The worldwide increase in tuberculosis is indicative of our failure to use modern medical advances to control an ancient enemy. The problems confronting us, now is due to decades of neglect partly fostered by the misconception that we had drugs to control the disease. HIV and tuberculosis have an unholy alliance that further compounds the diagnostic dilemmas. Today, newer rapid laboratory diagnosis of tuberculosis is becoming a vital aspect of management.

A. Microscopy

Given the poor specificity of Tuberculin Skin Test (TST), the importance of smear and culture confirmation cannot be overemphasized. Smear examination by Ziehl-Neelson (Z/N) stain has the advantage of simplicity, availability and rapidity, but the sensitivity is affected by the skill and experience of the microscopist, the number of specimens examined and the concentration of organisms in the sputum. Microscopy can detect up to 70% of culture positive samples with a lower limit of detection of $5 \times 10^3$ organisms / ml. Optimising microscopy includes methods as bleach, guanidinium hydrochloride etc as well using more sensitive methods as fluorescent microscopy.

Fluorochrome stains

The waxy mycolic acids in the cell walls of mycobacteria have an affinity for the fluorochromes, auramines and rhodamine. These dyes non-specifically bind to nearly all mycobacteria. The mycobacterial cells appear bright yellow or orange against a greenish background. This method can be used to enhance detection of mycobacteria directly in patients’ specimens. The advantage of this method is that lower magnification can be used, enabling rapid screening over a wider area. However the cost of microscope and reagents are high and only a highly skilled technician can screen the smears. Today cheaper adaptations of LED microscopes are being evaluated which will certainly help to reduce cost. The Strategic and Technical Advisory Group (STAG) to WHO in Sept 2009 acknowledged compelling demonstrating the superiority of direct fluorescent microscopy (FM) over direct light microscopy using Ziehl-Neelsen (ZN) staining in throughput, efficiency and improved sensitivity of diagnosis.

B. Culture Techniques

Conventional Tests

Agar based methods - Culture of mycobacteria is a far more sensitive test than smear examination and allows for biochemical identification of the species considerably enhancing the specificity. Unfortunately the slow doubling time of *M. tuberculosis* makes culture on agar / egg based solid media is slow and time consuming. Agar based media allow detection of colonies in 10-12 days, whereas the most commonly used Egg based Lowenstein Jensen Medium (L.J.) usually takes 18-24 days. Susceptibility testing can also be performed on LJ but the turn around time is approximately 3 months for culture and susceptibility.

Automated liquid culture methods
1. Mycobacterial Growth Indicator Tube (MGIT) 960 TB: The test employs a new state of the art fluorescent technology that enables result towards positivity as rapidly as 7-10 days. MGIT 960 TB is used for isolation & accurate identification of mycobacteria. The method employed currently for identification of culture positive isolates is by PNB (Para-NitroBenzoic Acid)

2. MB Bact is based on a colorimetric CO2 sensor that is altered by bacterial metabolism

Liquid culture systems when combined with DNA probes or the MPT 64 detection in a lateral flow format for rapid species identification are capable of producing positive results in 2 weeks for the vast majority of sputum smear-positive specimens, and within 3 weeks for smear-negative specimens.

Drug Susceptibility Tests - DST

Phenotypic methods

The in-vitro DST is a universally accepted way of selecting appropriate drugs for chemotherapy for TB patients. An attempt to standardize susceptibility tests for M. tuberculosis was made in 1961 at an international informal meeting under the aegis of the World Health Organization (WHO); this was followed up at a second meeting in 1969. The report of this group described the three methods viz. absolute concentration, resistance ratio and proportion method.

1. Absolute concentration method: In this method, growth is taken as the end point. It is also referred to as the MIC method. The method requires care in the choice of appropriate inoculum, since resistance on the part of the microorganisms is clinically significant only when at least 1% of the total bacterial population develops resistance at the critical concentration. The critical concentration is defined as the lowest concentration at which susceptible bacilli fail to grow in the presence of the drug. Smaller proportion of resistant microorganisms has no clinical significance.

2. Resistance ratio method: This determines the resistance ratio between the MIC of the strain of patients and MIC of reference strain (H37RV). This test also requires proper adjustment of inoculum size. Since reference strains are also included in this test, it is more accurate than the absolute concentration method as slight changes in the drug concentration are adjusted for.

3. Proportion method: The ratio of the number of colonies obtained on the drug-containing medium to the number on drug-free medium gives the proportion of resistant bacilli. Thus it is a qualitative as well as a quantitative method, as it gives the proportion of resistant bacilli to sensitive ones.

Standard methods require anywhere between 3 weeks to 6 weeks to report a susceptibility test from a positive culture. The BACTEC MGIT 960 1% proportion methods has been approved by the US FDA and is also considered to be the “gold standard” for the drug susceptibility testing to first-line anti-tuberculosis drugs. More recently the critical concentrations for second line drugs have also been tested successfully for most drugs in a multicenter evaluation.

Direct rapid methods for screening of MDR TB

i. Nitrate Reductase Assay (NRA)

The nitrate reductase assay (NRA) is quite a simple technique based on the ability of M.tuberculosis to reduce nitrate to nitrite, which is detected by adding a chemical reagent to the culture medium. M.tuberculosis complex is cultivated on LJ media in presence of an antibiotic and its ability to reduce nitrate is measured after 10 days of incubation. Resistant strains reduce the nitrate, which is revealed by a pink-red colour in the medium, while susceptible strains lose their capacity as they are inhibited by the antibiotic. The assay has been evaluated in several studies for first-line drugs and ofloxacin, with good results. The disadvantage of this assay is that, some NTMs can also reduce nitrate to nitrites and hence can not be differentiated from M. tuberculosis complex, when applied directly to clinical specimens.

ii. Microscopic observation broth-drug susceptibility assay (MODS)

Microscopic observation drug-susceptibility assay (MODS) is a novel assay based on the observation of the characteristic cord formation of M.tuberculosis complex that is visualized microscopically with the use of an inverted microscope. Anti-tubercular drugs can be added to the Middlebrook 7H9 broth to detect drug resistance quickly – if growth occurs in the presence of the drug, it is resistant. The MODS method uses a 24-well culture plate format. Patient sputum samples are placed in wells with culture broth, and in wells with broth to which anti tuberculosis drugs have been added. If mycobacteria grow in broth alone, but not in drug-containing wells, the diagnosis of drug-sensitive M.tuberculosis is made. In a recent, large study from Peru, MODS detected 94% of 1908 positive
sputum cultures, whereas the conventional L.J. culture detected on 87%. MODS also had a shorter time to culture positivity (average of 8 days) compared with LJ culture. The results obtained by direct susceptibility testing for INH and RIF using MODS were available at the same time as TB detection. This method has been proposed as a rapid, inexpensive, and specific method for *M. tuberculosis* drug susceptibility testing. As the test is performed in liquid medium, and needs to be handled often, it is more of a biosafety risk for laboratory staff, hence requires experienced personnel and level 3 biosafety facilities.

iii. Thin layer Agar (TLA):

Microscopic examination of growth on solid media using 7H11 Middlebrook agar in quadrant Petri plates containing isoniazid, rifampicin, para nitrobenzoic acid and one without additives. TLA was found to be concordant with the standard proportion method and showed a shorter turn around time (11days)

C. Immunologic Methods

1. Serological tests

A large number of antigens have been used in assessing the humoral response in tuberculosis, but the fact that none has emerged victorious underscores the poor sensitivity and specificity of serology in endemic areas.

A good serological assay should perform well in specific targeted populations especially childhood tuberculosis, HIV positive and extrapulmonary TB. Unfortunately, the sensitivity of most serological tests falls with smear negativity – a finding attributed to the lower burden of organisms. In addition, the fact that large number of environmental bacteria have cross reactive antigens and the antigens used so far are not very species specific contrive to confuse the diagnosis. These tests should **not be performed in high burden** countries and attempts should be made to do smear and culture instead.

The WHO has issued a negative policy recommendation in July 2011 for the use of any commercial serological assay in the diagnosis of active tuberculosis.

2. Interferon Gamma Release Assays (IGRAS)

The Interferon gamma assays are used in the diagnosis of latent tuberculosis. These assays are based on the principle that tuberculosis antigen sensitized T cells produce interferon gamma when they re-en-counter mycobacterial antigens.

These in vitro assays employ extremely specific antigens of *M. tuberculosis* as Early Secretory Antigenic Target-6 (ESAT-6) and culture filtrate protein 10 (CFP-10) called the RD1 antigens. These antigens are not shared by BCG strains and most Non Tuberculous Mycobacteria.

Two commercial assays are currently available

- The QuantiFERON GOLD assay with both ESAT 6 and CFP-10 uses whole blood to measure γ interferon response to the above antigens
- T SPOT –TB assay uses peripheral blood mononuclear cells (PBMCs) and detects the number of “spot forming T cells” by use of ELIS-POT in response to the above RD1 antigens.

The WHO has issued a policy statement in Oct 2011 stating that IGRAs should not be used for the diagnosis of active TB

D. Molecular Techniques

**Molecular diagnostics** for tuberculosis have evolved because the long turn around time for culture methods.

i. Direct detection form clinical samples

ii. Genotypic methods for detection of drug resistance

i. Direct detection by nucleic acid amplification directly from clinical samples allows for initiation of treatment if the clinical picture is consistent. Various *in house* PCR assays include amplification of genes encoding mycobacterial antigens, repetitive sequences, ribosomal RNA etc., Commercial FDA approved systems include Amplicor (Roche Diagnostics) and AMTD (GenProbe) Nucleic acid amplification

a. Polymerase chain reaction (PCR): PCR is an in vitro method for amplifying specific DNA sequence. Starting with extremely minute amounts of a particular nucleic acid sequence from any source, PCR enzymatically generates millions or billions of exact copies thereby making genetic analysis of tiny samples a relatively simple process. Nested PCR certainly enhances the sensitivity of PCR.

b. Transcription mediated amplification (TMA): TMA uses a species specific sequence of ribosomal RNA (rRNA) as the target for reverse transcriptase. The advantage of this technology being that the
dead cells have no transcription machinery hence only viable cells are picked up and amplified.

DNA amplification technology can amplify minute quantities of DNA to levels that can be readily seen following routine agarose gel electrophoresis. But amplification can amplify even minute quantities of contaminating DNA. False positive results are the major concern. Also the presence of an organism in a clinical specimen does not necessarily indicate disease. Various target antigens have been used IS6110, MPB64, 16S rRNA gene, 65kd, 38kD rpo B etc.

ii. Molecular methods for detection of drug resistance

Genotypic methods for drug resistance

These methods assess the genetic determinants of resistance rather than the resistance phenotype. PCR based sequencing is used to elucidate and study mutations as it allows for detection of both previously recognized and unrecognized mutations. Unfortunately it is not readily applicable in routine practice.

Solid-phase hybridization techniques: There are currently two commercially available solid-phase hybridization techniques for the rapid detection of drug resistance in tuberculosis: The Line Probe Assay (INNO-LiPA Rif TB Assay, Innogenetics, Belgium) for the detection of resistance to Rif and the GenoType MTBDR assay (Hain Lifesciences, Germany) for the simultaneous detection of resistance to isoniazid and rifampicin. An in house Reverse Line blot Hybridization assay to simultaneously speciate mycobacteria and detect resistance to all known mutations for rifampicin, isoniazid and streptomycin has also been developed that can be performed directly on smear positive clinical samples.

Real-time PCR techniques:

The main advantages of real-time PCR techniques are the speed of the test and a lower risk of contamination. Real-time PCR techniques have been applied to M.tuberculosis strains and, more recently, directly to clinical samples.

Newer methods: Biprob es are single probes which are fluorescently labeled at the 50 end. In real-time PCR, biprob es are included in the reaction mix together with Sybr-Green, a dye that emits fluorescence when it binds to double-strand DNA. Thus, at the end of each annealing step of the PCR, the SybrGreen excites the probe fluorophore by energy transfer, emitting fluorescence which can be measured by the real-time PCR instrument. The analysis of mutations involves monitoring of melting temperature of the probes. Deviations in the Tm of the biprobe indicate the presence of mutations. In one study, this kind of probe correctly assigned all mutations in 46 rifampicin resistant strains by using three independent bispobes which involve the performance of three independent reactions. Multiplex allele specific PCR, which are multiplex PCR that include the outer primers in the reaction to generate the amplicon for analysis and inner primer designed alternatively with certain nucleotide substitutions at specific codons. The length of the PCR fragment varies according to the direction of the inner primer which binds to the codon in analysis.

Molecular beacon assays are based on a stem and loop structure with the loop in the probe. Easier to perform, real time formats as GeneXpert are being currently evaluated directly from samples. These assays have huge potential as they are rapid, and can be performed in a real world setting out of the molecular lab.

Microarrays, also known as biochips or DNA chips, have been proposed as genotypic methods for detecting drug resistance in M.tuberculosis. They are based on the hybridization of DNA obtained from clinical samples to high density oligonucleotides immobilized on a solid support, such as miniaturized glass slides.

Despite the clear advantages that molecular methods offer for drug susceptibility testing in terms of turn around time, the cost implications have to be borne in mind for resource constrained settings as ours. Associated laboratory infra structure including proper design, and quality control issues to avoid cross contamination and amplicon generation is paramount. Lastly, wherever the genetic basis of resistance is not fully characterized, drug resistance should be confirmed by well standardized phenotypic methods.

REFERENCES


