

TB Laboratory Techniques for Diagnosing Tuberculosis

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Objectives

- Overview of laboratory role in TB diagnosis and control
- Techniques for diagnosing TB
- Additional lab results critical to effective case management
- Relevance of lab results to clinical and public health settings

Tuberculosis Past to Present

- 5000 BC Evidence of TB in Spine of Neolithic Man
- 400 BC Hippocrates first described TB
- 1882 Koch discovers TB bacillus
- 1882 Ehrlich describes acid-fast TB
- 1895 Roentgen – X rays
- 1908 Mantoux skin test
- 1921 BCG Vaccine
- 1932 PPD (purified protein derivative)
- 1944 Waksman – Streptomycin
- 1947 First Antibiotic treatment for TB
- 1949 PAS developed
- 1951 Isoniazid developed
- 1975 Bactec 460 TB rapid broth isolation system developed
- 1985 Genetic probes for TB developed (growth based)
- 1995 Direct specimen Genetic Probe for TB (PCR amplification)
- 2003 Gamma Interferon Blood Test for TB
- 2009 Molecular based susceptibility testing

Lab Testing - an integral part of TB diagnosis & control

- Might patient have TB?
- Might TB patient be infectious?
 - How infectious?
 - Is patient responding to therapy?
- Might patient have drug resistant TB?
 - Is patient developing resistance to therapy?
 - Which drugs to use?
- Might patient's TB be mixed with MOTT?
- What strain of TB does patient have?
 - Do other people have the same strain?
 - Reactivation or newly acquired?

To Diagnose TB "Consider The Big Picture"

- "History" of exposure or past treatment
- TB "skin test" or blood test
 - Indication of TB infection
- Clinical "signs/symptoms"
 - Suggestive of TB infection/disease
- Lab "test results"
 - Presence of AFB
 - Presence of TB (infection) or MOTT (casual occurrence, colonization or infection)
 - Mixed TB/MOTT infection?
 - Patient's relationship to other TB cases

Lab Test Results

- First evidence of AFB infection (TB)
- Support TB clinical diagnosis
- Clarify progress of TB infection/disease
- Determine infectivity of patient
- Determine appropriate drug regimen
- Monitor for development of resistance
- Confirm treatment success
- Determine relationship to other cases

CDC Recommended National Standards For AFB Testing Laboratories

- Promote rapid delivery of specimens to the laboratory (goal is delivery in lab within 24 hrs from collection)
- Use fluorescent acid-fast staining and microscopic examination (goal reporting AFB smear result within 24 hrs from receipt of specimen in lab)
- Use rapid broth system for primary culture detection of AFB (goal to reduce culture detection of AFB from 3-4 wks to 1-2 wks)
- Use rapid ID, i.e. HPLC/genetic probes (goal ID TAT* 14-21 days from receipt of specimen in lab)
- Use rapid broth susceptibility testing (goal TAT 21-28 days from receipt of specimen in lab)
- Report susceptibility results to attending physician as soon as available, i.e. by phone or FAX

*TAT = turn around time

Current Goals Promoted By CDC

- Use methods and attain or maintain the TATs cited in Tenover et al (1993), CDC/APHL recommendations and Healthy People 2010
- Decrease specimen transport to lab from 3 to 1 day
- Increase the number of patients tested using NAA-TB assays
- Reduce TAT of NAA-TB testing to 48 hrs from date of specimen collection
- Increase the % of new TB susceptibility results reported within 28 days
- Improve rapid communication and networking of AFB test results
- Promote the use of molecular methods of TB detection and susceptibility testing
- Refer TB isolates with resistance to primary TB drugs for secondary antibiotic susceptibility testing

Today's TB Lab Technology

- Chemical specimen decontamination
- Fluorescent acid-fast microscopy
- Rapid broth culture
- Indirect HPLC AFB identification
- Indirect genetic probe TB identification
- Direct specimen NAA TB testing
- Rapid broth based susceptibility testing
- Molecular based ID and susceptibility testing
- Interferon Gamma Release Assays

TB Specimen Sources

- Sputum (primary)
- Pulmonary aspiration (secondary)
- Gastric aspiration
- Body fluids
- Tissues
- Blood
- Stool
- Other

Specimen Collection (1)

- Collect in sterile, leak-proof containers
- Refrigeration of specimen is recommended to reduce overgrowth during transit to lab
- Deliver specimen to TB lab within 24 hrs
- Goal: to process specimens ASAP after collection to minimize effects of contaminating bacteria
- Always include patient name on both test request form and the specimen container (CLIA requirement)

Specimen Collection (2)

- Pulmonary specimen (usually sputum)
 - Early morning specimens = highest yield of AFB
 - Recommended 3 consecutive specimens in 18-24 hrs (at least 1 early morning specimen)
 - Recommended volume for testing is 5-10 ml, low volume specimens may compromise test results
 - If patient cannot produce sputum, then induce sputum, perform bronchial or gastric washing (less preferred)
 - 24 hr collections (pooled specimens) are not acceptable for testing

Specimen Collection (3)

- **Gastric Wash / Lavage**
 - Collect only when other pulmonary secretions cannot be collected
 - Early morning collection
 - Neutralize with sodium carbonate within 4 hrs of collection, must be neutralized to pH 6.0 – 8.0
 - Specimens must be received in the laboratory for testing within 72 hrs of collection
 - Recommended volume for testing is 5-10 ml, low volume specimens compromise test results
- **Urine**
 - 3-5 early morning specimens
 - Clean catch midstream to avoid contamination
 - No preservatives or pooling of specimens

AFB Tests

- AFB Smear Examination (24 hrs)
- Amplified Genetic Probe ID (24-48 hrs)
- Culture Isolation (7-10 da)
- Indirect Genetic Probe ID (8-14 da)
- Indirect HPLC ID – MOTTs (8-14 da)
- Indirect Susceptibility (12-18 da)
- ID Confirmation (3 wks)
- DNA Fingerprinting (1-2 wks)
- Interferon Gamma blood assays for *M.tb*
- Culture still requires 6 weeks for negative rpt.

Specimen Processing

- Requires Class II Biological Safety Cabinet
- Requires use of BSL 2 lab facility, using BSL 3 laboratory practices
- Low volume specimens reduce proportionally the sensitivity of AFB slide and delays AFB growth on culture
- Molecular testing requires a significant portion of the specimen

Processing Pulmonary Specimen

- Optimal volume of specimen - 7 mL
- Optimal # specimens – 3 early am on 3 successive days
- Digestion/Decontamination/Concentration
 - Liquefy using a mucolytic agent to dissolve mucin in the specimen
 - Decontaminate using a strong alkali which kills non-AFB much more readily than AFB, particularly *M. tuberculosis*
 - Concentrated by centrifugation at a minimum 3000 G's
 - Pour off supernatant leaving a concentrated sediment pellet
 - Concentrated (spun) sediment is used to prepare smear, inoculate media and perform direct molecular testing

Acid-Fast Smear Examination

- Purpose
 - Determine presence of acid-fast organisms in specimen
 - Assess infectivity of patient, i.e. AFB load
 - Monitor TB drug therapy
- General Comments
 - AFB smear result may assist with diagnosis of TB
 - AFB difficult to stain due to the high lipid content of cell wall (mycolic acids)
 - Unique capability of binding fuchsin stain which cannot be de-colored by acid-alcohol
 - AFB staining detects both viable and non-viable organisms
 - Recommended to be tested, read and reported within 24 hrs of specimen collection and/or receipt of specimen in laboratory

Acid Fast Staining

- Fluorochrome stain
 - Fluorochrome stained smears require a fluorescent microscope utilizing UV light source
 - Generally read at 250x-450x magnification which allows rapid scanning of the entire smear
 - Not all fluorescence is due to AFB
- Carbol fuchsin-based stain
 - Utilize a regular light source
 - Read at 1000X magnification (can observe only a portion of the smear, i.e. 300 fields)
 - Types: Ziehl-Neelsen and Kinyoun
 - Used to confirm acid-fast fluorescent bacilli

AFB Smear – Ziehl Neelsen Interpretation

<u>AFB Seen (1000X)</u>	<u>Report</u>
0	No AFB Seen
1-2 / 300F (3 sweeps)	Doubtful; repeat
1-9 / 100F	Rare (1+) AFB
1-9 / 10F	Few (2+) AFB
1-9 / F	Moderate (3+) AFB
>9 / F	Numerous (4+) AFB

AFB Smear

- Least sensitive of all AFB tests (20-75%)
- Requires 10,000 AFB/ml for a slide to be positive
- If positive, the patient can infect others
- Positive smear cannot determine AFB viability
- Positive smear does not determine whether TB or MOTT
- To be reported within 24 hrs of receiving the specimen in the laboratory

AFB Culture Examination (1)

- **Solid media**
 - Growth media (2 types):
 1. Egg-based, i.e. Lowenstein Jensen, etc
 2. Agar-based, i.e. Middlebrook 7H11, etc(Advantage: colony morphology can be visualized to assist with ID)
 - Selective media, i.e. Lowenstein Graft and Middlebrook 7H11S
(Advantage: Less overgrowth due to contamination)
 - Requires 14-21 days for AFB growth

AFB Culture Examination (2)

■ Liquid media

- Growth media, i.e. Dobois, Middlebrook 7H9, etc.
- Selective Media (several commercially available, i.e. Bactec/MB BacTAlert/VersaTrek)
(Advantages: allows rapid detection of AFB and used for rapid susceptibility testing of primary TB drugs)
(Disadvantage: more overgrowth due to contamination than solid media)
- Requires 4-14 days for AFB growth

AFB Culture Test (7-14 days)

- More sensitive than AFB smear
- Only 10 AFB/ml of specimen can produce a positive result
- Culture may be positive even though the smear was reported negative for AFB
- Rapid broth testing – normally positive within 1-2 wks, sometimes less
- Positive culture result may be either *Mycobacterium tuberculosis* complex or MOTT
- Requires 6 wks to report culture as negative
- AFB growth used for identification, susceptibility and genotyping testing

AFB Identification Testing

To help rule in or out TB as soon as possible:

- Direct "Amplified" Nucleic acid probe test (performed on patient specimen)
- Indirect Nucleic acid probe tests
 - DNA probe tests are species specific
- Indirect high performance liquid chromatography (HPLC)
 - HPLC uses a chromatography method to identify several mycobacterial species
 - This method is normally only available at reference laboratories

Nucleic Acid Amplification (NAA) Testing

- Qualitative test
- Rapid test (same day)
- Positive NAA and positive smear is sufficient for presumptive TB diagnosis
- Sensitive (95% sm-pos / 70% sm-neg)
- Specific (99% *M. tuberculosis* complex)
 - Incl. *M. bovis*, *M. kansasii*, *M. szulgai*, *M. marinum*
 - Not positive due to BCG
- Not a test of viability or cure
- Pulmonary specimens only
- Only available at reference labs
- Reduces subsequent test sensitivity
- Delays culture & susceptibility results

GEN-PROBE® MTD – Direct Amplified Probe for *M.tb* Complex

- Rapid test (24-48 hrs)
- Sensitivity – smear neg 72%/smear pos 97%
- Specific – smear neg 99.3%/smear pos 100%
- Non-bloody, pulmonary specimens only
- Specimen processing may cause inhibition
- Reduces subsequent test sensitivity
- Delays culture & susceptibility results
- Not a test of viability or effective therapy
- Not available in most laboratories
- Is available at some reference laboratories

New CDC Guidelines of Use of MTD MMWR January 16, 2009

“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.”

'09 Guidelines (continued)

At least one specimen, preferably the first diagnostic specimen, from each patient to be tested by NAA should be processed, suspended in a sufficient volume of buffer to ensure adequate sample volume for all planned tests (e.g. microscopy, culture, and NAA) and tested using an NAA test for TB. NAA testing should be performed in accordance with the manufacturer's instructions or a validated standard operating procedure.

'09 Guidelines (continued)

Interpret NAA test results in correlation with AFB smear results.

- If NAA + and AFB smear +, then TB
- If NAA + and AFB smear -, then re-test, i.e., 2 NAA+'s and AFB smear - = TB
- If NAA - and AFB smear +, test for inhibition
 - If inhibition, then no result
 - If no inhibition, then MOTT
- If NAA - and AFB smear -, then await culture (NAA testing only detects 50-80 % of AFB - and culture + TB's)

Susceptibility Testing (1)

■ When to test

- All new (initial) *M.tb* isolates
- Suspected drug resistance
- Repeat after 90 days if specimens continue to produce *M.tb*
- Relapse or failed therapy

Susceptibility Testing (2)

■ Direct

- Performed directly from specimens with positive smears
- More accurate assessment of resistance

■ Indirect

- Performed on TB growth from culture

Susceptibility Testing (3)

■ Methods

- "Rapid Broth" method for primary anti-TB drugs (results in 1-2 weeks)
- "Proportion Plate" method – "Gold Standard" for 1st, 2nd-line drugs (final read @ 3 weeks)
- "Molecular PCR" assays (not FDA-approved)

Primary Anti-TB drugs

- Isoniazid (INH)
- Rifampin (RIF)
- Ethambutol (EMB)
- Pyrazinamide (PZA)

Secondary Anti-TB Drugs

- Fluoroquinolone (Cipro/Oflox, etc.)
- Kanamycin
- Ethionamide
- Cycloserine
- Capreomycin
- Amikacin
- PAS
- Streptomycin

Susceptibility Testing (Report in 28 days)

- Performed as soon as growth from culture has been identified as *M. tuberculosis* complex
- Primary antibiotic susceptibility report may be expected 1 wk from the *M. tb* identification report
- PZA susceptibility report may be expected 1 wk after the primary drug report
- Secondary antibiotics may be tested by request and are reported approx. 3 wks after initiated

NAAT Testing for Drug Resistance (1)

- Detect genetic sequences which:
 - Block activity of drug
 - i.e. *rpoB* - prevents binding of RA to RNA polymerase and inhibition of transcription
 - Block conversion of drug to an active form
 - i.e. *katG* - causes loss of ability of catalase to activate INH
 - Cause binding or destruction of drug
 - i.e. *inhA* - increases *inhA* protein which binds INH and reduce effective concentration below inhibitory levels

NAAT Testing for Drug Resistance (2)

- Molecular beacons assay
 - Direct specimen testing
 - Detect *M. tb.*
 - Detect resistance to INH and Rifampin
 - Available at California Microbial Disease Laboratory (Edward Desmond)
 - Not FDA approved, i.e. "home brew in CA"
- Line probe assays
 - Not FDA approved
 - Commercially available in Europe

CDC – Molecular Testing for TB Drug Resistance (1)

- Testing for DNA sequences associated with 2nd-line drug resistance
- Testing available September 2009
- 3-4 day turn around time

CDC – Molecular Testing for TB Drug Resistance (2)

- Secondary drugs tested
 - Isoniazid
 - Rifampin
 - Kanamycin
 - Amikacin
 - Capreomycin
 - Fluoroquinolone

IGRA – Interferon Gamma Release Assays

- TB Antigen stimulates T cell (memory) to produce TB-specific IFN γ release (TB specific)
- Advantages:
 - No boosting
 - One patient visit
 - No reader variability
 - One day result
 - Latent TB
 - Not affected by BCG
- Disadvantages:
 - Availability
 - Universal acceptance of result
 - Requires lab skills and instrumentation
 - HD's tendency to target and batch
 - Cost
 - Need more studies in children, elderly and HIV-infected populations

IGRA – Interferon Gamma Release Assay Blood Tests for *M.tuberculosis* Two FDA Approved Tests

- Cellestis – QuantiFERON® TB Gold
- Oxford Immunotec – T-SPOT. TB® (May 2008 FDA Approval)
- Becoming more accepted and more available

QuantiFERON® TB Gold

- Antigen stimulation of fresh whole blood
 - ESAT-6 and CFP-10
- Measure interferon-gamma released after stimulation
- Positive reactions against:
 - *M. tuberculosis*
 - *M. bovis*
 - *M. kansasii*
 - *M. szulgai*
 - *M. marinum*
- Non-reactive to BCG
- Results are qualitative

TB DNA Genotyping Universally Offered by CDC

- CDC
- Michigan Department of Community Health – Eastern States
- California Department of Public Health – Western States

Genetic Typing

- Group isolates by “identical” genetic types (clusters)
- Companion to “regular” epidemiology or contact investigation
 - I.e., NOT a replacement for contact investigation
- Primary typing PCR methods (15 day TAT)
 - Spoligo - spacer oligonucleotide
 - MIRU - mycobacterial interspersed repetitive units
- Alternate non-PCR method (30 day TAT)
 - RFLP - Restriction Fragment Length Polymorphism

Genetic Typing

- MIRU & Spoligo typing performed on all new culture-confirmed isolates
 - Isolates w/ same patterns for both tests are considered a cluster
 - Cluster may indicate ongoing transmission – need to interpret in conjunction with epidemiologic or contact investigation data
- RFLP typing may be performed in certain circumstances to differentiate clusters

APHL's Stop TB Partnership

"Evidence-based Tuberculosis Diagnosis"
website information resource

CDC's Recommendations for NAA and IGRAS

<http://www.tbevidence.org>

Questions?
