

WHAT IS NEW IN THE DIAGNOSIS OF TUBERCULOSIS?*

PART 1 : TECHNIQUES FOR DIAGNOSIS OF TUBERCULOSIS

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Despite the discovery of the tubercle bacillus more than a hundred years ago, and all the advances in our knowledge of the disease made since then, tuberculosis still remains one of the major health problems facing mankind, particularly in developing countries. Presently, about one third of the world's population is infected with *Mycobacterium tuberculosis*. It is estimated that currently there are about 10 million new cases of tuberculosis every year with 3 million deaths occurring world-wide¹. Currently, more people die of tuberculosis than from any other infectious disease. Death from tuberculosis comprises 25% of all avoidable deaths in developing countries. Nearly 95% of all tuberculosis cases and 98% of deaths due to tuberculosis are in developing countries and 75% of tuberculosis cases are in the economically productive age group². In India, out of a total population of over 1 billion, each year about 2 million develop active disease and up to half a million die³. It implies that every minute, a death occurs due to tuberculosis in our country. It also imposes a cost on our economy in terms of current and future output losses because of premature deaths and ill health⁴. To add to the existing burden, the situation is compounded by the large scale increase of new TB cases associated with increasing HIV infection. India is estimated to have 3.5 million HIV patients, and about 1.8 million of these are co-infected with TB⁵. The HIV seroprevalence among TB patients in India ranges from around 2 to 20%, with an estimated 60% of HIV infected persons breaking down with active TB disease in their life-time⁶. The diagnosis of TB with HIV positive patients is more difficult than in those without HIV infection. These issues need to be scrutinized when laboratories are required to cater to this group of population.

Early diagnosis of tuberculosis and initiating

optimal treatment would not only enable a cure of an individual patient but will also curb the transmission of infection and disease to others in the community. Of the several distinct components of the TB Control Programme, case finding remains the corner stone for effective control⁷. However, there are no definite guidelines available as of today as to how to use optimally the number of diagnostic tests ranging from simple AFB microscopy to complex molecular biological techniques which have become available over a period, to establish or rule out diagnosis of tuberculosis in a given patient. The present write-up gives, in two parts, a brief description of the important techniques available for the (i) early diagnosis of tuberculosis, and (ii) drug susceptibility testing.

There are two basic approaches for the diagnosis of tuberculosis. The direct approach includes detection of mycobacteria or its products and the indirect approach includes measurements of humoral and cellular responses of the host against tuberculosis.

The diagnostic modalities should have certain desirable features *viz.* sensitivity, specificity, predictive value, speed, reproducibility, cost effectiveness, safety, simplicity, robustness and easy application for wider use. Ideally, the tests should be quantitative, at least in some measure, so that the infectiveness of the individual cases can be measured. This is especially important for decisions to isolate hospitalized patients and to provide preventive therapy to contacts. Diagnostic modalities must also be tailored to needs of the population and epidemiology of TB in that region. Epidemiologically, the countries can be grouped as non-endemic or endemic. The diagnostic algorithms are planned as

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per specific needs and resources available in individual countries.

The diagnostic needs in disease non-endemic countries include identification of latent infection in high risk groups, diagnosis of patients in early phase of disease, faster detection of outbreaks (nosocomial and community transmission), and finding out patients with non-tuberculous mycobacterial disease. The diagnostic needs in disease endemic countries include improved microscopy, usage of liquid culture for childhood and extra-pulmonary TB, chemical and physical detection of mycobacterial antigens in paucibacillary condition, antigen capture, antibody detection, cellular immune recognition, nucleic acid amplification, and phage assay.

The diagnosis in endemic countries depends more on the use of labour intensive, easy to use methodology with minimum infrastructure or equipment. The need is to find a viable alternative for smear microscopy. This method has to have the following desirable features: results within 2 hours, simple training, easy interpretation, should function well in HIV positive patients and allow start of treatment as early as possible. Currently, less than 20% of the nearly 10 million predicted annual cases of tuberculosis are identified as smear positive.

DIRECT APPROACH

Microscopy: Microscopy is the simplest and most rapid procedure currently available to detect acid-fast bacilli (AFB) in clinical specimens by Ziehl-Neelsen staining method or its modifications. The limit of detection with this method is that it requires at least 5×10^3 bacilli per ml of sputum⁸. Fluorescent staining method offers the advantage of screening the smear under low power where a large number of slides is screened in less time.

The results of smear microscopy can be influenced by the type of specimens, thickness of the smear, extent of decolorisation, type of counter stain used, and training and experience of the person examining the smear. Several approaches are being made to enhance the sensitivity of the smear

microscopy. Concentration of sputum sample by cytocentrifugation has been found to enhance the sensitivity to almost 100%⁹. Liquefaction of sputum with sodium hypochlorite followed by concentration of bacilli by overnight sedimentations enhances the sensitivity of smear microscopy close to 70% compared to culture. Similarly, treatment of sputum samples with Zwitterionic detergent, also known as C₁₈ carboxy-phosphobetaine (CB18) interferes with the innate buoyancy of the bacilli and enhances the result of smear microscopy¹⁰.

The main advantages of smear microscopy:

- (i) It is inexpensive, simple.
- (ii) It is relatively easy to perform and read and detects transmitters of tubercle bacilli.
- (iii) Results can be reported within hours of receipt of the sample. Provides reliable epidemiological indicators needed for the evaluation of the National Tuberculosis Control Programme.

For our country, the smear microscopy is likely to remain for the foreseeable future, the only cost effective tool for diagnosing patients with infectious tuberculosis and to monitor the progress of treatment. Under the Revised National Tuberculosis Control Programme (RNTCP), Government of India has ensured good quality sputum microscopy in all aspects in the entire RNTCP implemented areas of the country. This includes training, enhancing manpower, developing manuals, establishing uniform laboratory set up, ensuring supply of good quality reagents and microscopes and instituting quality control measures, right from the microscopic centres up to the state level laboratories. The various national tuberculosis institutes of the country are made responsible for monitoring the above-mentioned activities.

Culture

Isolation of mycobacteria from clinical samples by culture still represents the corner stone on which definitive diagnosis of tuberculosis and other mycobacterioses relies. At present, mycobacterial culture can be performed on conventional egg based solid medium such as Lowenstein-Jensen medium and agar based ones, such as Middlebrook 7H10 or 7H11 and liquid media such as Kirschner's or Middlebrook 7H9 broth. The major constraint of culturing mycobacteria in conventional media is its slow growth which necessitates a mean incubation period of at least 4 weeks¹¹. The drug susceptibility tests to anti-tuberculosis drugs require additional 4 weeks. Most of the laboratories in the developing world rely on solid media for culture of mycobacteria. The choice and preparation of specimens by various pretreatment procedures has tremendous influence on the sensitivity of results. The positivity of culture largely depends on the technique of decontamination used by various laboratories, viz the chemicals used for decontamination and the centrifugation method adopted for processing specimens for culturing mycobacteria by inoculating into solid or liquid media.

Although a combination of solid and liquid media is currently the gold standard for the primary isolation of mycobacteria, a few modern, rapid methods are also available. These include micro colony detection on solid media (including the rapid slide culture technique), septi-check AFB method, microscopic observation of broth culture (MODS), the BACTEC 460 radiometric system, BACTEC MGIT 960 system (Becton Dickinson), MB/Bac T system (Organon Teknika), and the ESP II culture system.

Micro colony detection on solid media: In this method, plates poured with thin layer of Middlebrook 7H11 agar medium are incubated and examined microscopically on alternate days for the first 2 days and less frequently thereafter. In less than 7 days, micro colonies of slow growing mycobacteria such as *M.tuberculosis* can be detected. Though this method is less expensive and requires about half the

time needed for conventional culture, the recovery of mycobacteria is less efficient and it is labour intensive. Since *M.tuberculosis* grows more rapidly in liquid medium forming strings and tangles, which can be observed under the inverted light microscope with 40x magnification, this method is a better alternative for culturing tubercle bacilli¹².

Septi-check AFB method : The septi-check AFB system consists of a capped bottle containing 30.0 ml of Middlebrook 7H9 broth under enhanced (5-8%) CO₂ a paddle with agar media enclosed in a plastic tube, and enrichment broth containing glucose, glycerine, oleic acid, pyridoxal, catalase, albumin, polyoxyethylene 40 stearate, azlocillin, nalidixic acid, trimethoprim, polymyxin B and amphotericin B. One side of the paddle is covered with non-selective Middlebrook 7H11 agar, the reverse side is divided into two sections: one contains 7H11 agar with *para*-nitro- α -acetyl-amino- β -hydroxypropionophenone (NAP) for differentiation of *M.tuberculosis* from other mycobacteria, the other section contains chocolate agar for detection of contaminants. This non-radiometric approach has the potential to expedite processing, obviate CO₂ incubation requirements and facilitates early detection of positive cultures. This method requires about 3 weeks of incubation. The unique advantage of this technique is the simultaneous detection of *M.tuberculosis*, non-tuberculous mycobacteria (NTM), other respiratory pathogens and even contaminants. A multicentric study conducted in the USA has shown that the system gives a better culture result when compared to other methods including BACTEC 460 TB system¹³.

Radiometric BACTEC 460 TB method : This technique is specific for mycobacterial growth, wherein ¹⁴C labelled palmitic acid in 7H12 medium is used. This system detects the presence of mycobacteria based on their metabolism rather than visible growth. When the ¹⁴C labelled substrate present in the medium is metabolized, ¹⁴CO₂ is produced and measured by the BACTEC system instrument and reported in terms of growth index (GI) value. The BACTEC system is also useful in the identification of *M.tuberculosis* using specific inhibitor, *para*-nitro- α -acetyl-amino- β -hydroxypro-

piophenone. Using the same system, drug susceptibility tests can also be performed for all the anti tuberculosis drugs when sufficient GI is observed. Mycobacteria in clinical samples can be detected in half the time compared to conventional culture methods¹⁴.

A comparison of the BACTEC radiometric method with the conventional culture and drug susceptibility testing methods undertaken at the Tuberculosis Research Centre (TRC), Chennai showed that the rate of isolation of positive cultures was significantly faster with the BACTEC method, with 87% of the positives being obtained by 7 days and 96% by 14 days. There was a good correlation in drug susceptibility tests and most of these results could be obtained within 8 days by the BACTEC method¹⁵. By facilitating early diagnosis, the BACTEC method may prove to be cost effective in a population with high prevalence of tuberculosis.

MGIT 960 mycobacteria detection system : It is an automated system for the growth and detection of mycobacteria with a capacity to incubate and continuously monitor 960 mycobacteria growth indicator tubes (MGIT) every 60 minutes for increase in fluorescence. Growth detection is based on the AFB metabolic O₂ utilization and subsequent intensification of an O₂ quenched fluorescent dye contained in a tube of modified MGIT. A series of algorithms are used to determine presumptive positivity and alert the operator to the presence and location of positive tubes.

In a multi-centre evaluation of the MGIT 960 system, three high volume testing sites in USA compared the growth and recovery of mycobacteria to that of BACTEC 460 TB and conventional culture methods¹⁶. Comparison of average time of detection between paired specimens showed that the BACTEC 460 and MGIT 960 systems were 8.7 versus 8.6 days for *M. avium* complex (MAC) and 13.4 versus 15.5 days for *M. tuberculosis* respectively. It was revealed that MGIT 960 system exhibits greater potential as a rapid, accurate and cost effective method for a high volume mycobacteriology laboratory.

MB/BacT system : This is a non-radiometric continuous monitoring system with a computerized database management. The system is based on colorimetric detection of CO₂.

Comparison of the performance of MB/BacT system with that of BACTEC 460 showed that the mean time for detection of *M. tuberculosis* by the BACTEC system was 11.6 days vs 13.7 days by the MB/BacT system. It was concluded that the MB/BacT with the computerized data management system is an acceptable alternative for BACTEC 460 method despite some minor disadvantages such as increased contamination and slightly longer time for detection of growth¹⁷.

ESP culture system II : This is a fully automated continuous monitoring system based on the detection of pressure changes within the headspace above the broth culture medium in a sealed bottle, i.e. either gas production or gas consumption due to microbial growth. A special detection algorithm is present in this system for the detection of very slowly growing mycobacteria.

The system was evaluated in clinical specimens for the detection of mycobacteria against BACTEC 460 and 7H11 agar solid medium. The mean time for recovery of all mycobacteria, *M. tuberculosis* complex and MAC was found to be 13.1, 15.5 and 10.9 days respectively. Hence the ESP II culture system seems to be a reliable non-radiometric less labour-intensive alternative to BACTEC 460 system for the growth and detection of mycobacteria. However, as with other liquid culture systems, ESP II should be used in combination with a solid medium, not as a stand-alone system¹⁸.

Microscopic observation of broth culture : This is a rapid and relatively inexpensive method which compares very well with other well established systems in terms of both sensitivity and specificity, and also to some extent with speed when compared to solid media. Although this technique may be appropriate for disease endemic high-burden countries, it requires P2 Bio-safety cabinets, relatively expensive Middlebrook 7H9 broth, oleic acid dextrose catalase (OADC) and anti-microbial supplements and

a relatively high technical skill¹⁹.

IDENTIFICATION OF MYCOBACTERIAL SPECIES

Mycobacterial speciation is carried out by various methods ranging from conventional biochemical tests to modern high-tech molecular biological methods. These can be broadly classified as:

- (i) Phenotypic characterization
- (ii) Biochemical typing
- (iii) Analysis of lipid: By gas chromatography, mass spectrum and high pressure liquid chromatography (HPLC).
- (iv) Probe based identification: It includes peptide nucleic acid (PNA) fluorescence *in situ* hybridization assay for identification of mycobacterial species. PNA is a novel DNA mimic in which sugar phosphate backbone of DNA has been replaced by a polyamide backbone. The uncharged nature and high conformational flexibility of PNA allows PNA probes to hybridize DNA or RNA with excellent affinity and specificity. The sensitivity of the *M.tuberculosis* (MTB) probe targeting MTB complex was reported to be 98%. The sensitivity of NTM probes targeting NTM species, however, was only 57% since these do not target all non-tuberculous mycobacterial species²⁰.
- (v) Sherlock mycobacteria identification system (SMIS): It uses computerized software to identify mycobacterial species on the basis of mycolic acid pattern generated by HPLC²¹.
- (vi) PCR restriction enzyme analysis: It exploits the 65 kDa hsp gene and 16s rRNA gene for identification

purposes.

- (vii) DNA chips: A technology still under development that appears promising involves oligonucleotide arrays or DNA chips (molecular biology coupled with computer technology), which have been designed to determine the specific nucleotide sequences diversity of the rpoB and 16S rRNA genes for species identification²².

Detection and identification of mycobacteria directly from clinical samples

Both genotypic (molecular) and phenotypic methods are available with newer modifications for the diagnosis of tuberculosis as an alternative for smear microscopy.

Genotypic methods

Polymerase chain reaction : PCR allows sequences of DNA present in only a few copies of mycobacteria to be amplified *in vitro* such that the amount of amplified DNA can be visualized and identified. If appropriate sequences specific for *M.tuberculosis* are selected, 10-1000 organisms can be readily identified. The PCR methodology is rapid; results are available within a day of DNA extraction from the sample. A number of target genes of mycobacterial DNA have been evaluated for diagnosis by PCR and various other genotypic methods. The most common target used in the PCR is IS6110. This sequence is specific for *M.tuberculosis* complex and is present up to 20 times in the genome, thus offering multiple targets for amplification. PCR detection of IS6110 in sputum (in pulmonary TB) and peripheral blood (in extra-pulmonary TB), when compared to culture has a sensitivity, specificity and positive predictability of 83.5, 99.0 and 94.2% respectively. A variety of PCR methods have been described in the search for a sensitive and reliable screening test for tuberculosis in clinical specimens. Species-specific and genus-specific PCR methods are being used with various targets and modifications of PCR. The following are some of the methods used for identification of

M.tuberculosis and NTM²³:

Transcription mediated amplification (TMA) and nucleic acid amplification (NAA): This approach identifies the presence of genetic information unique to *M.tuberculosis* complex directly from pre-processed clinical specimens. The NAA technique uses chemical, rather than biological amplification to produce nucleic acid, so that within a few hours these tests distinguish between *M.tuberculosis* complex and NTM in an AFB positive specimen. It is currently used only for respiratory specimens; use for non-respiratory specimens is likely in the near future.

A positive direct amplified test in conjunction with an AFB-positive smear is highly predictive of tubercular disease. However, the results of NAA are preliminary; mycobacterial culture is still needed for species identification/confirmation and for drug-susceptibility testing. A negative NAA with an AFB-positive smear indicates that the AFB is probably NTM. The *M.tuberculosis* direct test (MTD) and amplified mycobacterial direct test (AMDT) are highly sensitive (96%) and specific (100%) for *M.tuberculosis* on specimens that are smear positive for AFB. However, there are occasional false-negative or false positive results being reported, which are either due to the presence of fewer bacilli or due to contamination. Another disadvantage of the technique is that both viable and dead bacilli can give positive results as the DNA of both can be amplified.

The ligase chain reaction: It is a variant of PCR, in which a pair of oligonucleotides are made to bind to one of the DNA target strands, so that they are adjacent to each other. A second pair of oligonucleotides is designed to hybridize to the same regions on the complementary DNA. The action of DNA polymerase and ligase in the presence of nucleotides results in the gap between adjacent primers being filled with the appropriate nucleotides and ligation of the primers. The LCX *M.tuberculosis* assay kit (Abbot) is mainly being used for respiratory samples, and has a high overall specificity and sensitivity for smear positive and negative specimens.

Other modifications of the PCR include strand displacement amplification (SDA), nucleic acid sequence based amplification (NASBA), branched DNA (b-DNA) and line probe assay (LiPA). The list of the targets in commercially available kits is given in Table 1.

Table 1. Targets in commercially available kits

Sl.No.	Method	Target
1	PCR	IS 6110 65 kDa
2	TMA	16 S r RNA
3	SDA	IS 6110
4	NASBA	16 S r RNA
5	b DNA	As for PCR
6	LiPA	As for PCR

Phenotypic method

FAST Plaque TB™ : This is an original phage based test, which uses the mycobacteriophage to detect the presence of *M.tuberculosis* directly from sputum specimens. It is a rapid, manual test, easy to perform and has a higher overall sensitivity as compared to sputum smear microscopy, in newly diagnosed smear positive TB patients. The test has a specificity of 98.7 – 99.0% and a sensitivity of 70.3 – 75.2%, as compared to smear microscopy, which has a specificity of 97.3 – 97.4% and a sensitivity of 61.3 – 63.4%^{24,25}.

Serological diagnosis of Tuberculosis

Most of the serological tests have low turn around time, high negative predictive value and are useful as screening tests. The limitation of these tests is low sensitivity in smear negative patients, HIV positive cases, and in disease endemic countries with a high infection rate. The tests are also expensive, require trained personnel and often have difficulty in distinguishing between *M.tuberculosis* and NTM.

Development of antigen detection assay for diagnosis of TB using sputum samples

Capture ELISA : A quantitative test to detect lipoarabinomannan (LAM) has been developed for the detection of TB in urine specimens. Another test being used in a field trial is the dipstick method (semi-quantitative) for the detection of LAM in both pulmonary and extra-pulmonary specimens. Preliminary reports have shown a sensitivity and specificity of 93 and 95% respectively²⁶.

Detection of LAM in sputum : The test is based on the capture antibody derived from murine source (murine monoclonal antibody against LAM). The rabbit antiserum against *M.tuberculosis* is used as a source of detector of the antibody. The specific and sensitive assay for the detection of LAM in sputum is potentially useful for the diagnosis of TB.

Antigen detection in body fluids : The advent of nucleic acid amplification technology (especially PCR) has overshadowed recent developments in antigen detection. However, free mycobacterial antigen at a concentration of 3-20ng/ml can be detected in biological fluids such as cerebrospinal fluid (CSF) or pleural fluid. The most commonly used antigens include mycobacterial sonicates, extracted glycolipids, PPD, Ag5 (38kDa), Ag A60, 45/47kDa Ag, Ag Kp90, 30 kDa Ag, P32 Ag, cord factor (trehalose dimycolate) and lipoarabinomannan. Most of the tests use polyclonal antibodies raised against crude mycobacterial antigens except for antigen 5 and LAM. The sensitivity of tests ranges from 40-50% and specificity 80-95%. The methods used for antigen detection are: the sandwich ELISA, inhibition ELISA, latex agglutination and reverse passive haemagglutination tests²⁷.

INDIRECT APPROACH

Detection of Antibodies for diagnosis of TB

Antibodies to mycobacterial antigens in sera of patients are detected either by using monoclonal or polyclonal antibodies. Cross-reaction by environmental mycobacteria is likely to produce false-positive results. Reproducible methods for

purification of mycobacterial antigens have yet to be evolved; hence the results of most assays available at present are variable in different settings. It is also important to note that the immune response in mycobacterial disease appears to be associated with HLA Class II allotypes and different patients appear to recognize different antigens. It is thus unlikely that all tuberculosis patients will recognize a single antigen, and hence prove to be a handicap for the development of antibody-based detection systems for mycobacteria. Some of the newer approaches are as follows:

TB STAT-PAK : Immunochromatographic test based on the detection of antibodies has been evolved with a capability to differentiate between active or dormant TB infection in whole blood, plasma or serum. Its value in disease endemic countries such as India is yet to be ascertained²⁸.

Enzyme immuno assay for the detection of anti-mycobacterial superoxide dismutase antibody: Superoxide dismutase is an important secretory protein of *M.tuberculosis* and has been evaluated for the serodiagnosis of tuberculosis. It is found to be useful only in low prevalence countries (93-94% positive predictive value), compared to high prevalence countries like India and Egypt, where the positive predictive value drops to 77-88%²⁹.

Insta test TB : It is a rapid *in vitro* assay for the detection of antibody in active TB disease using whole blood or serum. The test employs an antibody binding protein conjugated to a colloidal gold particle and a unique combination of TB antigens immobilized on the membrane³⁰.

Some of the other commercially available antibody tests for pulmonary TB are listed in Table 2.

Miscellaneous diagnostic methods

To overcome the poor specificity of the existing skin test based on tuberculin, newer tests with defined antigens are needed to discriminate

between the infected individuals from those with active disease. The latest of these is the MPB 64 patch test.

Table 2. Some commercially available antibody tests for diagnosis of pulmonary TB

Name of the assays	Antigen used
MycoDot (Dot-blot)	Lipo arabinomannan (LAM)
Detect-TB (ELISA)	Recombinant protein Peptide
Pathozyme Myco (ELISA)	38 kDa (recombinant Ag) and LAM
Pathozyme TB(ELISA)	38 kDa (recombinant)
Antigen A60 (ELISA)	Antigen – 60
ICT diagnostics (membrane based)	38 kDa (recombinant)

TB MPB 64 patch test : MPB 64 is a specific mycobacterial antigen for *M.tuberculosis* complex. This patch test becomes positive in 3-4 days after patch application and lasts for a week. The test has a specificity of 100% and a sensitivity of 98.1%³¹. This promising test has been reported so far only in one setting in Philippines and needs to be carried out in other settings.

Another approach is the use of defined antigens for an accurate and rapid test for tuberculosis infection based on the detection of T cells sensitized to *M.tuberculosis* either by blood tests *in vitro* or skin tests *in vivo*³². Mononuclear cells from the peripheral blood are stimulated *in vitro* and production of IFN gamma from the sensitized T cells is measured by ELISA³³. The antigens used are ESAT 6 (early secretory antigen TB) and CFP 10 (culture filtrate protein), which are being used as alternatives for PPD, for use in skin test (tuberculin testing) *in vivo*.

Measurement of IFN gamma producing cells

The ESAT 6 (6 kDa) is a specific antigen and a strong inducer of IFN gamma production by

T cells of TB patients. The *M.tuberculosis* genome encompasses regions of differences (RD). These RD may encode potential antigens relevant for protection or diagnosis. The RD1 region is responsible for the secretion of ESAT-6 in response to TB. This antigen is recognized by T cells of TB patients and is not recognized by BCG vaccinated or healthy unvaccinated individuals. The levels of IFN gamma increases in treated compared to untreated patients, and is associated with improved immunity against TB. Hence this may be useful for monitoring TB patients³⁴.

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