World TB Day – Laboratory Aspects of Tuberculosis

Ohio Department of Health Laboratory

March 26, 2019

Mycobacteria Collection and Testing Guidelines

	Clinical Specimens	Reference Specimens	Direct Detection	Susceptibilities
Purpose	Culture for mycobacteria in clinical specimens with subsequent identification of isolates	Identification of acid-fast bacilli	Detection of *Mycobacterium tuberculosis* DNA in sediments prepared from sputum, bronchial specimens, or tracheal aspirates.	Antibiotic susceptibility testing of *Mycobacterium tuberculosis*
Tests Include	Acid Fast Stain, Nucleic Acid Amplification, Culture	Acid-fast stain; DNA probe; high performance liquid chromatography (HPLC)	Nucleic acid amplification (Polymerase Chain Reaction – PCR)	Non-radiometric broth susceptibility test for the following antituberculous drugs: Streptomycin, Isoniazid, Rifampin, Ethambutol and Pyrazinamide
Sample Requirements	Minimum 5 ml of unprocessed sputum, bronchial specimen, or tracheal aspirate. Collect in sterile 50ml screw-capped centrifuge tubes (BD Falcon 352070 or equivalent)	Pure subculture of organism on agar slant or liquid medium to support organism growth (tubed media preferred). Specimens should not be submitted on plates	At least 500 µL of sediment or a minimum 5 ml of unprocessed sputum, bronchial specimen, or tracheal aspirate. Collect in sterile 50ml screw-capped centrifuge tubes (BD Falcon 352070 or equivalent)	Pure subculture of TB on agar slant or liquid medium to support organism growth (tubed media preferred). Slant <14 days **OR** MGIT+ within 5 days of becoming positive.
Shipping Requirements	Ship overnight using Biological Substance, Category B classification and an insulated box with cold packs	Ship at ambient temperature using Infectious Substance, Category A classification for suspect *M. tuberculosis* isolates. Ship all others using Biological Substance, Category B classification.	Ship overnight using Biological Substance, Category B classification and an insulated box with cold packs	Ship at ambient temperature using Infectious Substance, Category A classification for suspect *M. tuberculosis* isolates.
Turnaround Time	1 day (AFB smear) 21 days (culture ID) 56 day No Growth (culture)	7 days	2 days	14 days from ID (isolates), 17 days from ID (culture)

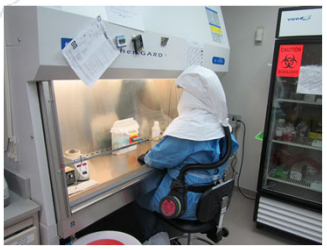
Current Algorithms for TB testing

- Specimen Processing- w/in 7 days of collection
- Nucleic acid amplification- TB PCR for new patients
- AFB Microscopy- 48 hr TAT
- Inoculate Media- Incubate up to 8 weeks
- Culture Positive
- Species Identification- 21 days from receipt
- Drug Susceptibility if MTBC- 7 to 21 days

Molecular Detection of Drug Resistance

Positive Air Pressure Respirators

Gowning up and Preparing the BSC



Processing or any manipulation of a sample is carried out inside a Biological Safety Cabinet (BSC) in a Biosafety Level 3 Laboratory

Specimen Processing (Decontamination/Digestion)

- Most respiratory specimens for Mycobacterial culture are contaminated by various organisms capable of outgrowing Mycobacteria.
- Generation time for MTB is 24-48 hr (40-60 minutes for common flora)
- The optimal recovery of Mycobacteria depends on striking a balance between eliminating contaminating organisms while releasing Mycobacteria trapped in mucin and cells
- Not necessary for aseptically collected specimens from normally sterile sites

Sodium Hydroxide (NaOH) /N-acetyl-L-cysteine (NALC)

- Decontaminant and mucolytic agent, only marginally less harmful to Mycobacteria
- High lipid content is protective, but NaOH may kill 20-90% of Mycobacteria present
- NALC liquefies sputum and enhances recovery by permitting the use of lower concentrations of NaOH
- Concentration by centrifugation (>3000 x g for 15min) necessary to sediment buoyant, high lipid content cells for staining and culture

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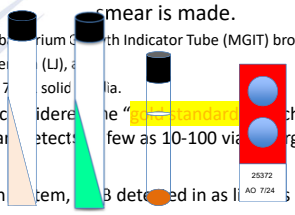
Culture Innoculation

After processing, 3 types of media are inoculated and a smear is made.

- Bactec Mycob rium th Indicator Tube (MGIT) broth system,
- Lowenstein Je (LJ),
- Middlebrook 7 solid lia.

Culture is still c idere e "gold standard" ch more sensitive than smear etect few as 10-100 via rganisms/ml)

With broth tem, B dete ed in as l s 6 to 10 days



Treatment of only a few days can slow or prevent the recovery of Mycobacteria

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Acid Fast smear

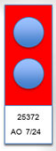
Read within 24 hrs of specimen receipt

Overall sensitivity low, est. (5,000-100,000 cells/ml needed for detection by smear)

Positive smear means patient more likely capable of transmission

Does not provide information on identity or viability

May be positive for 2-10 weeks post treatment



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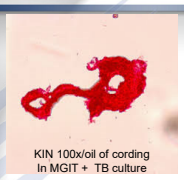
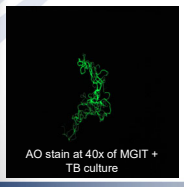
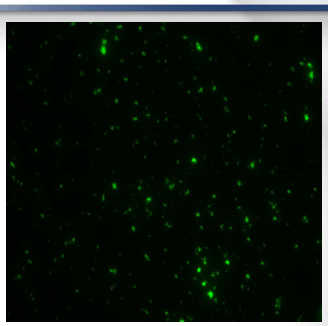

KIN 100x/oil of cording In MGIT + TB culture


AO stain at 40x of MGIT + TB culture


AO at 40x of 4+ clinical smear

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M.tb identification in clinical specimens

- PCR can be run on clinical samples.
- PCR is a nucleic acid amplification test (NAAT).
- Normal sample processing occurs in the BSL3. Following concentration and decontamination in the BSL3, samples are heated for 1 hr at 80ºC to kill all bacterium in the sample. Samples are then lysed using glass beads, heated briefly, centrifuged to remove cell debris and supernatants containing DNA are used in real-time PCR.

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PCR for Detection of Tuberculosis from Clinical Specimens

Polymerase Chain Reaction is a technique that copies and amplifies specific regions of DNA/RNA.

- Primers are used to amplify a specific region of interest. (IS6110 region)
- 1 to >25 copies per bacterium
- Should be interpreted in conjunction with other laboratory and clinical data.

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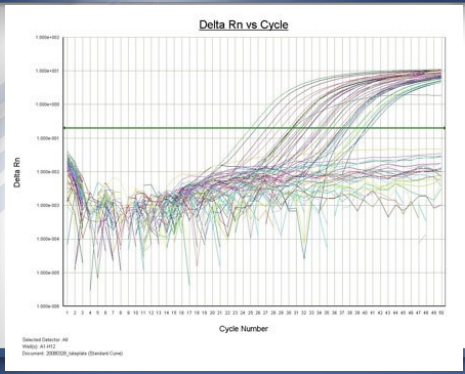
Results



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PCR-polymerase chain reaction

Summary

- Nucleic acid amplification based test
- Rapid (<2 days) direct detection in clinical samples, usually reported same day
- Used on sediments (at least 1/2ml) within 3 days of processing or raw specimen (5ml) within 7 days of collection.
- For patients who have not received treatment in past 12 months or less than 7 days of treatment
- For direct detection in clinical respiratory samples. Non respiratory sites are run with a disclaimer.

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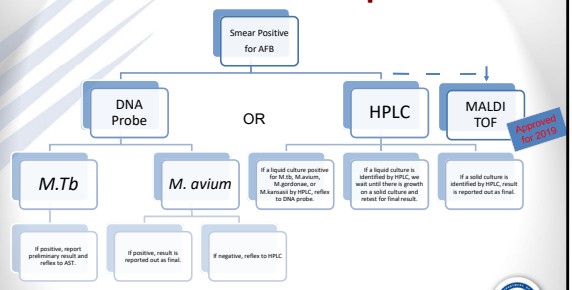
When a MGIT becomes positive. . .



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ASSAYS FOR IDENTIFICATION OF POSITIVE CULTURES (REFERENCE ISOLATES)

DNA Probe

-NOT amplification based.
-Targets ribosomal RNA sequences which are unique to MTB Complex organisms
-Does not differentiate between members of the MTB complex (M. tuberculosis, M. bovis, M. bovis BCG, M. africanum, M. microti, and M. canetti)
-Not for direct identification in clinical samples– requires an isolate

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DNA Probe

- Cells are lysed and an acridinium labeled single stranded DNA probe forms a stable complex with complimentary ribosomal ribonucleic acid (rRNA)of the target organism.
- Unbound probe is chemically destroyed
- Chemiluminescence of the bound probe is measured

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DNA Probe

- Fast - can be performed in as little as 2 hrs., depending on batch size
- Sensitivity 99.2%, Specificity 99.9%
- Currently batched and run once a week, usually on Monday
- Can be performed on liquid or solid culture, but does not guarantee a pure isolate for susceptibility testing
- Expensive, approximately $20-$50 per test (depends on batch size)

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High Performance Liquid Chromatography-HPLC

High Performance liquid chromatography (HPLC) is an identification test that can provide species specific identifications of microorganisms isolated in pure cultures on solid medium based on their mycolic acid composition profile.

Harvested cells are extracted and mycolic acid esters are separated using a reverse-phase cartridge column with a methanol/isopropyl alcohol gradient elution.

The resulting peaks are identified by their relative retention times, which are determined with a high molecular weight internal standard. The chromatographic patterns are then compared to a library of known patterns using a pattern recognition software that will give us a similarity index, or how well our unknown matches a known species map in the library.

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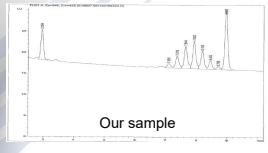
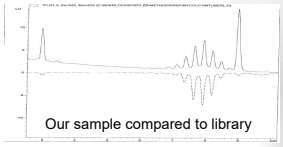
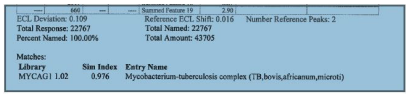
HPLC Chromatogram of MTBC



Our sample



Our sample compared to library



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MGIT 960 Drug Susceptibility Testing

- All MTB isolates from initial diagnostic specimens or isolates received for identification are tested
- Requires a pure isolate for testing
- Drugs tested are STR (1.0ug/ml), INH (0.10ug/ml), RIF(1.0ug/ml), EMB(5.0ug/ml), and PZA(100ug/ml)
- Sensitivity not repeated within 3 months unless requested
- Results in 4-21 days. Typically takes 6-7 days.

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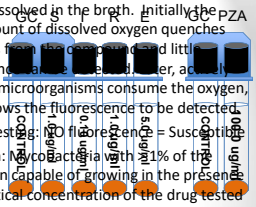
MGIT 960 Drug Susceptibility Testing

- A fluorescent compound is embedded in the silicone on the bottom of MGIT tubes. The compound is sensitive to the presence of oxygen dissolved in the broth. Initially the large amount of dissolved oxygen quenches emissions from the compound and little fluorescence is detected. However, actively respiring microorganisms consume the oxygen, which allows the fluorescence to be detected.
- For AST testing: NO fluorescence = Susceptible
- Definition: Mycobacteria with >1% of the population capable of growing in the presence of the critical concentration of the drug tested are considered **RESISTANT**



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Testing Available at CDC

MDDR- Molecular Detection of Drug Resistance

- Requires PCR + sediment or culture of MTBC
- Pyrosequencing- mutations associated with INH/RIF (48hrs.)
- Sanger sequencing- EMB,PZA,FQ,AMK,KAN,CAP, and the most effective second-line drugs. Run if a mutation associated with RIF resistance is found with pyro or previously known. (72 hrs.)
- Conventional agar proportion (also secondary DST)- solid media based (7H10) susceptibility testing method (1 month from date received)
- PZA by MGIT 960

Culture ID by 16S gene sequencing

Speciation of MTBC complex/ PZA confirmation

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Thank You, The Ohio Department of Health Laboratory

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