MYCOBACTERIA

General Features of Mycobacteria

- **Acid fastness** due to
- Mycolic acids in the cell wall & Integrity of the cell wall
- **Guanine plus cytosine (G+C) content of DNA of** Mycobacterium is 61–71 mol % (exception *M.leprae* G+C content 54 to 57 mol %)
- Weakly gram-positive, Obligate aerobes
- Non-motile, non-sporing, noncapsulated straight or slightly curved rod-shaped, sometimes show branching filamentous forms

Classification

- ***M. tuberculosis complex -*** tuberculosis in man
- ***M. leprae (Hansen's bacilli) - leprosy***
- **Nontuberculous mycobacteria (NTM)** –

- Saprophytes (*M.phlei*) or
- Commensals (*M.smegmatis*)
- occasionally cause opportunistic human infection

MYCOBACTERIUM TUBERCULOSIS COMPLEX

- **Causes tuberculosis and includes:**
- *M. tuberculosis (human tubercle bacillus)*
- *M. bovis (bovine tubercle bacillus)*
- *M. caprae (closely related to M. Bovis)*
- *M. africanum (isolated from a few West African cases)*
- *M. microti ('vole' bacillus, rare and less virulent)*
- *M. pinnipedii (infects seals in the Southern hemisphere* and recently isolated from humans)
- *M. canetti (a rare isolate from East African cases, that* produces unusual smooth colonies on solid media)

Pathogenesis

- **Source of Infection**
- Human cases of pulmonary tuberculosis
- Bovine source (unpasteurized infected milk)
- **Mode of Transmission**
- **Inhalational mode:** Droplet nuclei, generated while coughing, sneezing, or speaking
- Tiny dry droplets (<5–10 µm size) remain suspended for several hours & are easily inhaled
- **Other modes – Inoculation & Ingestion**

Risk Factors favouring transmission

- Sputum positive patients with **Bacillary load at least 10,000 bacilli/mL**
- C**avitary lesions** in lung - more bacillary load
- **Overcrowding** in poorly ventilated rooms
- **Low cell-mediated immunity – HIV**
- **Other comorbid conditions** - Post-silicosis, postransplantation, hemodialysis, diabetes, IV drug abuse, smoking, etc.
- **Age:** Late adolescence and early adulthood
- **Sex:** women at 25–34 years, men in older ages

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Sequence of Pathogenic Events

- **Droplet nuclei** inhaled →Majority trapped in upper airways and expelled out & usually <10% reach the alveoli
- **Adhesion to macrophages -** lipoarabinomannan (LAM) binds to complement receptors and mannose receptors → internalization
- **Phagocytosis by macrophages - enhanced by** complement (C3b) mediated opsonization of bacilli
- **Survival inside the macrophages -** cell wall LAM impairs phagosome-lysosome fusion →replicate inside the macrophage → macrophage ruptures & releases bacilli

Clinical Manifestations

- **Pulmonary Tuberculosis (PTB)**

- 80% of all cases of tuberculosis

- **Extrapulmonary Tuberculosis (EPTB)**

- 15–20% of all cases of TB
- Results from hematogenous dissemination.

Primary v/s Secondary Pulmonary TB

Features	Primary PTB	Post-primary/secondary PTB
Results due to	Initial exogenous infection with tubercle bacilli	i) Exogenous reinfection ii) Endogenous-reactivation of latent primary lesion
Age group affected	Children	Adults
Parts of lungs commonly affected	Sub pleural lesion affecting, upper part of lower lobe and lower part of upper lobe	Apical and posterior segments of the upper lobes (high oxygen tension)

Primary vs Secondary Pulmonary TB

Features	Primary PTB	Post-primary/secondary PTB
Lesions formed at the initial sites	Fibrotic nodular lesions (Ghon focus)	Calcified nodules (Assmann focus) Early hematogenous seedling in apex of lungs - Simon's focus
Lymph node	Ghon focus with associated hilar lymphadenopathy is common (called as primary complex)	Lymph node involvement is unusual

Primary vs Secondary Pulmonary TB

Features	Primary PTB	Post-primary/secondary PTB
Clinical feature	It may be asymptomatic or may present with fever, productive cough (with or without hemoptysis) and occasionally chest pain, night sweating, weight loss	Lesions undergoing necrosis and tissue destruction, leading to cavity formation. Symptoms are similar, but more pronounced.

Primary vs Secondary Pulmonary TB

Features	Primary PTB	Post-primary/secondary PTB
Fate	• Lesions heal spontaneously • Primary complex becomes calcified (Ranke complex)	• Bronchogenic spread to the same or opposite lung form satellite lesions → caseating pneumonia • Hematogenous spread to various parts of the body and granuloma formation. Rarely, heals spontaneously

Extrapulmonary Tuberculosis

- **Tuberculous lymphadenitis –** MC form (35% of all EPTB)

- Posterior cervical and supraclavicular lymph nodes - painless swelling in neck region without warmth or color change

- **Pleural tuberculosis** – (20%) - pleural effusion
- **Tuberculosis of the upper airways** - larynx, pharynx, and epiglottis
- **Genitourinary tuberculosis:** Renal tuberculosis

- Genital tuberculosis – Females: fallopian tubes & endometrium → infertility. Males: epididymis

Extrapulmonary Tuberculosis

- **Skeletal tuberculosis** - Weight-bearing joints (spine, hips & knees)

- Complications - collapse of vertebral bodies → kyphosis & paravertebral ‘cold’ abscess

-Tuberculosis of CNS – children, Tuberculous meningitis & tuberculoma

- **Tuberculous pericarditis - direct extension** from adjacent lymph nodes or following hematogenous spread

Extrapulmonary Tuberculosis

- **Gastrointestinal tuberculosis** - Terminal ileum and caecum

- Due to swallowing of sputum with direct seeding,hematogenous spread, or ingestion of cow's milk contaminated with *M. bovis*

- **Tuberculous skin lesions:**

- ***Scrofuloderma –*** skin involvement by direct extension from underlying tuberculous lymphadenitis

- ***Lupus vulgaris***: Apple jelly nodules are formed over face

- **Miliary or disseminated tuberculosis**: Hematogenous spread →yellowish 1–2 mm size granulomatous lesions resembling millet seeds in various organs

Laboratory Diagnosis

- **Specimen Collection**
- PTB: Sputum –
- S**pot sample & early morning sample,** at least 2–5 mL & preferably mucopurulent

Extrapulmonary specimens

Sterile site specimens collected aseptically	
Optimum specimens	CSF, spinal, pericardial, synovial, ascitic, blood and bone marrow, pleural biopsy, tissues (collected in sterile saline)
Suboptimal specimens (organism load is less)	Pleural fluid (20–50 mL is collected and centrifuged), Blood (indicated only for disseminated TB and HIV-TB coinfection)

Extrapulmonary specimens

Specimens containing normal flora	
Swabs	Only recommended swabs- - Laryngeal swabs: Collected early morning in empty stomach - Swab from discharging sinus
Urine	Three early morning specimens collected (500 mL/ specimen, centrifuged) on different days
Stool	For disseminated TB in HIV infected patients and infants

Extrapulmonary specimens

Specimens containing normal flora	
Other Resp specimens	Bronchial secretions (2–5 mL) Bronchoalveolar lavage (20–50 mL) Transbronchial and other biopsies
Gastric Lavage	Recommended for children (tend to swallow sputum), or ICU patients (aspiration)Early morning lavage should be collected and processed early (<4 hours)

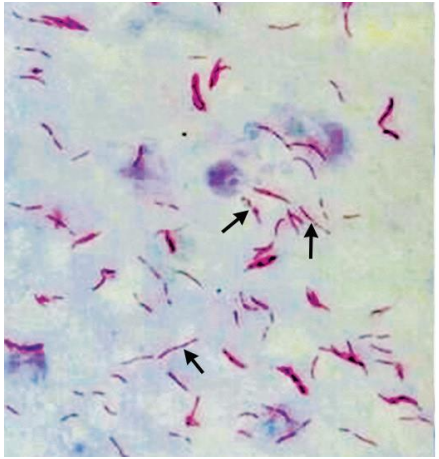
Digestion, Decontamination and Concentration

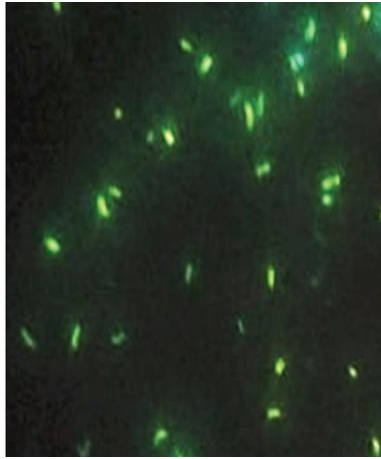
- Digestion - to liquefy the thick pus cells and homogenization
- Decontamination - to inhibit the normal flora
- Concentration - to increase the yield
- **Petroff's method -** 4% sodium hydroxide → centrifuged → neutralized with 8% HCl
- **NALC (N-acetyl-L-cysteine) + 2% NaOH:** NALC liquefies sputum and NaOH kills normal flora
- Superior & more compatible with automated culture systems

- **Acid Fast Staining Ziehl-Neelsen (ZN) technique**

Interpretation

- **Negative result**: ≥100 OIFs, 10–15 min
- **Positive result**: long slender, beaded, less uniformly stained red colored bacilli (AFB)

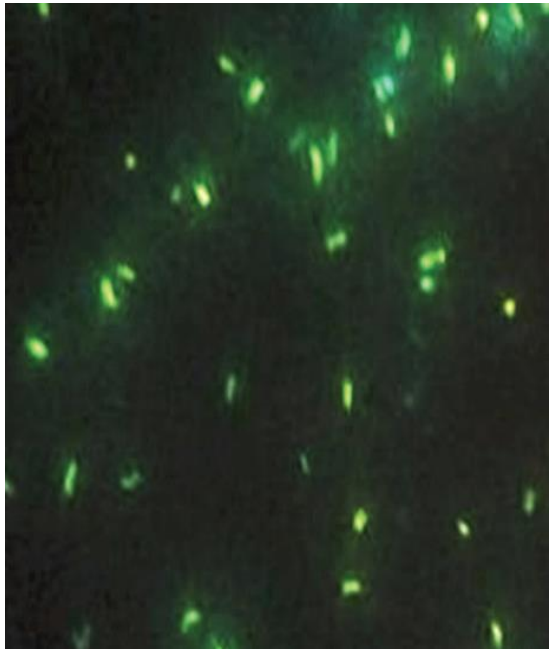




Direct Microscopy

- **Microscopy provides only** presumptive diagnosis
- **Advantages:** rapid, easy to perform & cheaper
- **Disadvantages:**
- **Less sensitive** than culture, Detection limit 10,000 bacilli/mL of sputum
- Cannot determine viability of bacilli
- Difficult to differentiate from saprophytic mycobacteria
- ***Acid alcohol*** – *M.tuberculosis* (acid & alcohol fast);*M.smegmatis (only acid* fast but not alcohol fast)

- **Kinyoun’s Cold Acid Fast Staining**
- Heating not required
- Phenol concentration in carbol fuchsin is increased
- **Fluorescence Staining**
- 0.1% auramine, fluorescent LED, 40X
- Brilliant yellow bacilli
- Screened faster (2 min for 100 fields)



RNTCP guidelines for grading of sputum smear

No. of bacilli seen	Grading	No. of OIF to be screened
>10/OIF	3+	20
1-10/OIF	2+	50
10-99/100 OIF	1+	100
1-9/100 OIF	Scanty	100
No AFB in 100 OIF	Nil	100

RNTCP guidelines for grading of sputum smear

- **RNTCP grading is useful for:**

- **Monitoring the treatment** response of the patients
- Assessing the **severity** of disease
- Assessing the **infectiousness** of the patient: Higher the grade more is the infectiousness. Smear negative patients (<10,000 bacilli/mL of sputum) are less infectious

Culture Methods

- **Advantages:**
- More sensitive (detection limit of 10–100 viable bacilli)
- Indicates viability
- Drug susceptibility testing can be performed
- **Conventional Solid Media (Lowenstein Jensen Medium)**
- Most widelyused and recommended by RNTCP
- Composition : coagulated hen's eggs, mineral salt solution, asparagine and malachite green (as a selective agent)
- Incubation: 6–8 weeks

- ***M.tuberculosis*** - typical rough, tough and buff colored colonies
- ***M.bovis*** - Smooth, moist and white colored colonies which break up easily when touched

Automated Liquid Culture

- **Monitor growth continuously** and offer a faster turnaround time
- 99% of positive growth gets detected within 3–4 weeks
- **Middlebrook 7H9 medium** supplemented with:
- **OADC enrichment growth media** (oleic acid, albumin, dextrose and catalase) - promote the growth *M. tuberculosis and*
- **PANTA antibiotic mixture** (polymyxin B, amphotericin B, nalidixic acid, trimethoprim, and azlocillin) to inhibit other organisms

- **BACTEC MGIT (Mycobacteria growth indicator tube):**
- Detects growth of mycobacteria
- Drug susceptibility testing
- **Principle:** O2 sensitive fluorescent compound in the broth quenches emissions from the fluorescent compound →quenching effect is lost if organisms grow & use O2

Automated systems

- **VersaTek system:**

- Detects change in pressure in headspace due to production of CO2 by *M.tuberculosis*
- Drug susceptibility testing against first line anti-tubercular drugs

- **BacT/ALERT:**

- Colorimetric detection of pH change due to CO2 liberated by *M. tuberculosis.*

Culture Identification

- Growth subjected to acid fast stain. Further testing if found AFB positive
- **MPT 64 antigen** detection by rapid immunochromatographic test
- **Biochemical tests -** niacin test, nitrate reduction and pyrazinamidase test - almost obsolete now
- **Rabbit pathogenicity test -** *M. bovis* is pathogenic to rabbit, whereas *M. tuberculosis* is not pathogenic. Obsolete now

Serology

- Serological methods (both antigen and antibody detection methods) are **NOT** recommended
- Low sensitivity
- Cross reactivity with other mycobacteria
- Variable antibody response against different epitopes
- **WHO has banned the use of serological tests**

Molecular Methods

- Take less time than culture
- More sensitive than culture. Useful for extrapulmonary samples
- Detect genes coding for drug resistance
- **Polymerase Chain Reaction**
- **Nested PCR** targeting IS6110 gene. Other genes MPT64 gene, 65 KDa and 38 Kda genes
- **Cartridge-based nucleic acid amplification test (CBNAAT)**

GeneXpert

- CBNAAT system endorsed by WHO and RNTCP
- **Rapid: 2 hours**
- **Principle: Real-time PCR** technique detects
- MTB complex DNA and
- Rifampicin resistance (mutations of the *rpoB gene)*
- **No contamination:** It employs single-use disposable cartridges
- **Procedure:**. The entire process (sample processing, nucleic acid extraction, amplification, and reporting of the result) is

GeneXpert

- **EPTB:** WHO recommends GeneXpert as the initial test using CSF, lymph nodes and other tissues specimens
- **Diagnostic utility:** Detection limit 131 bacilli/mL of specimen
- **Compared to culture:**

- Detection of TB bacilli - 88% sensitive and 99% specific
- Detection of rifampicin resistance: It is 95% sensitive and 98% specific

- **Disadvantages:** very expensive, cannot further speciate

GeneXpert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Line Probe Assay (LPA)

- Probe-based detection of amplified DNA in the specimen
- **Use of LPA in TB diagnostics:** Identification of MTB complex
- Detection of resistance to antitubercular drugs – 1st & 2nd line
- Speciation of MTB complex and NTM
- **Limitation**: Can be performed only on positive cultures or smear positive clinical specimens
- **Commercial Kits** - GenoType and INNO-LiPA
- **Systems** used to perform the assay - TwinCubator and GT-Blot

Line Probe Assay (LPA)

- **Principle:** 2–3 days of turnaround time

1. **DNA extraction** from clinical specimens
2. **Multiplex PCR** amplification with biotinylated primers
3. **Reverse hybridization***: C*hemical denaturation of amplicons → hybridization → streptavidin-conjugated alkaline phosphatase → detects hybrids by biotin-streptavidin moieties

Line Probe Assay (LPA)

Detection of	Sensitivity	Specificity
TB Bacilli	81.5%	87.5%
Rifampicin resistance	97%	99%
INH resistance	90%	99%

LPA is useful particularly in isoniazid mono-resistant cases of TB, which are not diagnosed by GeneXpert

Upcoming Methods for TB Diagnosis

❖ **TrueNat (Molbios) -** Chip Based Real Time PCR (TrueNat)

- Automated battery operated device; can be used at primary health center level
- **Unit 1** (sample preparation device) - DNA extraction Then
- **Unit 2** (analyzer) - extracted DNA is added to the chip (coated with the probes) and loaded for amplification
- **Disadvantages:** very expensive, cannot further speciate MTB complex and tests one sample at a time

Other Methods for TB Diagnosis

❖ **TB-LAMP (Loop-mediated isothermal amplification):**

- Alternative to smear microscopy for identification (WHO)
- Does not detect drug resistance

- **Next generation GeneXpert (Xpert Ultra):**
- **Ultra cartridge** with larger chamber for DNA amplification to accommodate **larger amount of sputum**
- Two additional molecular targets to detect TB
- More sensitive and specific with **detection limit of 16 bacilli/mL** compared to 131 bacilli/mL of first generation GeneXpert

Other Methods for TB Diagnosis

- **Breath biomarkers** such as volatile organic compounds
- **LAM (lipoarabinomannan) antigen detection** - urine and sputum
- **InterGam Rapid Immuno Suspension Assay** – IFN γ from extrapulmonary sites
- **Automated microscopy** and image detection
- **Non-commercial culture and drug-susceptibility testing**
- Microscopic observation of drug susceptibility
- Colorimetric redox indicator (CRI) methods

RNTCP Guideline 2016

- **Presumptive pulmonary TB:**

1. Cough>2 weeks
2. Fever >2 weeks
3. Significant weight loss
4. Hemoptysis
5. Any abnormalities in chest X-ray

RNTCP Guideline 2016

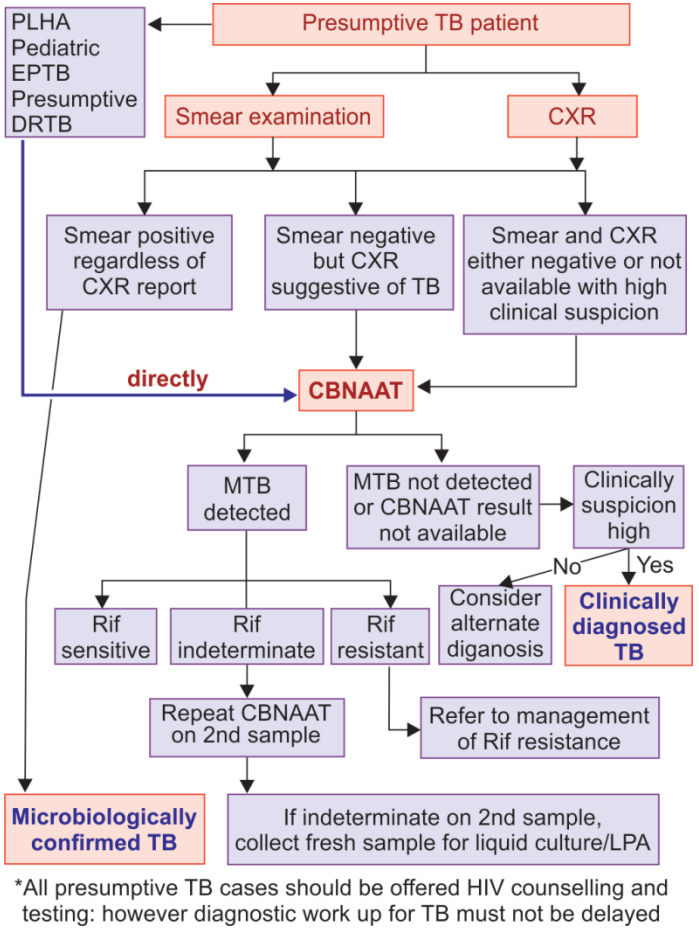
- **Presumptive DRTB (drug resistant TB) case:**

1. TB patients who have failed treatment with first line drugs
2. Pediatric TB non-responders
3. TB patients who are contacts of DRTB cases
4. TB patients found positive on any sputum smear examination during treatment withfirst-line drugs
5. Previously treated TB case
6. New TB patients with HIV co-infection

RNTCP Guideline 2016 for Diagnostic Algorithm for Tuberculosis – Adults

For extrapulmonary TB (EPTB):

- EPTB specimens are paucibacillary → CBNAAT/MGIT are directly performed



RNTCP Guideline 2016

- **For pediatric Presumptive pulmonary TB:**
- **CBNAAT** is directly performed on sputum
- If MTB is not detected/ CBNAAT not available→ X-ray & tuberculin test
- **Highly suggestive Chest X-ray is - miliary shadows,** hilar or mediastinal lymphadenopathy or chronic fibrocavitary shadows → perform CBNAAT on alternative specimens (gastric aspirate or induced sputum)

RNTCP Guideline 2016

- **Non-specific chest X-ray (e.g. consolidations)** → treat for bacterial pneumonia → If symptoms still persists, perform CBNAAT on alternative specimens
- If chest X-ray negative, TST positive—evaluate for EPTB
- If chest X-ray negative, TST negative—look for alternative cause

Diagnosis of Latent Tuberculosis – Tuberculin Test

- By demonstration of type IV hypersensitivity reaction against the tubercle bacilli antigens
- Discovered by Von Pirquet in 1907
- **Antigens used in tuberculin test:**
- **OT (Old tuberculin antigen)** - Crude preparation, not used now
- **PPD (Purified protein derivative antigen)** - purified preparation of the active tuberculoprotein
- PPD-RT-23 with Tween 80 PPD – Recommended by WHO

Tuberculin Test

- **Dosage: One TU** is equal to 0.01 mL of OT or 0.00002 mg of PPD
- **Procedure:**

- Mantoux test: Injected intradermally into flexor surface of forearm
- Heaf and Tine multiple puncture tests - not in use

Tuberculin Test

- **Reading:** after 48–72 hours. width of the induration

≥10 mm: Positive (tuberculin reactors)

6–9 mm: Equivocal/doubtful reaction

<5 mm: Negative reaction.

- **Interpretation of result:**

- **Adults**: Positive only indicates present or past exposure with tubercle bacilli . only used as an epidemiologicalmarker
- **Children**: positive test indicates active infection and used as **diagnostic marker**

Tuberculin Test

- **False-positive:**
- BCG vaccination (after 8–14 weeks)
- Nontuberculous mycobacteria infection.
- **False-negative:**
- Early or advanced TB, miliary TB, decreased immunity (HIV-infected people)
- **Two-step testing: In adults,** repeat test 1–2 weeks after the first test - booster effect → strong positive reaction (>20 mm)

Diagnosis of Latent Tuberculosis – IGRA

- **Interferon Gamma Release Assay (IGRA)**

- Highly specific *M. tuberculosis* antigens - CFP10 & ESAT6

- **Procedure:** an in vitro test

- Patient's Sensitized T lymphocytes exposed to ESAT-6/CFP-10 antigens → release of high level of IFNγ from the T lymphocytes
- QuantiFERON-TB Gold assay – commercially available ELISA format

- **Advantage:** highly specific; no false positive