The many roads to diagnosis of tuberculosis: Experience of a tertiary care centre in Southwest Coastal India

Jyoti Kini, Shrikala Baliga, Hema Kini, Suchitra Shenoy, Kausalya Sahu, Deepa Adiga

Department of Pathology and Microbiology,
Kasturba Medical College, Mangalore,
Manipal Academy of Higher Education (MAHE)
• Tuberculosis continues to be one of India’s major health problems
• Associated with significant morbidity and mortality.
• India alone accounts for one-fourth (2.8 million) of the global TB (10.4 million new cases) burden and 29% of the 1.8 million TB deaths.
• India also has a tremendous load of multidrug resistant TB (MDR-TB), 1.3 lakh MDR-TB of the estimated 480,000 new cases of MDR-TB worldwide.
• About 15% to 20% of all TB cases can be extrapulmonary tuberculosis (EPTB) with tuberculous lymphadenopathy the most common form.
M. tuberculosis

- Acid fast bacilli
- Thin, straight or slightly curved rod
- Approximately 0.4×3µm in size
- Non motile
- Non-sporing
- Non-capsulated
Classification

Based on their ability to cause disease mycobacteria are classified into-

1. Species always considered pathogenic
   - Examples: *M. tuberculosis*, *M. leprae*, *M. bovis*, *M. africanum*

2. Species potentially pathogenic
   - Moderately common e.g. *M. avium* complex
   - Rapid grower e.g. *M. chelonae*

3. Saprophytic species that rarely cause disease e.g *M. phlei*
   - Tubercle bacilli / *M. tuberculosis* complex (MTBC)
   - *M. tuberculosis*, *M. bovis*, *M. africanum*, *M. microti*
   - Non tuberculous mycobacteria (NTM)
Pulmonary tuberculosis

Extra Pulmonary tuberculosis

Tuberculous lymphadenitis
Genitourinary tuberculosis
Skeletal tuberculosis
Tuberculosis meningitis
Gastrointestinal tuberculosis
Pericardial tuberculosis
Specimen collection for microbiology/pathology
Depends on the organ or tissue infected.

**Pulmonary TB:**
1. Sputum/Induced sputum
2. Bronchoalveolar lavage / brushings
3. Laryngeal swab
4. Pleural fluid
5. Gastric aspirate – children less than 5
Specimen collection

Extrapulmonary TB:

1. CSF, body fluids, urine
2. Lymph node/Breast aspirate
3. Tissue biopsy, Lymph node, GIT, Breast, Endometrial biopsy
Methods of diagnosis

1. Microscopic smear examination
2. Histopathology examination
3. Isolation and identification
4. Molecular methods
5. Immunodiagnosis
Microscopic examination:

Staining methods-
  • Ziehl – Neelsen staining (Hot method)
    - Gabbet’s staining (Cold method)
  • Fluorescent staining – using fluorochromes
    Auramine or Auramine-Rhodamine
• In ZN and Gabbet’s stain, AFB are red to pink color with a beaded appearance against the blue background.

Fig:- ZN staining
• In fluorescent staining, AFB appears bright yellow/green against dark background

• Observed under low power (40X), therefore only 40 fields need to be examined

• LED microscopes are used

Fig:- Fluorescent stain
Advantages of microscopic examination:
1. Rapid diagnosis
2. Inexpensive, simple method
3. May be used to monitor treatment

Disadvantages of microscopic examination:
1. Low sensitivity – 25-75% as compared with culture and sputum should contain 5000-10,000 bacilli to be smear positive
2. Species identification and drug susceptibility testing not possible
2. Isolation and identification:

- Culture is the Gold standard method for diagnosis of TB with high specificity and sensitivity (10-100 bacilli/mL can yield growth)
- Species identification and drug susceptibility testing possible

Disadvantages of culture: Slow method: *M.TB* takes 4-8 weeks to grow

- Sputum sample should be processed for liquefaction (homogenization), decontamination (elimination of contaminants) and concentration (increase the number of bacilli) before culture.
- Body fluids like CSF, pleural fluid need concentration only.
3. Molecular methods

(a) Polymerase chain reaction: Amplifies pathogen DNA specific region IS 6110.
(b) Cartridge Based Nucleic Acid Amplification Test (CB NAAT) – also known as GeneXpert MTB/RIF - Detects *M. tuberculosis* DNA and rifampicin resistance.
Molecular methods

(c) Line Probe Assay (LPA): identification of *M. tuberculosis* and detection of mutations associated with drug resistance genes.

(d) TB LAMP: Loop mediated isothermal amplification, alternate to smear examination does not detect drug resistance.
Molecular methods

• Molecular methods are highly sensitive and specific. However, they can not differentiate between live and dead bacilli.

• Can be used to detect specific drug resistance
Immunodiagnosis

• Serological tests are not useful and should not be used for diagnosis of TB.

• LAM (Lipoarabinomannan) antigen detection in urine

• Latent tuberculosis can be diagnosed using Tuberculin skin test and Interferon Gamma Release Assay (IGRA)
Case – 1

- 39 years old female presented with complaints of fever since 1 month with no other associated complaints.
- No previous hospital admissions, no comorbidities.
- O/E- tachycardia and fever, systemic examination- unremarkable.
- Lab investigations: Hb-11, TC- 5800, Plt- 3.74 lakhs, ESR- 120, CRP- positive
- Chest x- ray- Normal
- USG Abdomen- Normal
Case – 1

- VIH, Widal, Weil Felix- negative

- 2 sets of Blood cultures- No growth

- CECT Chest- Few tiny microcystic foci with mild ground glass densities in the superior basal segment of left lower lobe.

- CECT Abdomen- Necrotic node in periportal, precaval region with multiple enlarged retroperitoneal and right common iliac lymph nodes- Koch’s v/s lymphoma
Case – 1

- LDH-247
- Mantoux- ulcerating, strongly positive
- Sputum AFB- negative
- Sputum Gene Xpert- Negative
- Fundus- no choroid tubercles
Case – 1

• Patient was **empirically started on ATT**

• Patient **stopped taking ATT after 2 weeks** l/v/o joint pains and myalgia and presented after 2 weeks with **persistent fever**.

• On 2nd admission: Hb- 10, TC- 5400, Plt- 3.35 lakhs, **ESR- 83**, uric acid- 10.9

• PET CT Scan- multiple enlarged metabolically active periportal, portocaval, left gastric, retroperitoneal, right common iliac, mediastinal and hilar lymph nodes. Prominent right posterior cervical lymph nodes.
Case – 1

• Exploratory laparotomy with excisional biopsy of periportal lymph node done.

• Biopsy- Stain for AFB- positive, Tubercular lymphadenitis.
Case – 1

• **ATT was restarted** and patient was discharged.

• LFT at follow up was normal.

• **Persistent fever spikes up to 2 months** after starting ATT.

• Patient has come for follow up visits.
Case 2

• 18 year old girl presented with chief complaints of:
  • Breathlessness
  • Swelling of left lower limb for 3-4 days and
  • Fever since 2 months on/off, and
  • Mild splenomegaly

• Doppler study showed acute DVT on left lower limb
• Past medical history of fever on/off 2 months prior to this admission.
Case – 2

• Blood culture no growth
• Bicytopenia
• Raised LDH
• Bronchoscopy: Mucosal plaques in the tracheobronchial tree
• CT chest with pulmonary angiogram
• Features s/o acute pulmonary embolism-bilateral lobar arteries thrombus
  • Consolidation in right lower lobe and medial segment of middle lobe
  • Nodular opacities in bilateral upper and left lower lobes
  • Mediastinal lymphadenopathy
Case – 2

- Clinical differential diagnosis of Acute Lymphocytic Leukemia/lymphoma VS autoimmune was considered.
- ICT and DCT was negative.
- Autoimmune workup, ANA profile and APLA study was negative
- C3 levels done: normal.
Case – 2
Case – 2
Case –2

Bronchial Lavage and USG guided Bronchial FNA

➢ Granulomatous lesion in favour of Tuberculosis.
Case – 2

- Findings from bone marrow study showing granulomas
- US-guided FNAC of lymph node with caseous necrosis, suggestive of Tuberculosis.
  BAL- AFB and Xpert-TB to be positive.
- Final Diagnosis of disseminated Tuberculosis.

Tuberculosis presentation as DVT and PTE is rare.
Laboratory diagnosis

- **Microscopy ZN/ Fluorescent** – detection
- **Isolation and identification:** Culture – detection, speciation, drug susceptibility
- **Molecular methods X-pert** – detection, speciation, drug resistance
- **Immunodiagnosis** – latent tuberculosis

*Mycobacterium tuberculosis*
Study 1: Evaluation of Auramine O with LED Fluorescent microscopy in cytodiagnosis of Tuberculosis

• 125 cytological specimens of patients with clinical suspicion of tuberculosis.
• Smears stained with Ziehl-Neelsen (ZN) and Auramine-O (AO) stains were studied for the presence of mycobacteria.
• The AO stained cytologic smears examined under LED fluorescent microscope.
• The overall positivity for tubercle bacilli with AO staining was 31.2% whereas it was 16% for ZN stain.
Study 1: Evaluation of Auramine O with LED Fluorescent microscopy in cytodiagnosis of Tuberculosis

• The kappa statistic is 0.592 (p<0.001) showing moderate agreement between the two staining methods.
• AO staining is more sensitive than ZN staining for demonstration of MTBC in various cytological specimens especially in paucibacillary cases.
• Screening is faster and effort is conserved.
• LED fluorescent microscopy is a simple, relatively inexpensive and valid option in resource poor laboratories with high case loads of tuberculous cytological samples.
The study compared the performance of PCR based assay with ZN staining, fluorescent staining and culture in diagnosis of tuberculous lymphadenitis.

94 samples from the nodes sent to Pathology department, 33 were in favor of tuberculous/ granulomatous lymphadenitis.

All the samples were concentrated and then used for smear microscopy, PCR and culture,

Specificity was 84.6%, 85.7% and 69.2% for ZN staining, fluorescent staining and PCR respectively. Sensitivity of these methods when compared with culture was 100%.
Study 2: A comparative study of different methods of laboratory diagnosis of TB lymphadenitis

• The specificity increased to 82.6% and 95.8% when PCR was combined with microscopy and compared with culture and pathology diagnosis respectively.

• PCR alone was not a reliable tool for diagnosis of TB lymphadenopathy as compared to a combination with microscopy and culture. However it had a high negative predictive value (100%) enabling the physician to rule out tuberculosis.
Study 3: Evaluation of Autopsy specimens for tuberculosis

- 40 cases of resected lung specimens Histopathological pattern analysis of PTB with associated changes and identification of *Mycobacterium tuberculosis* bacilli was done.
- Mean age of patients was 41 years with male predominance (92.5%).
- Tuberculosis was suspected in only 12.1% of cases before death.
- The presence of necrotizing granulomas was seen in 33 cases (82.5%).
- Acid fast bacilli were seen in 57.5% cases on Ziehl-Neelsen stain.
### Study 3: Evaluation of Autopsy specimens for tuberculosis

**Table**: Histopathological pattern of tubercular lesions in lungs

<table>
<thead>
<tr>
<th>Final diagnosis</th>
<th>Cases</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tubercular consolidation</td>
<td>11</td>
<td>27.5</td>
</tr>
<tr>
<td>Miliary tuberculosis</td>
<td>10</td>
<td>25.0</td>
</tr>
<tr>
<td>Fibrocaseous tuberculosis</td>
<td>07</td>
<td>17.5</td>
</tr>
<tr>
<td>Fibrocavitary tuberculosis</td>
<td>05</td>
<td>12.5</td>
</tr>
<tr>
<td>Caseating tuberculosis</td>
<td>03</td>
<td>7.5</td>
</tr>
<tr>
<td>Tubercular empyema</td>
<td>02</td>
<td>5.0</td>
</tr>
<tr>
<td>Tubercular bronchiectasis</td>
<td>02</td>
<td>5.0</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>40</strong></td>
<td><strong>100</strong></td>
</tr>
</tbody>
</table>
Study 3: Evaluation of Autopsy specimens for tuberculosis

• 19 cases of disseminated tuberculosis.
• Caseating epithelioid granulomas were seen in all cases of lungs (19/19 cases), 16 cases in liver, 12 cases in kidney and 17 cases in spleen, 2 cases of heart and 2 cases of pancreas.
• Disseminated tuberculosis accounted for 1.3% (n=19) of all
Study 3: Evaluation of Autopsy specimens with tuberculosis- Disseminated Tuberculosis
Study 3: Evaluation of Autopsy specimens with tuberculosis- Disseminated Tuberculosis
Road ahead: Dual color fluorescence in situ hybridization (FISH) assays in detecting Mycobacteria

- Two rapid dual color FISH assays for detecting M. tuberculosis and related pathogens in cultures.

- The MN Genus-MTBC FISH assay: orange fluorescent probe specific for MTBC and a green fluorescent probe specific for the Mycobacterium and Nocardia genera (MN Genus) to detect and distinguish MTBC from other Mycobacteria and Nocardia.

Study 4: Dual color fluorescence in situ hybridization (FISH) assays in detecting Mycobacteria

• A complementary MTBC-MAC FISH assay uses green and orange fluorescent probes specific for the MTBC and M. avium complex (MAC) respectively to identify and differentiate the two species complexes.
• The assays are performed on acid-fast staining bacteria from liquid or solid cultures in less than two hours.
## Study 4: Dual color fluorescence in situ hybridization (FISH) assays in detecting Mycobacteria

<table>
<thead>
<tr>
<th>Cultures</th>
<th>MN Genus—MTBC FISH assay</th>
<th>MTBC—MAC FISH assay</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MN Genus probe</td>
<td>MTBC probe</td>
</tr>
<tr>
<td></td>
<td>(green fluorescence)</td>
<td>(orange fluorescence)</td>
</tr>
<tr>
<td>MTBC</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>MAC</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Non-MAC NTM</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Mix of MTBC and MAC</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Nocardia</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>
Study 4: Dual color fluorescence in situ hybridization (FISH) assays in detecting Mycobacteria

• Forty-three of 44 reference mycobacterial isolates were correctly identified by the MN Genus-specific probe as Mycobacterium species.

• A total of 248 cultures of clinical mycobacterial isolates also tested by FISH assays.

• DNA sequence of a part of the 23S ribosomal RNA gene amplified by PCR was obtained from 243 of the 248 clinical isolates.

• All 243 were confirmed by DNA sequencing as Mycobacterium species, with 157 and 50 of these identified as belonging to the MTBC and the MAC, respectively.
Study 4: Dual color fluorescence in situ hybridization (FISH) assays in detecting Mycobacteria

- The accuracy of the MN Genus-, MTBC-and MAC -specific probes in identifying these 243 cultures in relation to their DNA sequence-based identification was 100%.
- All 10 isolates of Nocardia, (three reference strains and seven clinical isolates) tested were detected by the MN Genus-specific probe but not the MTBC- or MAC-specific probes.
- Specialized equipment: A standard light microscope fitted with a LED light source and appropriate filters.
- The two FISH assays meet an important diagnostic need in peripheral laboratories of resource-limited tuberculosis-endemic countries.
Road ahead: Study 5. Rapid method for detecting and differentiating MTBC and NTM in sputum by FISH with DNA probes.

- This study examined the utility of a rapid, simple fluorescence in situ hybridization (FISH) assay to identify and differentiate MTBC from NTM in sputum.
- MN Genus/MTBC FISH assay: 2 DNA probes, hybridize with 16S rRNA of Mycobacterium and 23S rRNA of MTBC
- Parallel culturing and AFB staining
- DNA sequencing of cultured bacilli

Road ahead: Study 5. Rapid method for detecting and differentiating MTBC and NTM in sputum by FISH with DNA probes.

- 202 sputum samples from 143 patients
- 67 reacted with both probes
- 22 with MN genus
- Sensitivity, specificity, PPV, NPV: 89.7%, 95.5%, 88.0%, 92.6%

Road ahead: Study 5. Rapid method for detecting and differentiating MTBC and NTM in sputum by FISH with DNA probes.
MTBC FISH assay findings in sputum in relation to Ziehl–Neelsen staining of MTBC culture-positive samples derived from sputum (n = 68).

<table>
<thead>
<tr>
<th>Sputum ZN positive</th>
<th>MTBC DNA sequence positive in culture</th>
<th>MTBC FISH positive in sputum</th>
<th>Diagnostic sensitivity for sputum MTBC FISH based on DNA sequencing of cultured mycobacteria</th>
<th>Mean % (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sputum ZN positive</td>
<td>60</td>
<td>58</td>
<td>Sensitivity</td>
<td>96.7 (66.9–98.2)</td>
</tr>
<tr>
<td>Sputum ZN negative</td>
<td>8</td>
<td>3</td>
<td>Sensitivity</td>
<td>37.5 (NA)</td>
</tr>
</tbody>
</table>

MTBC FISH assay: Affordable, rapid (2 hrs), minimal technical equipment
## Diagnostic modalities

<table>
<thead>
<tr>
<th>Diagnostic modality</th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>AFB smear and culture</td>
<td>67.5%</td>
<td>97.5%</td>
</tr>
<tr>
<td>FNAC</td>
<td>96.1%</td>
<td>96%</td>
</tr>
<tr>
<td>PCR</td>
<td>97.2%</td>
<td>72.4%</td>
</tr>
<tr>
<td>Gene Xpert</td>
<td>93%</td>
<td>98.3%</td>
</tr>
</tbody>
</table>
Diagnostic modalities

- Sputum - AFB smears and culture (MGIT, BACTEC)
- Fine needle aspiration cytology and biopsy
- GeneXpert and PCR – Nucleic acid amplification technique
References


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