracheal aspirate</td>
<td>Stool</td>
</tr>
<tr>
<td></td>
<td>Gastric lavage</td>
</tr>
<tr>
<td></td>
<td>Urine</td>
</tr>
</tbody>
</table>

Swabs are generally not acceptable
Collect specimens before chemotherapy is begun

Collect 3-10 ml of specimen in a labeled sterile 50 ml conical tube

Collect a minimum of 3 single patient specimens
- Early morning specimens collected on successive days
- Specimens collected 8 hours apart with at least one being an early morning collection

Refrigerate and transport specimens to the laboratory as soon as possible

Seal and package specimen containers carefully to avoid leakage and breakage in transit
<table>
<thead>
<tr>
<th>Site</th>
<th>Collection Time</th>
<th>Volume</th>
<th>Frequency</th>
<th>Storage and transport</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gastric Aspirate</td>
<td>Early morning before eating and while in bed</td>
<td>5–10 ml</td>
<td>One specimen per day on three consecutive days</td>
<td>Room temperature; if delayed &gt;1 hour, neutralize with 100 mg sodium carbonate</td>
</tr>
<tr>
<td>Urine</td>
<td>First morning specimen (mid-stream)</td>
<td>40 ml</td>
<td>One specimen per day on three consecutive days</td>
<td>Refrigerate</td>
</tr>
<tr>
<td>Stool</td>
<td></td>
<td>Min. 1 gram</td>
<td>-</td>
<td>Refrigerate</td>
</tr>
<tr>
<td>CSF</td>
<td></td>
<td>10 ml</td>
<td>-</td>
<td>As soon as possible at room temperature; do not refrigerate</td>
</tr>
<tr>
<td>Other Body Fluids (pleural, peritoneal, pericardial, synovial)</td>
<td></td>
<td>10-15 ml</td>
<td>-</td>
<td>Refrigerate</td>
</tr>
<tr>
<td>Tissues or Lymph Nodes</td>
<td></td>
<td>As much as possible; add 2-3 ml sterile saline</td>
<td>-</td>
<td>As soon as possible at room temperature</td>
</tr>
<tr>
<td>Blood</td>
<td></td>
<td>10ml; SPS/heparin</td>
<td>-</td>
<td>As soon as possible at room temperature</td>
</tr>
</tbody>
</table>
Decontamination, digestion and concentration

- Majority of the specimen types (sputum) are contaminated with normal flora
- Many different procedures developed to decontaminate specimens using acid or alkaline chemical agents to kill contaminants
  - NALC-NaOH, Z-TSP, NaOH, oxalic acid, sulfuric acid, CPC
  - Commercial and lab-made reagents
- High lipid content of cell walls (mycolic acid), mycobacteria are able to resist bacteriocidal effects of decontamination agents
Decontamination, digestion and concentration

- Decontamination procedures digest or liquefy mucoid specimens, free mycobacteria from clumps of protein, and allow for organisms to sediment during centrifugation.
- Decontamination/digestion procedures use centrifugation to concentrate the specimen before smear culture.
- Normally sterile specimens (e.g. CSF) can be concentrated by direct centrifugation of the specimen without decontamination.
- Concentration increases the sensitivity of both smear and culture.
Mycobacteria have unique cell wall structure made of mycolic acid

- In addition to peptidoglycan, the acid-fast cell wall of *Mycobacterium* contain a large amount of glycolipids, especially mycolic acids.

- Stain poorly because cell wall structure and lipids interfere with gram stain.

- Acid fast stains are used to force complex of dyes into mycobacterial cells.
How do AFB stains work?

Ziehl-Neelson Stain
Kinyoun Modification

Acid Fast Organisms

- A small amount of organism suspended in saline solution is fixed on a slide.
- Slide is flooded with Carbol Fuchsin and phenol for 3 minutes, and gently rinsed with water.
- Slide is decolorized with 3% HCl in 70% alcohol until color appears to be removed (approx. 2 mins), and rinsed with water.
- Slide is flooded with methylene blue counterstain for 30 secs, rinsed with water and air-dried.

Not Acid Fast Organisms

- non-acid-fast bacteria (Staphylococcus epidermidis)
- acid-fast bacteria (Mycobacterium gordonae)
AFB smear microscopy

• Examination of specially stained smears for acid-fast bacteria
  – Stains allow detection of organisms containing mycolic acid in the cell wall; resist decolorization with acid-alcohol and retain primary stain
• Direct and concentrated smears
• Two main types of AFB stains
  – Fluorescent
    • Auramine O, Auramine Rhodamine
  – Brightfield
    • Kinyoun
    • Ziehl-Neelsen
How can AFB smear results be used?

• Clinical management
  – Initiate patient therapy based on smear result and clinical presentation; presumptive diagnosis, allows dx weeks before culture results
  – Monitoring response to therapy

• Laboratory testing
  – Interpretation of NAAT tests

• Public health interventions
  – Smear results identify most infectious cases
  – Prioritization of contact investigations
  – Decisions regarding respiratory isolation
Limitations of AFB microscopy

• Does not distinguish between live and dead organisms
• Follow-up specimens from patients on treatment may be smear positive yet culture negative
• Limited sensitivity and requires relatively high bacillary load for detection
  – AFB stain much less sensitive than culture
    • 10-100/ml needed to detect by culture
    • 5,000-50,000/ml to detect by AFB stain
• Limited specificity as all mycobacteria are acid fast; cannot determine species ID by AFB smears alone
• PPV of positive smears for TB should be taken into consideration with local prevalence of MTB and NTM in the patient population
## AFB smear reporting

<table>
<thead>
<tr>
<th>Number of AFB observed by fluorochrome at 250x</th>
<th>Report</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>No AFB Seen</td>
</tr>
<tr>
<td>1-2/30 Fields</td>
<td>Doubtful, Repeat test</td>
</tr>
<tr>
<td>1-9/10 Fields</td>
<td>1+</td>
</tr>
<tr>
<td>1-9/Field</td>
<td>2+</td>
</tr>
<tr>
<td>10-90/Field</td>
<td>3+</td>
</tr>
<tr>
<td>&gt;90/Field</td>
<td>4+</td>
</tr>
</tbody>
</table>

**AFB microscopy reports should indicate magnification, scoring, and method of staining**

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 CDC Scale for AFB Microscopy
Isolation of mycobacteria

• Most species are slow growing and require extended incubation times

• Can easily be overgrown; Require appropriate pre-treatment and processing

• Digestion and decontamination of non-sterile specimens vs. normally sterile specimens

• Isolation requires variety of media including selective and non-selective broth and solid media
Culture Media

• Solid media
  – Egg based
    • Lowenstein-Jensen
    • Petragnani
    • American Thoracic Society (ATS) medium
    • Selective variants such as LJ-Gruft and Myctosel-LJ
  – Agar based
    • Middlebrook 7H10, Middlebrook 7H11
    • Selective variants such as Mitchison’s selective 7H11 (7H11s)
• Liquid media
  – Mycobacteria usually grow faster in liquid media and sensitivity for recovery is higher
    • 7H9, 7H12, 7H13
Examples of media types

LJ  
7H10  
MGIT (7H9)
MGIT 960 instruments

- Used for liquid media tubes and to incubate cultures for primary growth and drug-susceptibility testing
- Middlebrook 7H9 Broth base with supplements
- Processes 960 tubes at once
- Uses fluorescent testing technology that detects the growth of mycobacteria in clinical specimens
- Continuously monitors samples to detect positive growth tubes every 60 minutes and check for fluorescence.
Biochemical identification

• Phenotypic identification of mycobacteria is based on the characteristics of the culture and biochemical features

• Comprises a large variety of tests and divides mycobacteria into two separate groups: TB complex and NTM

• Examples of biochemical tests
  – Growth rate, colony morphology, pigment production, niacin test, nitrate reduction, catalase activity, formation of the cord factor, urease test, pyrazinamidase test, growth in the presence of p-nitrobenzoic acid, growth in the presence of hydrazide of thiophene-2-carboxylic acid, pigment, growth in MacConkey without crystal violet, growth in 5% NaCl, Tween-80, acid phosphatase test, arylsufatase test
Runyon classification

Photochromogen  Scotochromogen  Nonphotochromogen  Rapid Grower
Examples of biochemical tests

CDC Public Image Library
Kent and Kubica. Public Health Mycobacteriology
DNA probe-based methods

- Target rRNA and performed on organisms grown in culture
- Identifies 4 groups: M. tuberculosis cplx, M. avium cplx., M. kansasii, or M. gordonae
HPLC analysis of mycolic acid

- FDA-approved method for mycobacterial species ID based on mycolic acid chain length
- Library contains >30 entries of TB complex and NTM and also includes other mycolic acid containing species of bacteria
- Utilizes liquid or solid growth and consists of a 2 day process
- Can separate MTB and M. bovis BCG
- Relatively inexpensive method
- Used often in conjunction with other methods

HPLC method for mycobacterial ID

DNA sequencing

- rRNA (rrs) gene sequencing commonly used for bacterial taxonomy and identification
  - 100% = genus and species
  - 99-99.9 % = genus
  - > 95% = unable to identify by 16s rRNA sequencing

- Liquid cultures or colonies from solid media

- Issue is that 16s rRNA gene nucleotide differences may not discriminate enough between species to provide identification

- Additional and alternative targets are needed for mycobacterial species (rpoB, hsp65, ITS, dnaA, gyrB, and recA)
MALDI-TOF MS for microbiology

- Accurate, rapid, cost-effective, and reproducible method for identification of bacteria, fungi, and mycobacteria
- Ability to analyze organisms with relatively little sample preparation
- Potential for performing better than conventional biochemical systems for correct species identification and less mis-identifications
- Commercial databases are available and updated frequently; currently only RUO for mycobacteria
How does MALDI-TOF MS work?

- Used on liquid cultures and colonies from solid growth
- Mycobacteria require inactivation and extraction using heat, EtoH, and bead beating
- Extract is spotted on target plate and coated with matrix to ionize proteins
- Compare to RUO system/databases and/or in-house reference spectral libraries

Comparison of common ID methods

Rapid and accurate species-level identification may aid in guiding management decisions

- Increased confidence value for identification from liquid media
- Use of Mycobacterium-specific library had higher overall mean confidence scores for all groups/species tested compared to std library
- Highly concordant with DNA sequencing based methods
BD MGIT TBc

- BD MGIT TBc identification test is an immunochromatographic assay that utilizes growth positive MGIT liquid culture (not avail. in US: CE-approved)
- Detects MPB64 in liquid cultures using an MPT64-specific monoclonal antibody.
- LOD = 5x10^5 CFU/mL

GenoType MTBC

- Not avail. in US; CE-marked
- Solid or liquid culture
- Allows differentiation of all *M. tuberculosis* complex members
- PCR-based assay with hybridization of amplicon to probe and detection by addition of conjugate

http://www.hain-lifescience.de/
• Not avail. in US; CE-marked
• Solid or liquid culture
• ID TB complex and 43 NTM species
• Able to identify organism from low growth and differentiation of mixed cultures from fast- and slow-growing mycobacteria
• PCR-based assay with hybridization of amplicon to probe and detection by addition of conjugate

http://www.hain-lifescience.de/
Why are molecular methods needed for ID?

- Rapid diagnosis and detection
- Difficult to culture
- Inadequate biochemical tests
- Resistance screening/confirmation
- Patient management
- Cost effectiveness
- Relatively few FDA-approved tests for identification
• NAATs have several advantages over culture:
  – Higher positive predictive value (>95%) with AFB smear-positive specimens in settings in which NTM are common
  – Rapidly confirm presence of MTB in 50%–80% of AFB smear-negative, culture-positive specimens
  – Can detect TB in a specimen weeks earlier than culture for 80%–90% of patients suspected to have pulmonary TB
• FDA approved, modified FDA-approved, and LDTs
  – Depending on test, may be used on direct patient specimens, concentrates, or isolates
  – Pulmonary and extra-pulmonary specimens
Why use NAATs?

- Assist in decision to start therapy
- Reduce unnecessary treatment
- Respiratory isolation
- Cost savings

NAA testing should be performed on at least one respiratory specimen from each patient with signs and symptoms of pulmonary TB for whom a diagnosis of TB is being considered but has not yet been established, and for whom the test result would alter case management or TB control activities.

-CDC recommendations
Collect respiratory specimens and perform AFB smear and culture. Testing should not be delayed to await NAAT.

At least one specimen should be tested by NAAT.

Interpret NAAT results in conjunction with AFB smear.

Use of NAATs lead to earlier treatment initiation, improved patient outcomes, and increased opportunities to interrupt transmission.
Important points to remember
Interpret results on basis of clinical presentation
Single negative NAAT cannot be used to rule out TB
Cx and smear should always be ordered with NAAT
Sputum specimens may contain inhibitors
NAATs detect live/dead TB bacteria

LA County TBCP NAAT Guidelines, 2012
Table 2: Interpretation of NAAT Result on 1st Respiratory Specimen and Case Management Actions

<table>
<thead>
<tr>
<th>AFB Smear Result</th>
<th>NAAT Result</th>
<th>Presume TB?</th>
<th>Case Management</th>
</tr>
</thead>
<tbody>
<tr>
<td>POSITIVE</td>
<td>POSITIVE</td>
<td>YES</td>
<td>• Begin anti-TB treatment</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>• Continue to implement airborne isolation precautions and contact investigation (CI) activities while awaiting culture results</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>• Must confirm diagnosis with cultures</td>
</tr>
<tr>
<td>POSITIVE</td>
<td>NEGATIVE</td>
<td>Cannot be ruled out</td>
<td>• Request a 2nd NAAT to rule out false negatives-result</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>• Refer to Table 3 to interpret 2nd NAAT result and for recommended case management actions</td>
</tr>
<tr>
<td>POSITIVE</td>
<td>INCONCLUSIVE</td>
<td>Cannot be ruled out</td>
<td>• Request a 2nd NAAT to determine if inhibitors are present in more than one specimen</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>• Refer to Table 3 to interpret 2nd NAAT result and for recommended case management actions</td>
</tr>
<tr>
<td>NEGATIVE</td>
<td>POSITIVE</td>
<td>YES</td>
<td>• Begin anti-TB treatment while awaiting culture results</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>• Must confirm diagnosis with culture results</td>
</tr>
<tr>
<td>NEGATIVE</td>
<td>NEGATIVE</td>
<td>Cannot be ruled out</td>
<td>• Use clinical judgment whether to begin anti-TB treatment while awaiting culture results</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>• Testing for the presence of inhibitors will not be performed in this situation</td>
</tr>
</tbody>
</table>

Table 3: Interpretation of NAAT Result on 2nd Respiratory Specimen and Case Management Actions

<table>
<thead>
<tr>
<th>AFB Smear Result</th>
<th>NAAT Result</th>
<th>Presume TB?</th>
<th>Case Management</th>
</tr>
</thead>
<tbody>
<tr>
<td>NEGATIVE</td>
<td>Not Done$^7$</td>
<td>Cannot be ruled out</td>
<td>• Use clinical judgment whether to begin anti-TB treatment and/or to continue contact investigation (CI) activities while awaiting culture results</td>
</tr>
<tr>
<td>POSITIVE</td>
<td>POSITIVE</td>
<td>YES</td>
<td>• Begin anti-TB treatment</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>• Continue to implement airborne isolation precautions and CI activities while awaiting culture results</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>• Must confirm diagnosis with culture results</td>
</tr>
<tr>
<td>POSITIVE</td>
<td>NEGATIVE</td>
<td>Cannot be ruled out</td>
<td>• Use clinical judgment whether to discontinue isolation and CI activities while awaiting culture results</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>• Must confirm diagnosis with culture results</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>• If 1st specimen was smear-positive and NAAT-negative, presume the patient has a probable non-tuberculous mycobacteria infection</td>
</tr>
<tr>
<td>POSITIVE</td>
<td>INCONCLUSIVE</td>
<td>Cannot be ruled out</td>
<td>• Inhibitors were present</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>• NAAT is of no diagnostic help for this patient episode</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>• Use clinical judgment whether to begin anti-TB treatment and/or to continue CI activities while awaiting culture results</td>
</tr>
</tbody>
</table>
Amplified MTD

- Target-amplified nucleic acid probe test for detection of MTB complex rRNA in AFB smear positive and negative concentrated sediments
- Induced or expectorated sputum, BAL, BA, TA
- Useful for TB suspects who have not received therapy or less than 7 days treatment or have not received therapy in last 12 months
- Must be performed in addition to culture and smear; Neg. results do not exclude possibility of positive culture

MTD test can detect all TB complex
Xpert MTB/RIF

- Fully automated cartridge-based NAAT that uses real-time PCR
- Detects TB complex and mutations associated with RIF resistance
- Direct or concentrated sputum
- Use in conjunction with AFB smear and mycobacterial culture to address the risk of false negative results; recover MTB for further characterization and DST

Resistance mutations detected by RT-PCR should be confirmed

DNA Sequencing for Confirmation of Rifampin Resistance Detected by Cepheid Xpert MTB/RIF Assay

Allison J. McAlister, Jeffrey Driscoll, Beverly Metchock
Division of Tuberculosis Elimination, Centers for Disease Control and Prevention, U.S. Department of Health and Human Services, Atlanta, Georgia, USA

DNA sequencing of rpoB and culture-based drug susceptibility results were evaluated for samples referred for confirmation of rifampin resistance detected by the Cepheid Xpert MTB/RIF assay. Silent mutations and mutations associated with low-level resistance were found in the study population. These data support CDC recommendations to confirm Xpert rifampin resistance results.

• Culture-based DST is necessary to complement molecular results
  – Clinical relevance of some mutations is unknown
    – Not all mechanisms of resistance are understood
• Potential low PPV for detection of RIF
• Assay does not provide the specific rpoB mutation detected

Key points laboratories and clinicians must consider when adopting or performing the Xpert® MTB/RIF assay:

- Specimens received in the laboratory for TB testing are strongly recommended to receive both AFB smear and culture regardless of the NAA test result to confirm the presence or absence of MTBC.
- Specimens in which rifampin resistance is detected are strongly recommended for immediate referral to your state or local public health laboratory to receive confirmation of rifampin resistance by DNA sequencing.
- Isolates are required for all positive MTB patients in order to perform culture-based susceptibility testing and for genotyping.
- If AFB smear and/or culture are not available in-house, specimens should be sent to a reference laboratory.
- For optimal interpretation of results, perform AFB smear and culture on the same specimen. If this is not possible, collection of an additional specimen will be required for AFB smear and culture.
# Molecular methods for resistance prediction

<table>
<thead>
<tr>
<th>Method</th>
<th>FDA approved</th>
<th>Where performed?</th>
<th>Specimen requirement</th>
<th>Resistance detection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cepheid Xpert MTB/RIF</td>
<td>Yes</td>
<td>Clinical and public health labs</td>
<td>Sputum</td>
<td>Rifampin</td>
</tr>
<tr>
<td>Pyrosequencing</td>
<td>No</td>
<td>State PHLs</td>
<td>Smear positive primary specimen or isolate</td>
<td>Rifampin, Isoniazid, FQs, Injectables</td>
</tr>
<tr>
<td>Sanger sequencing</td>
<td>No</td>
<td>CDC</td>
<td>Smear positive primary specimen or isolate</td>
<td>Rifampin, Isoniazid, Ethambutol, Pyrazinamide, FQs, Kanamycin, Amikacin, Capreomycin</td>
</tr>
</tbody>
</table>

http://www.cdc.gov/tb/topic/laboratory/default.htm
### Example of mechanisms of drug resistance

**Table 1**  Mechanisms of drug resistance in *Mycobacterium tuberculosis*

<table>
<thead>
<tr>
<th>Antimycobacterial agent</th>
<th>Mechanism of action</th>
<th>Genes involved in resistance</th>
<th>Frequency of mutations associated with resistance</th>
<th>Mechanism of resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isoniazid</td>
<td>Inhibition of mycolic acid biosynthesis</td>
<td>(i) <em>katG</em> (catalase-peroxidase)</td>
<td>(i) 42–58%</td>
<td>(i) Mutations in <em>katG</em> result in failure to generate an active intermediate of isoniazid</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(iii) <em>ahpC</em> (alkyl hydroperoxide reductase)</td>
<td>(iii) 10–15%</td>
<td>(iii) <em>ahpC</em> mutations may just serve as a marker for lesions in <em>katG</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>rpoB</em> (β subunit of RNA polymerase)</td>
<td>96–98%</td>
<td>Mutations in <em>rpoB</em> prevent interaction with rifampicin</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>Inhibition of transcription</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Streptomycin</td>
<td>Inhibition of protein synthesis</td>
<td>(i) <em>rpsL</em> (ribosomal protein S12)</td>
<td>(i) 52–59%</td>
<td>Mutations prevent interaction with streptomycin. Resistance not associated with mutation in <em>rpsL</em> or <em>rrs</em> is usually low level</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(ii) <em>rrs</em> (16S rRNA)</td>
<td>(ii) 8–21%</td>
<td></td>
</tr>
<tr>
<td>Ethambutol</td>
<td>Inhibition of arabinogalactan and lipoarabinomannan biosynthesis</td>
<td><em>embC</em>AB (arabinosyl transferase)</td>
<td>47–65%</td>
<td>Over expression or mutation of EmbB allow continuation of arabinan biosynthesis. Resistance not associated with EmbB mutation is usually low level</td>
</tr>
<tr>
<td>Pyrazinamide</td>
<td>Unknown</td>
<td><em>pncA</em> (pyrazinamidase-nicotinamidase)</td>
<td>72–97%</td>
<td>Loss of pyrazinamidase activity results in decreased conversion of pyrazinamide to pyrazinoic acid, the putative active moiety</td>
</tr>
<tr>
<td>Fluoroquinolones</td>
<td>Inhibition of the DNA gyrase</td>
<td><em>gyrA</em> (DNA gyrase subunit A)</td>
<td>75–94%</td>
<td>Mutations in <em>gyrA</em> prevent interaction with fluoroquinolones. Mutations in <em>gyrB</em> and efflux may contribute to resistance</td>
</tr>
</tbody>
</table>

## Gene targets for molecular resistance

<table>
<thead>
<tr>
<th>Drug</th>
<th>Category</th>
<th>Gene Target</th>
<th>Mutation(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rifampin</td>
<td>1&lt;sup&gt;st&lt;/sup&gt; line</td>
<td><em>rpoB</em></td>
<td>81 bp core region</td>
</tr>
<tr>
<td>Isoniazid</td>
<td>1&lt;sup&gt;st&lt;/sup&gt; line</td>
<td><em>inhA</em>&lt;br&gt;<em>katG</em>&lt;br&gt;<em>ahpC-oxyR</em></td>
<td>Promoter region&lt;br&gt;Ser315&lt;br&gt;Intergenic region</td>
</tr>
<tr>
<td>Pyrazinamide</td>
<td>1&lt;sup&gt;st&lt;/sup&gt; line</td>
<td><em>pncA</em></td>
<td>Promoter and coding region</td>
</tr>
<tr>
<td>Ethambutol</td>
<td>1&lt;sup&gt;st&lt;/sup&gt; line</td>
<td><em>embB</em></td>
<td>Met306, Gly604</td>
</tr>
<tr>
<td>Fluoroquinolones (Ofloxacin)</td>
<td>2&lt;sup&gt;nd&lt;/sup&gt; line</td>
<td><em>gyrA</em></td>
<td>Gly88, Ala90, Ser91, Asp94</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>2&lt;sup&gt;nd&lt;/sup&gt; line</td>
<td><em>eis</em></td>
<td>Promoter region</td>
</tr>
<tr>
<td>Capreomycin</td>
<td>2&lt;sup&gt;nd&lt;/sup&gt; line</td>
<td><em>tlyA</em></td>
<td>Coding region</td>
</tr>
<tr>
<td>Kanamycin, Amikacin, Capreomycin,</td>
<td>2&lt;sup&gt;nd&lt;/sup&gt; line</td>
<td><em>rrs</em></td>
<td>nt1401, 1402, 1484</td>
</tr>
</tbody>
</table>

*Database of TB mutations can be found at: [https://tbdreamdb.ki.se](https://tbdreamdb.ki.se)*
GenoType MTBDRplus/ GenoType MTBDRsl

- Not available in US (CE-marked)
- Pulmonary specimen and from culture isolates
- Detects mutations in genes for drug resistance (MDR/XDR)
- Nucleic acid isolation and amplification followed by product denaturation and hybridization to probe; detection by addition of conjugate
- Results in ~5 hrs. and can be used to confirm DST

http://www.hain-lifescience.de/
Example of other methods in development

• Non-molecular
  – MODS (Microscopic Observation Drug Susceptibility) assay is a broth microtiter method where wells are periodically examined for growth

• Molecular
  – Loop mediated isothermal amplification is a form of nucleic acid amplification where DNA is generated to enable detection by visual fluorescence
  – Oligonucleotide arrays allows for the simultaneous detection of multiple genetic sequences and may be useful to detect microorganisms or mutations conferring resistance

Purpose of TB molecular genotyping

• Detect and control TB outbreaks
• Identify incorrect TB diagnoses based on false-positive culture results
• Discover unknown relationships between cases
• Identify new and unusual transmission settings
• Detect transmission between patients who reside in different jurisdictions
• Evaluate routine contact investigations and progress toward TB elimination by monitoring measures of recent TB transmission
PH applications of molecular epi tools

- Supplement traditional contact tracing
- Enhance TB control activities through identification of unrecognized chains of transmission, monitoring disease trends, and allocation of resources
  - Patient management
  - Transmission dynamics
  - Strain lineage and pathogenesis

For detailed information on TB genotyping methods, visit http://www.cdc.gov/tb/programs/genotyping/manual.htm
When is TB genotyping used?

- To detect or confirm an outbreak of TB
- When it is suspected that a cross-contamination has occurred in the TB laboratory, or other source of false-positive result
- To detect previously unsuspected patterns of transmission or outbreaks
- Three main methods currently used:
  - Spoligotyping (spacer oligonucleotide typing)
  - Mycobacterial Interspersed Repetitive Units (MIRU/VNTR)
  - Restriction fragment length polymorphism (RFLP)

Laboratories that perform TB genotyping

Michigan Department of Community Health and at the CDC perform genotyping for all TB isolates submitted from California.
Spoligotyping

- Spoligotyping is a PCR-based method which relies on the amplification of a highly polymorphic Direct Repeat (DR) locus.
- DR region consists of multiple copies of a conserved 36-41bp sequence (direct repeats) separated by multiple unique spacer sequences; used for MTB complex strain differentiation.
- Different *M. tuberculosis* strains have various complements of 43 spacers.
- Isolates of MTC bacteria can be differentiated by the presence or absence of one or more spacers- Octal code designations.
- Spoligotypes evolve by deletion of a single or multiple contiguous DVRs.
Spoligotyping

[Diagram showing DR and spacer regions with detailed patterns and labels]

Example 1:
- Original banding pattern
- Binary code
- 14+1 grouping
- Odal designation

http://www.cdc.gov/tb/programs/genotyping/chap3/3_CDCLab_2Description.htm
Multiple interspersed repeat unit (MIRU) typing is based on differences in repeats at specific loci.

Variable number of tandem repeat (VNTR) typing is based on analysis of DNA segments containing “tandem repeated” sequences in which the number of copies of the repeated sequence varies among strains.

Method relies on PCR amplification and calculation of the number of repeats on the basis of the size of the amplified product.

Total of 41 MIRU loci and 24 have been selected for genotyping; reported as 24-character designations, each character corresponding to the number of repeats at one of the 24 MIRU loci- 223225163324561333245623
Ex. 12- MIRU designation: 232234253322

<table>
<thead>
<tr>
<th>MIRU locus name</th>
<th>02</th>
<th>04</th>
<th>10</th>
<th>16</th>
<th>20</th>
<th>23</th>
<th>24</th>
<th>26</th>
<th>27</th>
<th>31</th>
<th>39</th>
<th>40</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of repeats</td>
<td>2</td>
<td>3</td>
<td>2</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>2</td>
<td>5</td>
<td>3</td>
<td>3</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>

H37Rv

Locus X

392 bp

BCG Pasteur

Locus X

288 bp
IS6110 RFLP

- Southern blot of digested DNA
- IS6110-based RFLP genotyping detects variations generated by the insertion element IS6110
- Insertion elements are capable of making copies of themselves and then inserting the copy anywhere in the genome in a process known as transposition
- Strains can differ in both the number of copies of IS6110 and the positions of IS6110 in the bacterial DNA
- Performed when strains match by MIRU and spoligotyping
The future of molecular TB epidemiology

- Current methods are dependent on determining whether strains are clonal and if they could have originated from the same source or are genetically distinct and reflect independent transmission events
- Retrospective analysis performed months after initial patient dx
- Real-time molecular epi data is currently a challenge
- Ideal methods would lead to:
  - Better understanding of genetic markers and association with clinical consequences and dynamics of epidemics
  - Development of databases containing molecular, epidemiologic, sociological, and environmental data may lead to predictions of future outbreaks or predominant strains
  - Patient and population level benefits
Whole genome sequencing and epi investigations

- Represents a relatively new method for molecular epidemiology
  - Increasingly accessible means for tracking disease outbreaks
  - Can distinguish strains with single nucleotide polymorphisms and may provide greater resolution than current methods

- Current challenges include:
  - No single platform/method protocol available
  - Read length, data assembly, and analysis
  - Criteria to define indistinguishable, closely related, possibly related, and different strains incl. outbreak thresholds
  - No current informatic infrastructure for data processing and integration of results
  - Technology remains out of reach for many clinical and public health lab due to cost and bioinformatics expertise needed
Will WGS be the future of TB contact tracing?

Using WGS, several additional cases were recognized as related.
Rapid WGS of M. tb directly from clinical samples

- Utilized biotinylated RNA baits designed specifically for M. tb DNA, captured genomic DNA directly from infected sputum samples; sequencing without culture
- Performed on 24 smear positive (1+ - 3+) sputum specimens and compared to culture and conventional molecular methods
- Sequence data was highly concordant for phenotype and predicted resistance based on genotype

• TB complex mycobacteria are slow-growing organisms that can be identified using growth characteristics, biochemical tests, and molecular methods
• Rapid identification methods for TB and determination of drug resistance is important for TB control
• New molecular methods can be used for both species identification and TB epidemiology
• Communication between lab and TB control is important for results interpretation and follow-up
Acknowledgments

• Ed Desmond, PhD, D(ABMM)

• Hector Rivas, MPH and the LA County Public Health Laboratory Mycobacteriology and Molecular Epidemiology Units

• LA County Tuberculosis Control Program

• Brian Baker, MD, MPH

Our Mission: To protect health, prevent disease, and promote health and well-being
Questions?

biosafety
Lowenstein
probe
acid
Maldi sequencing respirator
ziehl neelsen
microscopy Middlebrook
TOF PCR sputum
fast mycobacteria
DNA HPLC Tuberculosis
MS kinyoun
extrapulmonary
Jensen
mycolic