CHAPTER

21

Available Diagnostic Tools in Mycobacteriology: A Recap on Utility

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ABSTRACT

India accounts for a quarter of the world's annual incidence of Tuberculosis. So much work has been done for the diagnosis and treatment of this disease. Other than routine blood tests like ESR, imaging modalities, sputum examination and culture techniques, the efforts are ongoing to develop more useful tests both at peripheral level & in highly equipped laboratories. We will try to discuss the diagnostic tests and their utility from a clinician's perspective in this chapter.

INTRODUCTION

India is not only world's second most populous country; it also accounts for a quarter of the world's annual incidence of TB. Every year around two million people develop TB in India & 3, 00,000 die.[1]

The aim of the National Strategic plan is to extend TB care in such a way that is particularly relevant in Indian context, acceptable to the medical fraternity in both public and private sectors in India.[2] In this regard, 26 standards have been formulated to cover all aspects of TB care. In this chapter, we will focus on diagnostic plan of TB patient.

Table 1: Diagnostic Tools in Mycobacteriology

DIRECT:	INDIRECT:
1. Microscopy	1. Imaging modalities
2. Culture	a. Chest X-ray
3. Genotypic methods	b. Ultrasound
a. PCR/CBNAAT*	c. CT/ MRI
b. LiPA*	2. Serological assays: (Antibody based Elisa Test) banned in 2012 [Figure 1])
4. Histopathological examination (FNAC, tissue biopsies)	3. Hypersensitivity Reaction assay
	a. Humoral mediated:
	b. Tuberculin sensitivity test (TST)
	c. Cellular mediated:
	- TB feron Gold/IGRA*
	- ADA*
	4. TB LAM Assay*
	5. Analysis of body fluids (pleural fluids, CSF, ascites, synovial): To look for exudative character.
	6. ESR

*CBNAAT- Cartridge Based Nucleic acid amplification Test, *LiPA- Line Probe Assay, *IGRA- Interferon gamma release assay, *ADA- Adenosine Deaminase Assay, *TB LAM Assay- Tuberculosis Lipoarabinomannan Assay

TESTING AND SCREENING OF PULMONARY TUBERCULOSIS

The recommended guidelines are as below.[3-6]

TESTING

1. Any person with symptoms and signs suggestive of TB including cough >2 weeks, fever >2 weeks, significant weight loss, haemoptysis etc. and any abnormality in chest radiograph must be evaluated for TB.
2. Children with persistent fever and/or cough >2 weeks, loss of weight/ no weight gain, and/or h/o contact with pulmonary TB cases must be evaluated for TB.

SCREENING

1. People living with HIV (PLHIV), malnourished, diabetics, cancer patients, patients on immunosuppressant or maintenance steroids therapy, should be regularly screened for signs and symptoms suggestive of TB.
2. Enhanced case finding should be undertaken in high risk populations such as healthcare workers, prisoners, slum dwellers and certain occupational groups such as miners.

PROBABLE TB

Patients with symptoms suggestive of TB without microbiological confirmation (sputum smear microscopy, culture and molecular diagnosis), but with strong clinical and other evidence (e.g. X-ray, Fine Needle Aspiration Cytology (FNAC), histopathology may be diagnosed as "Probable TB". [7,8]

BASIC APPROACH TO DIAGNOSIS

There are two basic approaches for the diagnosis of tuberculosis.[9]

1. Direct approach- Includes detection of mycobacteria or its products.

Table 2: Future Tools

Type	Pipeline
Microscopy	TBDx (Signature Mapping Medical Sciences) automated system for smear microscopy that automatically loads and reads slides. Needs Optimization.
Phenotypic - Culture	A colorimetric thin layer agar method (TLA) to detect TB and screen for isoniazid, rifampicin, and ciprofiloxacin- resistance. The TREK Sensititre MYCOTB MIC- Microtitre-plate based liquid system for first and second DST.
Molecular Diagnosis	**LAMP test**- manual NAAT test at microscopy centre level.* **TruNAAT Test:** "micro-PCR handheld device" (bigTec & Molbio Diagnostics PVT ltd, India)-result in 30 mts – 1 hr geneDirve (Epistem)- genotyping and sequencing test in a hand-held device. Result within 1 hr **B-SMART-** detects TB and DR (first-line) detection limit (<1000) at present and being refined to <50 bacilli in sputum to be useful for smear negative cases. Other molecular tests like legase chain reaction medofication of PCR_ SDA, NASBA, b-DNA (ICMR)
Non Molecular tests	Breath Analysis Test Detection of Volatile organic compounds Alere TB LAM- Urine LAM- Lateral flow test-rule in TB with 71% Sensitivity along with smear especially useful in HIV+ve
Serological Tests	MBio and FIND-developing a series of antignes for detection of active TB as a POC platform. Field Evaluation by late 2012 or 2013

* Recommended by World Health Organization (Geneva 11.08.2016)

2. Indirect approach- Includes measurements of humoral and cellular responses of the host against disease (Tables 1 & 2).

DIRECT APPROACH

1. SPUTUM EXAMINATION/ MICROSCOPY:- Sputum smear microscopy is the most commonly used method for bacteriologic diagnosis of TB for the last 70 years but has limited sensitivity (Tables 3 & 4), especially in patients with non cavitatory pulmonary disease; paucibacillary TB (e.g. in HIV positive patients) and in children.[10]

Two staining methods are used to observe acid fast bacilli: - Zeihl-Neelsen staining and fluorescent auramine staining. The staining procedure depends on the ability of mycobacteria to retain these dyes when treated with acid and alcohol solutions (Figures 1 & 2).

DESIGNATED MICROSCOPY CENTRES have been established in India under RNTCP for efficient networking of sputum examination and reporting.

2. CULTURE METHODS: All clinical specimens suspected of containing mycobacteria should be inoculated on to culture media for four reasons:[11]

1. Culture
2. Growth
3. Drug sensitivity testing (DST)
4. Genotyping

Indication for sputum culture includes:

- To diagnose paucibacillary disease in TB suspects (e.g. HIV positive patients) who have two negative smears.
- For drug susceptibility testing in TB suspects with a history of previous TB treatment (interruption, failure, relapse) patients who remain smear positive at the end of the intensive/continuation phase of treatment or who fail to improve clinically during treatment.[12]
- For drug susceptibility testing in people at high-risk such as MDR and XDR- TB contacts, health care personnel and prisoners.
- Speciation of mycobacteria in case of atypical mycobacterial disease.
- Follow up examination in MDR and XDR TB patients.
- Confirmation of TB in case of invalid results in CBNAAT and LiPA.
- Rifampicin resistance in case of indeterminate resistance shown by CBNAAT and LiPA.

Disadvantages

1. Expensive
2. Slow diagnostic technique
3. Not accessible to all patients.

Types

At present, the mycobacterial cultures used in our country are:[13-15]

Table 3: Sputum Examination

Method	No of Bacilli required (per ml of sputum)	Sensitivity	Specificity
1. Microscopy a. Conventional ZN Stain b. Fluorescent Stain	1000-10000	50-80%	98%
2. Culture a. Liquid b. Solid	10-100	80-85%	98%
1. Molecular assay a. CBNAAT b. LiPA	1-30	85%	99%

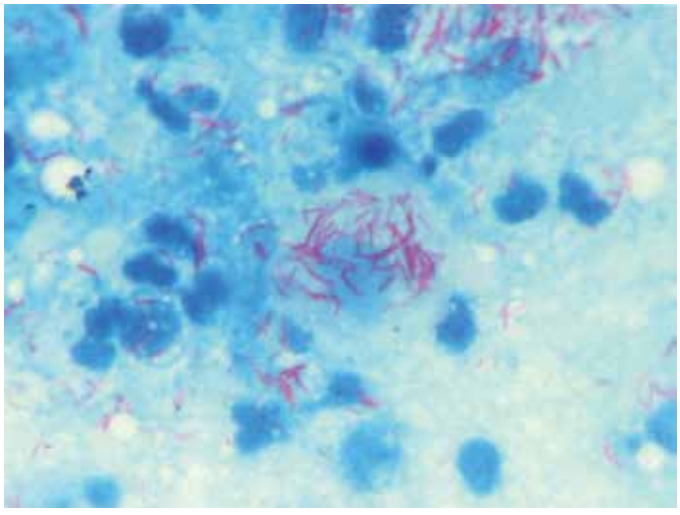

Fig. 1: Zeihl-Neelsen Stain

1. Liquid Media:- MGIT960 and BACTEC 460 Radiometric system.

- MGIT 960 is fluorometric assay (ICMR Research)
- BACTEC 460 on the other hand is radiometric system. The major disadvantage of this medium is DISPOSAL of material.

2. Solid Media:- Conventional egg based solid medium such as Lowenstein Jensen medium and agar based ones are used.

Major constraint of culture is its slow growth which necessitates a mean incubation period of at least 4 weeks.[16] The drug susceptibility test takes another 4 weeks. The other limitations are cost factor and non accessibility to all patients.

Drug Sensitivity Test (DST)

The commonly used method is broth based DST, using either BACTEC 460 cr MGIT 960 system.[12] The

Table 4: Grading Table for Sputum staining

ZN Staining		Auramine Staining	
Number of bacilli seen on smear	Results reported	Number of bacilli seen on smear	Results reported
No AFB per 100 oil immersion field	0	No AFB on slide	0
1-9 AFB per 100 oil immersion field	Scanty	<1 AFB per field	+
10-99 AFB per 100 oil immersion field	+	1-9 AFB per field	++
1-10 AFB per 1 oil immersion field (min 50 fields)	++	10-99 AFB per field	+++
>10 AFB per 1 oil immersion field (min 20 fields)	+++	>100 AFB per field	++++

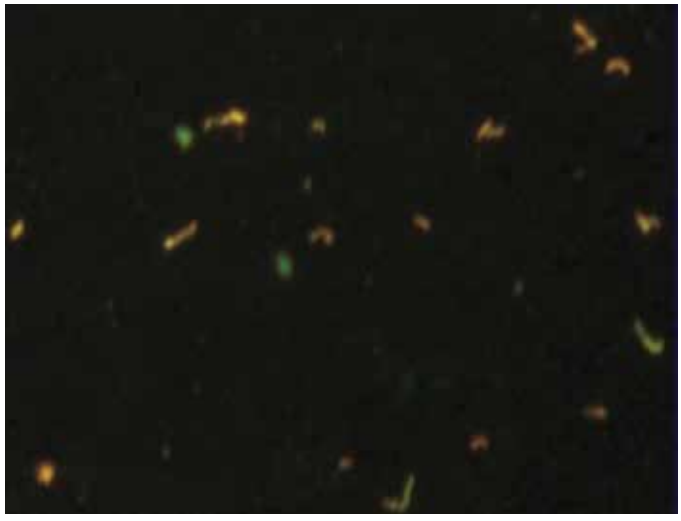

Fig. 2: Auramine Stain

drug free & drug containing tubes are inoculated with a homogenous suspension of MTB bacteria & incubated. The fluorescence of the drug containing tube is then compared with the drug free tube.

The phenotypic DST methods use critical concentrations for each TB drug. An isolate is considered to be resistant to a drug when growth in the presence of a critical drug concentration exceeds growth of the same isolate diluted 1:100 in drug-free media (Table 5). DST for isoniazid should be performed using two critical concentrations: low and high level. Isolates resistant to the low-

Table 5: Drug resistance in tuberculosis

Drug	Proportion method	NRA result			
		No. of resistant strains	No. of susceptible strains	% Sensitivity	% Specificity
RIF	Resistant Susceptible	56 0	0 44	100	100
INH	Resistant Susceptible	57 1	1 41	98	97.6
STR	Resistant Susceptible	49 8	2 41	96	83.67
EMB	Resistant Susceptible	38 1	13 48	74.5	98
Total	Resistant Susceptible	200 10	16 174	92.59	94.56

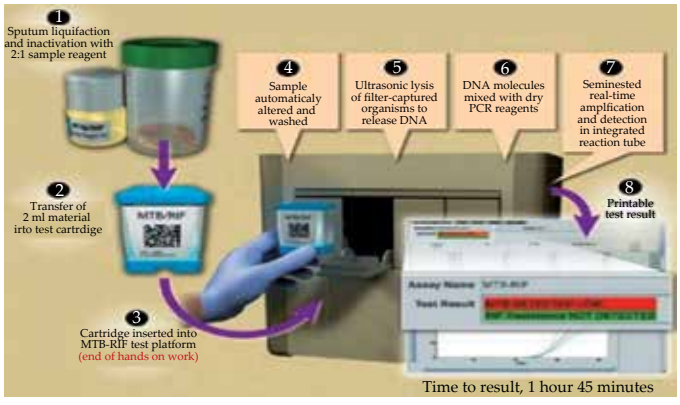

Fig. 3: Steps for CBNAAT

level critical concentration but susceptible to the high-level critical concentration should be reported as having low level resistance to isoniazid. These patients will benefit from higher dosage of isoniazid in their treatment.

Phenotypic DSTs require a positive culture (4-6 weeks) before testing for drug susceptibility can be performed. This takes an additional 2-3 weeks. Phenotypic DSTs is performed on Rifampicin, Isoniazid (high and low), Ethambutol, Pyrazinamide, Streptomycin, Amikacin, kanamycin, Capreomycin, Ethionamide, Ofloxacin and Moxifloxacin.[17]

3. Genotypic/Molecular Methods:- (ICMR)[9]

a. CBNAAT (Cartridge based nucleic acid amplification test):

b. LiPA (Line Probe Assay)

CBNAAT or Gene xpert MTB/RIF is a semi quantitative nested real time PCR in vitro diagnostic test which is useful for rapid diagnosis and has the potential to replace microscopy as first line diagnostic test. In additional it allows rapid screening of rifampicin resistance.[18,19]

Important features

1. Fully automated with 1 step external sample preparation.
2. Time to result- 11/2 hour (walk away test)
3. No biosafety cabinet
4. Closed system (No contamination risk)
5. Scalable technology
6. Specific for MTB
7. Sensitivity similar to culture
8. Detection of RIF-resistance via rpoB gene. (Acts as surrogate marker for MDR TBC)
9. Useful yield even in sputum negative samples.
10. Test is applicable to any body fluids except blood, Urine & stool.
11. Useful for both diagnosis of TBC and MDR TBC in paediatrics age gp, HIV patients (PLHIV), paucibacillary cases & extrapulmonary tbc.
12. It is specific for MTB complex (It can differentiate MTB from other Mycobacteria.)

Steps of the Test (Figure 3)

1. Treatment of sputum sample with sodium hydroxide & Isopropanol containing sample reagent to liquify and inactivate sample.
2. Transfer of 2 ml of liquified sputum to cartridge.
3. Loading the cartridge into Genexpert device.

Limitations:

1. It cannot be used for monitoring treatment because it does not distinguish between live & dead bacilli & hence its use is for diagnosis only.
2. The Rifampicin result can only be reported if MTB complex is detected as it is MTB complex specific test.

b. Line Probe Assay (LiPA)

This is again PCR based hybridization assay (Figure 4).[20] The commercial test available in our country is the Genotype® MTBDRplus assay.

Features

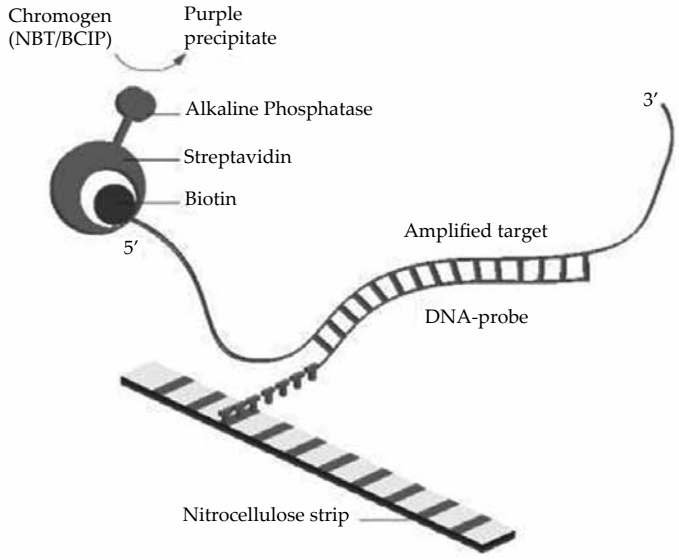

Fig. 4: Molecular depiction of line probe assay

Table 6: Comparison between Tuberculin test and Quantiferon

TST	Quantiferon
Delayed type hypersensitivity RxN	Cell mediated immune response.
Affected by BCG	Not affected
Two visits required	Single patient visit
Subject to reader bias and error	Less subject to reader bias and error
Response boosted in patients already tested once with TST	Does not boost response measured by subsequent test.

1. Approved for direct testing on smear positive specimens and on Isolate from solid & liquid cultures.
2. Simultaneously detects MTB complex and specific mutations in the rpo B gene conferring rifampicin resistance
3. Also detects mutations on KatG gene associated with higher levels of INH resistance and Inh A gene, linked with lower levels of INH resistance.
4. It reduces time to diagnosis of MDR Tbc to 7 days.

Limitations

1. Can be performed on smear positive specimen only.
2. Cannot be used for monitoring treatment
3. Test is labour intensive, prone to contamination and human error.
4. It requires a lot of space.

INDIRECT APPROACH

These are already listed in the table. We will be discussing the following ones only.

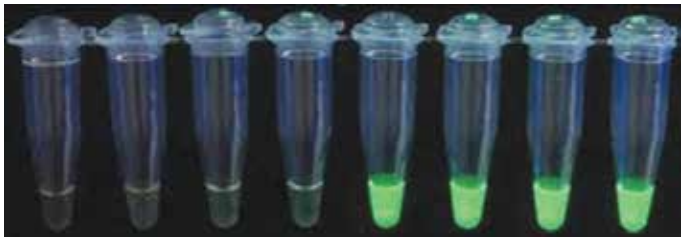

Fig 5: Loop Mediated Isothermal Amplification for tuberculosis

a. Tuberculin Skin test: It is a delayed type hypersensitivity reaction to tuberculin protein. TST has limited value in clinical setup especially in TB endemic area.[11] A positive test doesnot indicate TB disease but only infections. So, it is particularly useful in Paediatric age group patients. False negative results are seen in immunosuppressive conditions e.g. early stage of TBC, malignancy and HIV infections. Specificity of TST also varies depending on timing of BCG vaccination and whether repeated (Booster) doses are given (Table 6).

b. TB feron Gold/IGRA:- This a third generation in vitro test which is based on quantification of interferon gamma (IFN-r) released from sensitized lymphocytes in whole blood from patients having LATENT tuberculosis.[21] It uses peptides from three TB antigens (ESAT-6, CFP-10 & TB-7.7) in a tube format. The result is reported as IU/ml.

A positive IGRA may not necessarily indicate active TB, however a negative IGRA result rules out possibility of both active & latent tuberculosis.

Adenosine Deaminase Assay (ADA):- ADA level is a marker of cellular immunity in a patient with tuberculosis. it is an enzyme which contributes in purine metabolism. It is essential for proliferation and differentiation of T lymphoid cells.[22] The cut off level of ADA for pleural fluid is >30 U/l. The sensitivity and specificity of ADA is 92.8% and 90% respectively. The cutoff value of serum ADA in extrapulmonary TB patient reported was 24 U/L with sensitivity 94.3% and specificity 92.2%. Also a decline in serum ADA level was found as treatment was started.[23,24]

Test in Near Future

TB LAM (Lipoarabinomannan) detection in urine

a. LAM is a component of the outer cell wall that is shed from metabolically active or degrading cell & Is detectable in urine

b. In tube elisa and dipstick method

c. May be of particular value in HIV coinfected patients.

d. Rapid and simple diagnostic technique which can be performed at peripheral level

TB LAMP (Loop Mediated Isothermal Amplification):- Recommended by WHO in policy guidance on 11th August 2016. It is a unique temperature independent way of amplifying DNA from TB organisms, takes less

than one hour and results can be read with the naked eye under ultraviolet light (Figure 5).[25] The robust TB-LAMP instrument can be used at peripheral health centre level.

A few other points to be remembered

1. Extra Pulmonary TB:- For all patients (adults, adolescents and children) with presumptive extra-pulmonary TB, appropriate specimens from the presumed sites of involvement must be obtained for microscopy/culture/CB-NAAT/molecular test/ histopathology examination and drug sensitivity testing (DST).[26]

2. Diagnosis of HIV in TB patients:- All diagnosed TB patients should be offered HIV counseling and testing.

3. Diagnosis of Multi-Drug Resistant TB (MDR-TB):- Prompt and appropriate evaluation should be undertaken for patients with presumptive MDR-TB or Rifampicin ® resistance in TB patients who have failed treatment with first line drugs, paediatric non-responders, TB patients who are contacts of MDR-TB (or R resistance), TB patients who are found positive on any follow-up sputum smear examination during treatment with first line drugs, diagnosed TB patients with prior history of anti-TB treatment, TB patients with HIV co-infection and all presumptive TB cases among PLHIV.[27] All such patients must be tested for drug resistance with available technology, a rapid molecular DST (as the first choice) or liquid/solid culture-DST (at least for R and H; and at least for Ofloxacin (O) and Kanamycin (K), if MDR.

   Wherever available DST should be considered and offered to all diagnosed tuberculosis patients prior to start of treatment.

4. Diagnosis of Extensively Drug Resistant TB (XDR-TB):- On detection of Rifampicin and isoniazid resistance , patient must be offered sputum test for second line DST using quality assured phenotypic or genotypic methods, wherever available.

5. Contact Investigation:-

1. All care providers to patients with TB should ensure all household contacts and other persons who are in close contact with TB patients are screened for TB as per defined diagnostic standards.

2. In case of paediatric TB patients, reverse contact tracing for search of any active TB case in the household of the child must be undertaken.[28]

The highest priority contacts for active screening are:

- Persons with symptoms suggestive of tuberculosis.
- Children aged <six years.
- Contacts with known or suspected immune-compromised patient, particularly HIV infection.
- Contact with Diabetes Mellitus.
- Contacts with other higher risks including pregnancy, smokers and alcoholics etc.
- Contacts of patients with DR-TB. In case of contact with a DR-TB index case, close clinical monitoring should be provided, as there is no evidence that treatment of latent infection with available drugs is presently effective.

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