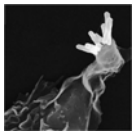
Laboratory Methods



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TB Program Managers' Workshop
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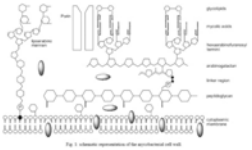
Cell Cycle Lengths

	Generation time (hrs)	Days needed for 26 generations (colony)
E. coli	**0.33**	**0.36**
M. smegmatis	**2.5**	**2.7**
M. tuberculosis	**22.0**	**24.0**

Mycobacterial cell wall

- Mycobacteria have a cell envelope with a high lipid content
- This characteristic accounts for the difficulty in staining them with conventional techniques.
- Mycobacteria have N-glycolylmuramic acid in the place of N-acetyl muramic acid in their peptidoglycan (cell wall)



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Outline of Laboratory Methods

1. Processing
2. Staining
3. Growth characteristics
4. Susceptibility testing
5. Safety in the laboratory

Laboratory Principles

- Digestion / Decontamination (non-sterile specimen types)
- Concentration
- Smear Exam
- Culture
- Susceptibility
- Molecular techniques (PCR, genotyping)

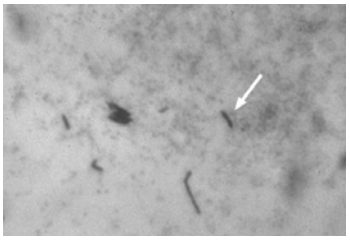
Digestion/Decontamination

- Traditional method: 4% NaOH
  - Decontamination: time of exposure must be carefully controlled
- Most common method:
  - N-acetyl-L-cysteine (NALC) + 2% NaOH - mild decontamination solution (NaOH) with mucolytic agent (NALC) to free trapped mycobacteria from mucus
- 4% Sulfuric acid
  - Often used for decontaminating urine specimens
- 5% Oxalic acid
  - Most useful for processing specimens that contain *Pseudomonas aeruginosa* as a contaminant

Smear - Stains

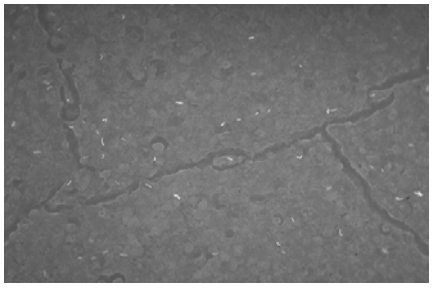
- Acid-fastness (mycolic acids)
  - Cannot be decolorized with acid alcohol
- Stains:
  - Ziehl-Neelsen - carbolfuchsin hot stain
  - Kinyoun - carbolfuchsin cold stain
    - Higher concentration of phenol instead of heat
  - Auramine Fluorochrome stain
    - Most sensitive

Mycobacteria tuberculosis **Ziehl-Neelsen Stain**



Mycobacterium tuberculosis is a slim (1-4µm), unencapsulated, strongly acid-fast rod that frequently shows **irregular beading** due to vacuoles and polyphosphate granules.

Mycobacteria tuberculosis
Auramine Stain



AFB Smear Interpretation
Fluorochrome

(read minimum of 30 fields[F]-about 2 mins)

AFB Seen (40X)	**Report**
0	No AFB
1-2 / 70F (1.5 sweeps)	Doubtful
2-18 / 50F (1 sweep)	Rare (1+) AFB
4-36 / 10F	Few (2+) AFB
4-36 / F	Moderate (3+) AFB
>36 / F	Numerous (4+) AFB

AFB Smear Interpretation
Kinyoun - Specimen

(read approximately 300 fields[F] - 15 mins)

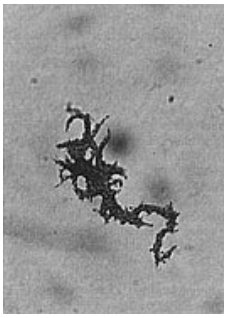
AFB Seen (100X)	**Report**
0	No AFB
1-2 / 300F (3 sweeps)	Doubtful
1-9 / 100F	Rare (1+) AFB
1-9 / 10F	Few (2+) AFB
1-9 / F	Moderate (3+) AFB
>9 / F	Numerous (4+) AFB

Growth Requirements

- One of the least fastidious of pathogenic microorganisms
- Aerobes, prefer 5% to 10% CO_2 for primary recovery
- Microaerophilic metabolism during latent infection
  - Recent drug/vaccine development focuses on this characteristic
- Multiply slowly, dividing only every 18-24 hours
- Serpentine growth, cord factor- 6,6-dimycolytrehalose
  - Cord factor is virulence factor

Cording

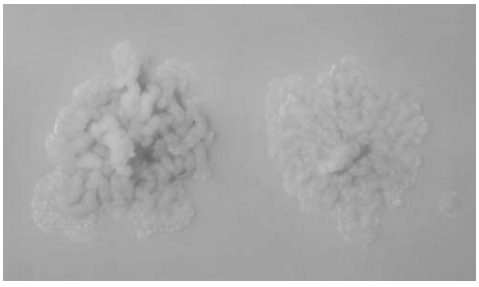
Caused by 6,6-dimycolytrehalose ("cord factor")



Culture Media

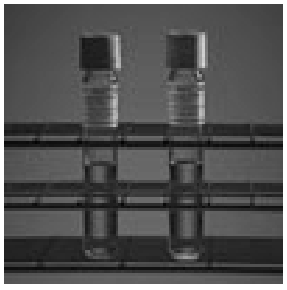
- Four traditional culture media:
  - Egg-based (Lowenstein Jensen)
  - Agar-based (Middlebrook agar)
  - Liquid (Middlebrook broth)
  - Selective media (susceptibility assays)
- TB is a slow growing, rough, buff colony on solid media

Lowenstein Jensen



M. tuberculosis - Crumbly buff colored colonies

MGIT Media



Identification

- Traditional - biochemicals (indirect)
  - Niacin positive, nitrate negative
  - Isolate from solid media required, test takes days to weeks
- Rapid Direct - Nucleic Acid Amplification
  - Can be performed within hours of specimen receipt
- DNA probe of isolate
  - Can be performed in hours on pellet from rapid broth culture
  - Commercially available probes:
    - *M. tuberculosis, M. avium-intracellulare, M. kansasii, M. gordonae*

Drug Resistance and Susceptibility Testing

Anti-tuberculosis Drugs

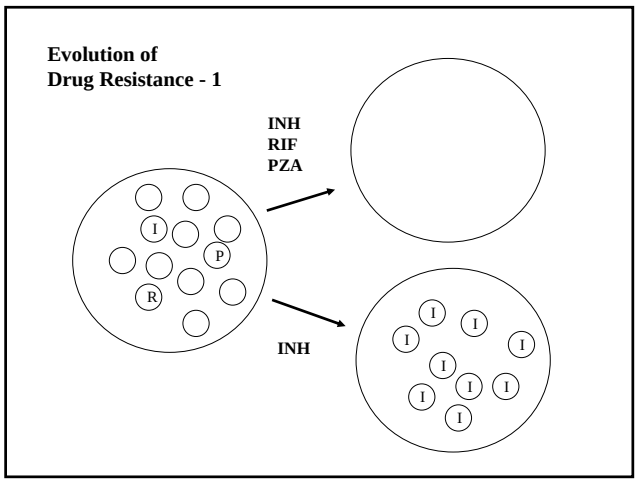
First-Line Drugs
- Isoniazid
- Rifampin
- Pyrazinamide
- Ethambutol
- Rifabutin*
- Rifapentine

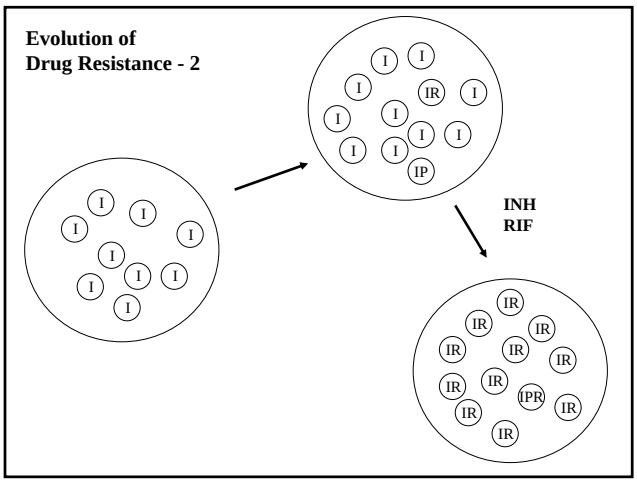
Second-Line Drugs
- Cycloserine
- P-Aminosalicylic acid
- Ethionamide
- Amikacin or kanamycin*
- Levifloxacin*
- Moxifloxacin*

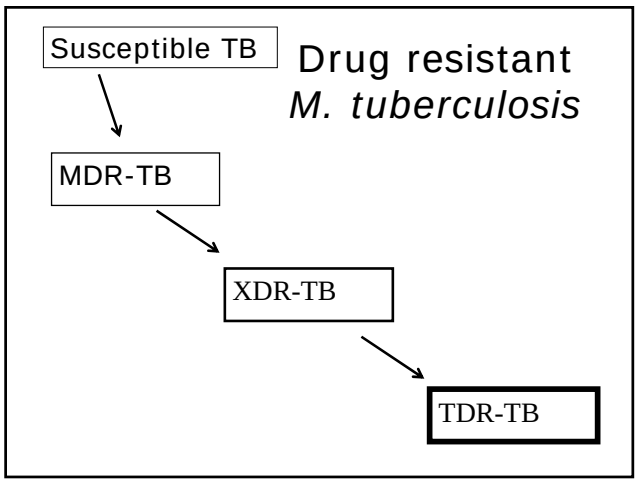
*Not approved by the US Food and Drug Administration for the treatment of TB

Drug-resistant TB

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





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Emergence of New Forms of Totally Drug-Resistant Tuberculosis Bacilli
Super Extensively Drug-Resistant Tuberculosis or Totally Drug-Resistant Strains in Iran

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This Article

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Abstract

Background The study documented the emergence of new forms of resistant bacilli (totally drug resistant (TDR) or Super XDR TB strains) among multi drug resistant tuberculosis (MDR TB) patients.

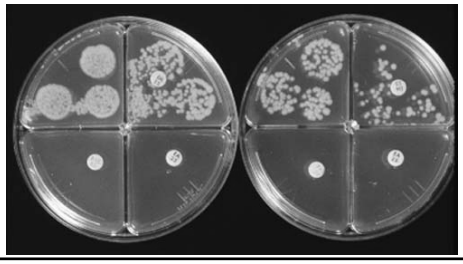
Methods The susceptibility testing against first and second line drugs was performed on isolated *M. tuberculosis* strains. Subsequently, the strains that were identified as XDR or TDR *M. tuberculosis* were subjected to spoligotyping and variable number of tandem repeats (VNTR).

Susceptibility Testing

- ***Direct***
  - Performed directly from specimens with positive smears
  - More accurate than indirect, faster turnaround
- ***Indirect***
  - Performed on recovered isolate
  - Resistant isolates often grow more slowly, and are overgrown by more sensitive isolates

Susceptibility Testing - 1

- Proportional method most common method
  - >1% growth represents resistance



Susceptibility Testing - 2

- Bactec method
  - Uses liquid media, faster than proportional method
  - 90% to 100% agreement with proportional method
  - Media contains ^{14}C-fatty acids: bacteria release ^{14}C- CO_2, which is detected

TB Turn Around Times

AFB smear:	24 hours
Growth detection:	14 days
TB identification:	21 days
Susceptibility testing:	28-30 days

False Negatives

- Infection with low numbers of organisms common
- Too small a sample could miss organisms
  - Sputum 10 mL
  - No swabs from wounds - tissue biopsies
  - Urine (1st morning total void)
  - Large volume of pleural fluid dilutes out the few numbers of organisms (as much as possible of sample should be collected and processed)
- Too few specimens
  - At least 3 sputums
  - 3-5 urines
- Wrong site

False Positives

- *M. tuberculosis* very hardy & survives well under very harsh conditions
- Cross Contamination possible from:
  - Other positive specimens
  - Quality control isolates
- Common scenario
  - Specimen with 3+ or 4+ smear processed at same time as questionable patient specimen
  - Patient has multiple specimens, none have positive smear and only one culture grows
  - DNA fingerprinting can be helpful to determine possible cross contamination

Biosafety in the TB Laboratory

How can I tell if I am being exposed to a hazardous substance?

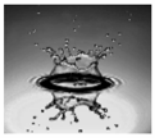
- Know what you are working with
- Ask questions
- Look at the container labeling
- Review the Material Safety Data Sheet
- Call Safety Officers

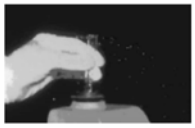
Use Your Senses

- Odors
- Eye Irritations
- Visible Clouds or Fumes
- Spills
- Look for leaking containers, dripping liquid, puddles, etc.

What are we protecting against?

Splashes
Needlesticks
Aerosols
Chemical spills
Other accidents





Ways to minimize exposure - 1

1. Substitution: Use of a less hazardous or attenuated material to reduce or eliminate hazard

2. Engineering Controls: Use of available technology and devices to isolate hazards from the worker

3. Administrative Controls: Monitor compliance, provide accessibility of control methods, investigate exposures to prevent future occurrence

Ways to minimize exposure - 2

4. Work Practice Controls: Manner in which task is performed to reduce exposure:

-Wash hands after removal of gloves
-Disposal of needles without recapping
-No lab coats outside of lab

5. PPE: Personal protective equipment: Specialized clothing or equipment used to protect workers from exposure: lab coats, gloves, face shields, eye protection, fluid resistant aprons, head and foot covering

Exposure Incident

A specific incident of contact with potentially infectious material, body fluid, or chemical

Procedures Following an Exposure

- Immediately wash affected area(s)
- Recover & save the specimen; refrigerate if possible
- Notify your supervisor
- Report all accidents involving body fluids or chemicals
- Getting treatment within a few hours of injury reduces the chance of sero-converting to HIV

Summary

- The TB lab works hard with a difficult organism
- TB laboratory diagnosis and treatment is extremely challenging:
  - TB bacteria are notoriously slow-growing and culture takes time
  - Molecular methods are under development but are not yet reliable
  - Culture remains the gold standard of identification and susceptibility testing