TB Laboratory Methods

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Objectives

- General overview of mycobacteriology (TB) lab practices in a U.S. public health setting
  - How labs function
  - Methods/Procedures used
  - Meaning of results
  - Challenges/Problems

Role of Lab

- Mycobacterium tuberculosis
  - Use rapid methods to detect, identify, and perform drug susceptibility testing (DST)
  - >TB vs. not TB
  - Issue rapid, clinically useful, and reliable reports
  - Develop and maintain 2-way communication - clinicians, caregivers, TB program, referring laboratories, etc.
- Non tuberculous mycobacteria (NTM)
  - Provide accurate /clinically relevant information (accurate ID IF clinically relevant/ appropriate DST IF clinically relevant)
  - Communication with clinicians (interpretation/consultation)

Types of Labs that do TB testing in U.S. (not mutually exclusive)

- Hospital/medical center laboratories (TB lab part of microbiology)
- Public health laboratories (e.g., State, county, city)
- Commercial laboratories (e.g., LabCorp, Quest, ARUP)
- Reference Laboratories (Nat. Jewish, CDC, Mayo)

- Mycobacteriology laboratory services are often dispersed
  - Work is often piecemeal; specimens/isolates referred from one lab to another
  - Communication between labs may be a problem
- Communication with care-giver/TB program a problem especially when testing becomes further removed from originating lab
### Step-by-Step

**“Typical” TB smear and culture (1)**
- Specimen received in lab
- Specimen accessioned (assigned lab number; entered into lab computer/worklog, etc.)
- Specimen stored appropriately (refrigerated) until processed – usually 1X/workday
- Specimen processed (digested/decontaminated) usually by NALC/NaOH method in batch with other specimens
- Smear prepared
- Culture media inoculated (usually 1 broth and 1 solid) and put into incubator/instrument
- Smear stained and examined and results reported same day as specimen processing

**“Typical” TB smear and culture (2)**
- Nucleic acid amplification test (NAAT) set up if appropriate/if lab offers test; some labs also do “molecular DST”
- Culture media examined as prescribed by method (automated/manual) for 6-8 weeks
- If growth detected, smear made and stained to confirm presence of AFB (acid fast bacilli)
- If AFB, go onto identification (e.g., HPLC, nucleic acid probe)
- If TB, make appropriate notifications and perform drug testing as appropriate
- If no growth, keep 6-8 weeks and sign out as “negative for mycobacteria”
- Turn-around-times depend on how these “steps” are put together

### Specimen collection

**(Garbage In = Garbage Out)**
- Sterile, leak-proof, disposable, non-breakable, appropriately-labeled lab-approved containers
- Collect aseptically, or bypass contamination as much as possible
- Avoid contamination with tap water (NTM may be in water)
- Collect prior to therapy if possible
- No swabs, fixatives, preservatives
- Transport immediately or refrigerate

### Sputum collection

- Early morning; different days; at least 3 but not more than 5-6 (new recommendations 3 within 48 h)
- 3 specimens to rule out TB
- Follow-up specimens at 2 months appropriate therapy (for smear, culture, and AST if culture +)
- Patient must be instructed about proper collection
Processing Pulmonary Specimens - Digestion/Decontamination

- Facilitate optimal recovery of mycobacteria
- Specimens are a complex organic matrix contaminated with a variety of organisms that can rapidly outgrow mycobacteria in/on media
- Liquefaction (digestion) often necessary - organic debris can protect mycobacteria from decontaminant
- NALC (liquefaction) and NoOH (decontamination) most commonly used in USA
- 30% or more mycobacteria killed during process!!!!

Concentration by Centrifugation

- After digestion/decontamination, material is concentrated
- At least 3,000 X g (rcf not rpm); can increase speed if use refrigerated centrifuge
- Safety "aerosol-containing" buckets/carriers/rotors
- In U.S., most microscopy is done on concentrated specimens

Acid-Fast Microscopy

- Acid-fastness
  - Resistance to decolorization with acid-alcohol
  - Cell-wall mycolic acid residues retain the primary stain (contains phenol)
- Methods
  - Fuchs-in-based (Light microscope)
    - Ziehl-Neelsen (heat); Kinyoun
  - Fluorochrome (Fluorescence microscope)
    - Auramine O; auramine rhodamine
    - Recommended staining procedure

AFB Microscopy

- Not very sensitive
  - 50-70% for pulmonary TB; correlates with disease severity and infectiousness; sensitivity much less in extrapulmonary TB
- Not specific for Mtb (PPV depends on the prevalence of NTM)
- Inexpensive and quick
- Value for TB
  - Rapid; 1st bacteriologic evidence of TB
  - Infectious patients
  - Follow therapy (AFB in smear are quantified)
  - Determine need for additional testing (e.g., NAA)
- Primary method for TB diagnosis in developing countries (http://www.phppo.cdc.gov/dls/afb/default.asp)
Microscopy vs. Culture

- 5,000 to 10,000 AFB/ml for smear
- 10 to 100 AFB/ml for culture (much more specimen goes into culture)

Significance of culture
- Confirm TB/mycobacteriosis; obtain isolate for DST, typing; evaluate therapy (if culture is + at 2 months, increase length of therapy)
- Only 85-90% cases of pulmonary TB are culture + (culture negative TB; clinical diagnosis)

Culture on Solid Media

- Middlebrook agar
- Lowenstein-Jensen media (egg-based)
- 6—8 weeks

- Advantage – can see colonies on surface of media

Broth-based Culture Methods

- Broth-based systems - some are highly automated
  - BACTEC 460; MGIT; TREK; MB/BacT
  - More rapid recovery than solid media
  - Current recommendations are to use at least one piece of solid media with the broth (mixed culture detection; increased sensitivity)

BACTEC 460 Instrument

- Semi-automated; needles
- Laboratory work-horse
- 12B media
- Radiometric
- Detects CO₂ production by mycobacteria
- DST for INH, RMP, EMB, PZA
Mycobacteria Growth Indicator Tube (MGIT)
- Fluorescence quenched by $O_2$ in $O_2$-rich media
- If mycobacteria present, $O_2$ used up, no quench, fluoresces under UV light
- DST for INH, RMP, EMB, PZA

BacT/Alert 3D bioMerieux
- Colorimetric sensor and detection technology
- Tracks CO$_2$ production (sensor turns yellow)

VersaTrek TREK Diagnostic Systems
- Unique growth matrix - cellulose sponge material
- Monitors rate of $O_2$ consumption
- INH, RMP, EMB, PZA DST available

Identification of Mycobacteria
- Presumptive or preliminary ID based on growth characteristics on solid media
  - Colony morphology
  - Pigment
  - Rate of growth
Identification of Mycobacteria

- Conventional biochemical tests (all mycobacteria) - 2-21 d (may not necessarily be accurate for NTMs)
- HPLC of cell wall mycolic acids ("all" mycobacteria) - 2 h - reference labs
- FDA-approved commercially available genetic probes (ACCUPROBE, GenProbe, San Diego, CA; www.gen-probe.com)
  - probes for MTb Complex, MAC, M. kansasii, M. gordonae)
  - 2-4 h - clinical labs
- RUO (Research Use Only) commercially available products
- "in-house" PCR/RE analysis/genetic sequencing/etc. - 1-2 d - reference labs/clinical labs

Susceptibility Testing of M. tuberculosis (NCCLS Standard M24-A; 2003)

Primary Antituberculosis Drugs

- Use a rapid method (e.g. BACTEC 460 or MGIT)
- Perform on all initial isolates from patients
- In addition, test isolates from relapse or re-treatment cases and if drug resistance is suspected

Test primary drugs:
- Isoniazid (INH)
- Rifampin (RIF)
- Ethambutol (EMB)
- Pyrazinamide (PZA)
- Streptomycin (SM)

Susceptibility Testing of M. tuberculosis Complex – Recommended Secondary Testing Panel (NCCLS M24-A)

- Secondary Drugs (test if R to rifampin or any 2 primary drugs)
  - Ethambutol hydrochloride (higher concentration)
  - Capreomycin
  - Ethionamide
  - Kanamycin (class for amikacin*)
  - Ofloxacin (class for quinolones)
  - p-Aminosalicylic acid
  - Rifabutin
  - Streptomycin (2 concentrations)
  - (Cycloserine testing not recommended – hard to reproduce)
- * new recommendations – test amikacin as well
DST of MtbC in U.S.
Molecular Methods

- Molecular assays for RIF and INH
  - Available in a few jurisdictions
  - Performed directly on clinical specimens or on culture isolates
  - Results available within 1-2 days
- In-house developed assays: molecular beacons, pyrosequencing, RT PCR
- Commercial assays: HAIN and INNO-LIPA line probe assays

Susceptibility Testing of M. tuberculosis

- Problem/Challenge
  - Rapid methods for first line drug testing is routine
  - No standardized methods for rapid testing of second-line drugs – some laboratories have validated in-house

Molecular Detection of Drug Resistance (MDDR) Service at CDC: Rationale

- Clinical/Program
  - Make rapid confirmation of MDR TB available
  - Make laboratory testing data available to clinicians about second-line drug resistance in cases of RIF-resistant or MDR TB
- Development
  - Continuous correlation of molecular (genotyping) results and DST (phenotypic) results
  - Addition of new drugs and alleles
- Research
  - Determination of mechanisms of resistance

Recommended Methods/ Turn-Around Times

- Specimen transport - 24 hours from collection
- Smear - fluorescent acid-fast microscopy - 24 hours from receipt
- Culture - broth-based system
- Identification - rapid (DNA probes; HPLC) - TB vs. NOT TB - 21 days from specimen receipt (faster if isolate)
- Primary drug susceptibility testing - broth system - 28 days from specimen receipt (much faster if isolate)

- Pieces need to fit together!!!!!!
Direct Detection of *M. tuberculosis* in Clinical Specimens - Nucleic Acid Amplification (NAA) Tests

- **Objectives:**
  - Detect/identify Mtb directly from clinical specimens
  - Avoid the weeks required for culture
- **Commercially-available tests:**
  - Amplified Mycobacterium Tuberculosis Direct test (AMTD); Gen-Probe
    - FDA-approved for detection of Mtb in AFB sm (+) AND sm (-) respiratory specimens from suspected TB patients
  - AMPLICOR; Roche
    - FDA-approved for detection of Mtb in AFB smear (+) respiratory specimens ONLY from suspected TB patients
  - In-house validated tests (RUO and Laboratory Developed Tests [LDT])
  - **CANNOT** replace clinical judgment or be relied on as the ONLY guide for therapy or isolation practices (less than perfect sensitivity and specificity)

Nucleic Acid Amplification Test (NAAT) for TB Diagnosis

- Becoming standard of practice (especially in certain clinical situations)
- Laboratory should have protocols in place for which specimens/patients to test; how to report results (qualifications) – requests for “off-label” use are common
- Batching may “defeat” purpose of offering rapid test
- Potential benefits – impact on therapy, public health measures, respiratory isolation, invasive procedures
- New recommendations: *MMWR* January 16, 2009/58(01);7-10

Accuracy problems in the Mycobacteriology Lab – False-negative and False-positive results

- **False-negative cultures** - over-decontamination; improper collection/transport; overheating during transport/centrifugation; media not inoculated
- **False-positive results** - Test result on a patient’s specimen (smear and/or culture) that is positive for a species of mycobacteria that in reality is not infecting the patient
  - Occur sporadically or as outbreaks
  - May result in misdiagnosis, unnecessary and costly therapy and medical treatment, unnecessary public health interventions

Review of False-Positive Cultures for Mtb; Recommendations for Avoiding Unnecessary Treatment

- Review of published studies
- Median false-positive rate = 3.1%
- 158/236 (67%) patients treated – toxicity, unnecessary hospitalization/testing/contact investigations
False Positive Cultures

- Cross-contamination - Source may be another patient's specimen/isolate, Proficiency Testing specimen/isolate, QC isolate; splashes, transfer on tools, aerosols during processing; contaminated reagents
- Specimen problem - improper specimen collection/labeling; specimen mix-up (not necessarily in the lab); AFB in water
- Clerical errors
- Lab should have protocol in place to detect
- Rapid genotyping can help
- Can cause a lot of problems!!!!!

Clues: False-Positive Results

- Smear
  - Negative control for AFB smear shows AFB
  - Increase in number of smear (+), culture (-) specimens
- Culture
  - Late appearing cluster of positive cultures which have scanty growth
  - Large number of isolates of a species that is rare in your lab -- may be drug-resistant Mtb
  - Large number of isolates of an "environmental" bug
  - Only 1 positive culture from multiple specimens submitted
- The clinician/TB controller/etc. calls you and says "this is impossible!"

Contamination Episode(s)

- 3 cases MDR-TB within a 2 day period
  - 1 case was sm + X2 - specimens processed on consecutive days
  - 2 cases sm -. Both patients had 3 specimens cultured, but only 1/3 culture + (7H11 only; BACTEC -). The positive cultures processed on consecutive days; right after one of the sm + specimens
- Smear (+) specimen grew Mtb. Only 1 specimen submitted on the patient.
  - Patient seen in ED for broken arm - no sputum specimens taken.
  - Labeling mix-up in ED
  - Not really "contamination!!"

Work-up of Suspected False Positive

- Review records
- COMMUNICATE - clinician, public health, lab director, etc. (easier to be pro-active with plan in-hand).
- Genotyping may help (especially if real-time and need to make treatment decisions); Genotyping cannot prove it!!
- Correct the "problem"
- Document, document, document!!!!
Summary of Challenges

- Decrease in TB will make it difficult for laboratories to maintain proficiency and/or efficient testing algorithms; status of NTM is continually changing
- Despite new technologies, Mycobacteriology still requires referral and different levels of service - sometimes the “pieces do not fit”
- There is a need for greater coordination between the public and private sectors for referral services -> 2-way coordination

- National Plan for Reliable Tuberculosis Laboratory Services Using a Systems Approach - MMWR 2005; 54 (RR-6)