



## LATEST LABORATORY DIAGNOSTIC TECHNIQUES AND ANTIBIOGRAM TESTING OF TUBERCULOSIS INFECTION

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### ABSTRACT

Tuberculosis (TB) ranks as the second leading cause of death from a single infectious agent, after the human immunodeficiency virus (HIV). Millions of people die because of this disease. Tuberculosis is a treatable disease, patients can be saved provided the disease is timely diagnosed. With the ambitious World Health Organization (WHO) goal to end the global TB epidemic, by 2035, with the targets of a 95% reduction in TB deaths and a 90% reduction in TB incidence (both compared with 2015). It becomes essential to focus on rapid laboratory diagnosis of the disease and subsequently starting effective first line treatment.

The techniques reviewed in this paper, are conventional to automated culturing procedures. Moreover, among rapid techniques included Loop Mediated Isothermal Amplification, Line Probe Assay, Ligase chain reaction, Real time PCR. The serological techniques include Gamma interferon release assays, Enzyme-linked immunospot (ELISPOT) assays.

The old conventional manual techniques for culture have been replaced by more rapid and reliable automated techniques. As long as speed of reporting is concerned the automated culturing procedures have been supplemented by real time PCR which gives report of culture and antibiogram testing report within hours. It has been justified, in order to achieve the goal set by WHO, for the eradication of TB, it is essential to improve detection techniques, including latent forms of the disease as well and timely identification of resistance strains. Real time PCR would fulfil this criteria, which can be performed even on OPD basis. This technique can be used as commonly as Acid Fast Bacilli (AFB) staining techniques were once used.

**Keywords:** Tuberculosis; Real time PCR; Line Probe Assay; Gamma interferon.

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## INTRODUCTION

Tuberculosis (TB) is a contagious and airborne disease. It is caused by *Mycobacterium tuberculosis*, which mostly affects lungs and also other organs of the body (Traore et al. 2006). As per the WHO Global TB report (WHO, 2015), 9 million people fell ill with TB, including 1.1 million cases with HIV in 2013. Of these, 1.5 million people died from TB, including 360,000 among HIV-positive cases. Among 510,000, women who died from TB in 2013, 180,000 were HIV-positive. Of the overall TB deaths among HIV-positive people, 50% were among women. TB is one of the top killers of women of reproductive age. An estimated 550,000 children became ill with TB and 80,000 children who were HIV-negative died of TB in 2013.

Globally (WHO, 2015) in 2013, an estimated 480,000 people developed multidrug-resistant TB (MDR-TB) and there were an estimated 210,000 deaths from MDR-TB. The number of people diagnosed with MDR-TB tripled between 2009 and 2013, and reached 136,000 worldwide.

Progress in the detection of drug-resistant TB has been facilitated by the use of new rapid diagnostic techniques. Timely and accurately detection of TB infection, has considerably reduced mortality and incidence rate of tuberculosis, which is documented by the TB death rates which has dropped to 45% between 1990 and 2013. An estimated 37 million lives were saved through timely TB diagnosis and treatment between 2000 and 2013 (WHO, 2015).

## MICROSCOPY

The diagnosis of TB relies primarily on the identification of AFB in sputum smears using a conventional light microscope. The sputum specimens are smeared directly onto the slides (direct smears) and subjected to Ziehl-Neelsen (ZN) staining (Smithwick, 1975). Although all mycobacterial species are acid fast, this assay is highly specific for *M. tuberculosis*. In spite of the high specificity, the sensitivity of the test has been reported to vary from 20 to 80%, and its usefulness is questionable for patients with reduced pulmonary cavity formation or reduced sputum bacillary load, such as children and HIV-coinfected patients. The sensitivity of the direct smear assay has been found to be dependent on staff that has been well trained so that sufficient time is spent on preparing, staining, and reading each smear, with a well-functioning EQA program in place.

Many studies (Trusov et al. 2009; Hendry et al. 2009) with culture as the reference standard indicated that the sensitivity of fluorescence microscopy ranged from 52 to 97%, with the fluorescent method being on average 10% more sensitive than light microscopy (Steingart et al. 2006). For HIV-positive patients, few studies reported between 26 and 100% increases in the detection of AFB using fluorescence microscopy. Because fluorochrome-stained smears can be examined at lower magnifications, it takes less time to examine these smears than to examine smears stained with ZN stain and still results in a higher sensitivity and a similar specificity.

### 1- LED (Light-emitting diodes)

Light-emitting diodes (LED) have been developed to offer the benefits of fluorescence microscopy without the associated costs. In 2009, the evidence for the efficacy of LED microscopy was assessed by the WHO (WHO, 2009), on the basis of standards appropriate for evaluating both the accuracy and the effect of new TB diagnostics on patients and public health. The results showed that the accuracy of LED microscopy was equivalent to that of international reference standards, it was more sensitive than conventional Ziehl-Neelsen microscopy and it had qualitative, operational and cost advantages over both conventional fluorescence and Ziehl-Neelsen microscopy (WHO, 2009).

On the basis of these findings, WHO (WHO, 2010) recommends that conventional fluorescence microscopy be replaced by LED microscopy, and that LED microscopy be phased in as an alternative for conventional Ziehl-Neelsen light microscopy. One has to introduce appropriate quality assurance programme and monitor the effect on TB case detection rates and treatment outcomes.

Accuracy of LED in comparison with reference standards: LED microscopy (WHO, 2010) showed 84% sensitivity (95% confidence interval [CI], 76–89%) and 98% specificity (95% CI, 85–97%) against culture as the reference standard. When a microscopic reference standard was used, the overall sensitivity was 93% (95% CI, 85–97%), and the overall specificity was 99% (95% CI, 98–99%). A significant increase in sensitivity was reported when direct smears were used rather than concentrated smears (89% and 73%, respectively).

- \* Accuracy of LED in comparison with Ziehl-Neelsen microscopy: LED microscopy was statistically significantly more sensitive by 6% (95% CI, 0.1–13%), with no appreciable loss in specificity, when compared with direct Ziehl-Neelsen microscopy.
- \* Accuracy of LED in comparison with conventional fluorescence microscopy: LED microscopy was 5% (95% CI, 0–11%) more sensitive and 1% (95% CI, -0.7% - 3%) more specific than conventional fluorescence microscopy.

## CULTURE

T.B culturing is done under following categories: Conventional (Manual) and Automated.



## 1- Conventional

Among conventional procedures, solid media culturing has been widely used. The most common media is egg based media called Lowenstein Jensen Media slants. Among agar based media widely used is Middle-Brook 7H10/11 agar media, in this category, plates are widely used. The liquid media commonly used is Middle-Brook 7H12 broth media.

## 2- Solid media

Brief procedure, the sediment obtained after processing clinical samples for Modified Petroff's procedure, the two slopes of LJ medium (Liu et al. 1973) are inoculated, using a sterile cool 5 mm inoculation loop made up of Nichrome wire (22 SWG). Individually wrapped, disposable 10mm loops can be used, if available. Caps are labelled with the lab serial number of the specimen and name them as 1 and 2. One loopful of sediment for each inoculation is used to incubate all the LJ media slopes at 37°C. The growth is checked weekly for eight weeks.

## 3- Culture of *M. tuberculosis* on Middlebrook 7H11 agar plates

Culture on Middlebrook 7H11 (Metchock et al. 1999). Agar Plates is done by using petri plates (100 mm x 15 mm) divided in two compartments. Half of the plate contains 7H11 and the other half 7H11+PNB. Decontamination and centrifugation of the sample is done according to the recommended procedures [NALC-NaOH (Bruchfeld et al. 2000) or Petroff method (Petroff, 1915)]. 100 µl of the sample is inoculated on one 7H11 compartment. Other half of the plate which contains TLA+PNB medium is inoculated with 100 µl of the sample. The Petri dishes are closed with parafilm, leaving a space of about 1 or 2 cm to allow the exchange of CO<sub>2</sub>. Plates are put in the incubator until the inoculum on the surface of the medium is absorbed (overnight). Plates are then incubated upside down the day after if the inoculum is absorbed. Plates can be incubated in 5-10% CO<sub>2</sub> (CO<sub>2</sub> incubator or hermetically closed candle jar) at 37°C. The plates are incubated for four weeks.

Plates are read after 48 hours, to check for contamination. The plates are observed twice a week to detect growth using a conventional microscope (objective 10X) for up to 6 weeks, identifying micro colonies as *M. tuberculosis* by their appearance and morphology. Any observation of growth or contamination is registered in a record sheet.

The growth on TLA medium is compared with the growth on TLA+PNB, as PNB inhibits the growth of *M. tuberculosis* complex. Growth of *M. tuberculosis* complex on TLA in the first few days looks like small cords. After a few days, the colony of *M. tuberculosis* complex on TLA assumes its typical appearance: colonies are bigger in size and are constituted by cords. At the same time the compartment TLA+PNB does not show any growth or cord formation. Both compartment TLA and TLA+PNB are compared. The reading is made using a conventional microscope (10X objective).

Plates are read after 48h (identification of rapid growing species), regularly two times a week to detect growth. If growth on TLA is observed, the growth is compared with that on TLA with PNB.

This is confirmed with Ziehl-Neelsen staining technique, if growth is seen on PNB and has the same appearance that on TLA without PNB, it could represent the presence of atypical mycobacteria.

## 4- Liquid media

Mycobacterial culture can be performed on conventional liquid media such as Middlebrook 7H9/7H12 broth (Middlebrook et al. 1977). The major problem with this media is that, it takes 6 to 8 weeks for growth. The drug susceptibility tests also takes additional 4 weeks. The liquid media has been successfully used for TB-automated culturing techniques, for MODS and also for Septi-check AFB.

## 5- Septi-check AFB

Septi-check AFB (Roche Diagnostic Systems, Nutley, N.J.) (Isenberg et al. 1991) is a test which allows simultaneous detection of *M. tuberculosis* and non-tuberculosis mycobacteria and it consists of a capped bottle containing 30 ml of Middlebrook 7H9 broth under enhanced CO<sub>2</sub> and a paddle with agar media enclosed in a plastic tube.

The paddle is covered on one side with non-selective Middlebrook 7H11 agar and on the other side it is divided into two sections, one which allows the differentiation of *M. tuberculosis* from other mycobacteria (7H11 agar with NAP – para-nitro- $\alpha$ -acetylamino- $\beta$ -hydroxy-propionophenone), and the other which ensures detection of contaminants (chocolate agar). The bottle also contains enrichment broth with glucose, glycerin, oleic acid, pyridoxal, catalase, albumin, polyoxy-ethylene 40 stearate, azlocillin, nalidixic acid, trimethoprim, polymyxin B and amphotericin B. This method requires about 3 weeks of incubation, but the non-radiometric approach has the potential to expedite processing, obviate CO<sub>2</sub> incubation requirements thus facilitating early detection of positive cultures.

Septi-check AFB can be used for the detection and isolation of mycobacteria from sputum, broncho-alveolar lavage or aspirate, urine, stool, body fluids, biopsy tissues, wounds and skin. The unique advantage of this technique is the simultaneous detection of *M. tuberculosis*, non-tuberculosis mycobacteria (NTM), other respiratory pathogens and even contaminants. A historical multicentric study conducted in four medical centers has shown that the system gives better results compared to conventional mycobacterial isolation media and BACTEC 460 TB System, supporting the growth of bacteria from small inocula and shortening the time required for recovery of mycobacteria from clinical specimens.



## 6- TB cultures – automated

Radiometric BACTEC 460 TB Method - This technique is specific for mycobacterial growth, wherein  $^{14}\text{C}$  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  $^{14}\text{C}$  labelled substrate present in the medium is metabolized,  $^{14}\text{CO}_2$  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- $\alpha$ -acetylamino- $\beta$ -hydroxypropiofenone. 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 methods. 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 method. By facilitating early diagnosis, the BACTEC method may prove to be cost effective in a population with high prevalence of tuberculosis.

## 7- MGIT 960 Mycobacteria detection system

The MGIT (Mycobacteria Growth Indicator Tube) (Tortoli et al. 1997; Siddiqi and Rusch-Gerdes, 2006) consists of liquid broth medium that is known to yield better recovery and faster growth of mycobacteria. The MGIT contains 7.0 ml of modified Middlebrook 7H9 broth base. The BACTEC MGIT growth supplement is added to each MGIT tube to provide substances essential for the rapid growth of mycobacteria. Oleic acid is utilized by tubercle bacteria and plays an important role in the metabolism of mycobacteria. Albumin acts as a protective agent by binding free fatty acids which may be toxic to mycobacterium species, thereby enhancing their recovery. Dextrose is an energy source. Catalase destroys toxic peroxides that may be present in the medium. Addition of the MGIT PANTA is necessary to suppress contamination. A fluorescent compound is embedded in silicone on the bottom of 16 x 100 mm round bottom tubes. The fluorescent compound is sensitive to the presence of oxygen dissolved in the broth. Initially, the large amount of dissolved oxygen quenches emissions from the compound and little fluorescence can be detected. Later, actively respiring microorganisms consume the oxygen and allow the fluorescence to be detected.

Tubes entered into the BACTEC MGIT instrument are continuously incubated at 37°C and monitored every 60 min for increasing fluorescence. Analysis of the fluorescence is used to determine if the tube is instrument positive; i.e., the test sample contains viable organisms. An instrument positive tube contains approximately  $10^5$  to  $10^6$  colony forming units per milliliter (CFU/mL). Culture vials are held a minimum of 42 days (up to 56 days) and if it shows no visible signs of positivity, are removed from the instrument as negatives and discarded.

## 8- Versa-TREK System

The Versa-TREK (Mirrett et al. 2007) System offers four FDA-cleared tests, blood culture, sterile body fluids, mycobacteria detection, mycobacterium tuberculosis susceptibility testing. Versa-TREK system is capable of detecting any gas produced or consumed by organisms. It is not limited to  $\text{CO}_2$  production, like other systems, Versa-TREK is able to detect a wider range of both common and fastidious organisms. The system uses only two bottles to recover organisms from adults, pediatrics and patients on antibiotics, The machine has many distinct features including: it works on as low as 0.1ml blood, without additional supplements; perfect for pediatric patients. Largest dilution ratio, allowing dilution of serum host factors.

## 9- MB/BacT

BacT/ALERT MB culture bottles in combination with the MB/BacT enrichment fluid are designed for the cultivation of mycobacterium spp. commonly isolated from blood. The medium will support growth of other aerobic organisms, including yeast, fungi, and bacteria. This complete system includes a lytic agent (Saponin), Sodium polyanetholesulfonate (SPS) and other media supplements which eliminate the processing step, prevent clotting of blood, and enhance the growth of mycobacteria. A 3ml-5ml volume of blood can be inoculated directly into the culture bottle. Inoculated bottles are placed into the instrument where they are incubated (35°C-37°C) and continuously monitored for microbial growth. MB/BacT (OrganonTeknika, Turnhout, Belgium) is a non-radiometric continuous monitoring system designed for the isolation of mycobacteria from clinical specimens. It utilizes a colorimetric sensor and reflected light to continuously monitor the  $\text{CO}_2$  concentration in the culture medium.

The MB/BacT Mycobacteria Detection System and the BacT/ALERT (Siddiqi and Rusch-Gerdes, 2006), utilize a colorimetric sensor and reflected light to monitor the presence and production of carbon dioxide ( $\text{CO}_2$ ) dissolved in the culture medium. If microorganisms are present in the test sample, carbon dioxide is produced as the organisms metabolize the substrates in the culture medium. When growth of the microorganisms produces  $\text{CO}_2$ , the color of the gas permeable sensor installed in the bottom of each culture bottle changes from blue-green to yellow. The lighter color results in an increase in reflectance units as monitored by the system. Bottle reflectance is monitored and recorded by the instrument every 10 minutes.

When comparing the performance of MB/BacT with that of BACTEC 12B media for BACTEC 460 (Pfyffer, 2007), a Swiss study showed that the mean time for the detection of *M. tuberculosis* from sputum, cerebrospinal fluid (CSF) and urine samples was 17.5 ( $\pm 6.4$ ) days for MB/BacT, 14.3 ( $\pm 8.2$ ) for BACTEC and 24.2 ( $\pm 7.5$ ) days for egg-based media cultures.



The study concluded that MB/BacT is an acceptable alternative for BACTEC 460 despite some minor disadvantages such as increased contamination and slightly longer time for detection of growth.

One other study performed in the Philippines showed that the detection time for MB/BacT system (Dinnes et al. 2007) was shorter with two weeks compared to the conventional Lowenstein-Jensen (LJ) egg culture.

## IDENTIFICATION OF MYCOBACTERIAL SPECIES (MOLECULAR TECHNIQUES)

### 1- TB PNA FISH

Fluorescence in situ hybridization (FISH) (Rodriguez-Nunez et al. 2012) using peptide nucleic acid (PNA) (Hongmanee et al. 2001) probes allows differentiation between tuberculous and nontuberculous mycobacteria in smears of mycobacterial cultures. PNA molecules are pseudopeptides with DNA-binding capacity in which the sugar phosphate backbone of DNA has been replaced by a polyamide backbone. The *M. tuberculosis* complex-specific PNA probes showed sensitivities of 84-97% while the non-tuberculous mycobacteria-specific PNA probes showed diagnostic sensitivities of 64-91%. Both types of probes showed diagnostic specificities and predictive values of 100%.

### 2- Nucleic acid amplification (NAA)

Nucleic acid amplification allows both detection and identification of *M. tuberculosis* through enzymatic amplification of bacterial deoxyribonucleic acid (DNA). The most widely used technique is PCR (Shaw and Taylor, 1998), but transcription mediated amplification (TMA) (Shenai et al. 2001) and strand displacement amplification (SDA) (Hellyer et al. 1996) are also commercially used. The sensitivity of this test is higher than that of smear microscopy but it is slightly lower than that of culture techniques.

Nucleic acid tests are currently used mostly for confirmation of smear-positive results or for primary case diagnosis, when combined with other methods. The main advantage of these tests is that they offer quick results, paired with a high level diagnostic accuracy. Because of their price and complexity, the use of these methods is still limited to developed countries, but their introduction to developing countries is improving gradually.

A positive direct amplified test in conjunction with smear positive for acid-fast bacilli are highly predictive for tuberculosis, but mycobacterial cultures are still needed for species identification, confirmation or susceptibility testing.

The advantages of nucleic acid amplification tests include the fact that results are available quite rapidly, in a matter of hours, with high specificity (98-100%), and sensitivity [higher than 95% acid-fast bacilli positive sputum, and between 60-70% in smear-negative, culture-positive (Shaw and Taylor, 1998; Hellyer et al. 1996) specimens]. Newer amplification tests may display better sensitivity in smear-negative specimens while retaining high specificity.

Nucleic acid amplification tests could also be used for detecting *M. tuberculosis* in specimens other than sputum (e.g., blood, lymph, cerebrospinal fluid, urine, bronchial aspirate and lavage, bone marrow, gastric aspirate), although, to date, results have varied widely. The disadvantages include the high costs and a possibly lower specificity in clinical conditions.

### 3- Cepheid Xpert MTB/RIF assay

Xpert® MTB/RIF, is manufactured by Cepheid, this has been endorsed by WHO (WHO, 2011). WHO recommends this machine for diagnosis of pulmonary TB, paediatric TB, extra pulmonary TB and rifampicin resistance. The Xpert MTB/RIF is a self-contained and cartridge-based technological platform that integrates sputum processing, DNA extraction and amplification, TB and MDR-TB diagnosis. It has similar sensitivity to culture, targets *M. tuberculosis* specifically and enables simultaneous detection of rifampicin resistance via the *rpoB* gene.

The system is fully automated for identification of *M. tuberculosis* complex and mutations in *rpoB*. Uses real-time PCR with molecular beacons, 5 probes for wild-type RRDR in *rpoB* and 1 probe for amplification control, decontamination, digestion, DNA extraction, amplification, and detection in same cartridge. It uses hemi-nested real-time PCR assay, to amplify a specific sequence of the *rpoB* gene, which is then probed with molecular beacons for mutations within the rifampin-resistance determining region, providing results within less than two hours. It is technically simple and platform is random access.

### 4- PCR

Polymerase Chain Reaction (PCR) (Shaw and Taylor, 1998), is an exponentially synthesizing a defined target DNA sequences *in vitro*. In this procedure the following things are needed -thermocycler, primers: 17 to 30bp, GC content >50%, Target DNA, primers can be for universal conserved sequences (16S rDNA, dNTPs). Buffer solution, maintains pH and ionic strength of the reaction solution suitable for the activity of the enzymetypical PCR, go through three steps, denaturation, annealing, extension.

PCR allows sequences of DNA to be amplified *in vitro* even when only a few copies of mycobacteria are present, so that the amount of amplified DNA can be rapidly visualized and identified. The most common target used for PCR is insertion sequence IS6110. This sequence is specific for *M. tuberculosis* and offers multiple targets for amplification.



## 5- IS-6110 probe

It is a transposon, which are self-replicating stretches of DNA, functions not known. This sequence has been found in MTB-complex organisms (*M. africanum*, *M. Microti*, *M. Bovis*). IS-6110 (Garberi et al. 2011) sequence generally occurs only once in *M. bovis*, but is found upto 20 times in certain MTB, thus offering multiple targets for amplification, thereby making it effective probe.

## 6- Loop mediated isothermal amplification (LAMP) for TB

LAMP (Iwamoto et al. 2003) assay is a first generation manual NAAT. It is a novel nucleic acid amplification method for the detection of MTB Complex, *M. avium*, *M. intracellulare*, directly from the sputum sample or from liquid culture (MGIT) or solid culture media. In this method the reagents react under isothermal conditions, with high specificity, efficiency and rapidity. LAMP does not require any special equipments like thermal cycler. The reaction can be performed in a water bath or a heating block. LAMP is cost effective technique.

The LAMP, employs DNA-Polymerase and a set of four specially designed primers, that recognizes a total of six distinct sequences of target DNA. Species specific primers are designed, by targeting *gyrB* gene. It is a simple procedure starting with, mixing of all reagents in a single tube, followed by an Isothermal reaction during which the reaction mixture is held at 63°C for 60 mts. The large quantity of DNA generated, is detected by visual inspection for fluorescence or turbidity, depending on the techniques used.

## 7- Transcription mediated amplification (TMA) - AMPLIFIED MTD (Mycobacterium tuberculosis Direct) Test

TMA (Shenai et al. 2001) is an RNA transcription amplification system using two enzymes: RNA Polymerase and reverse transcriptase. TMA is isothermal, the entire reaction is performed at the same temperature in a water bath or heat block. This is in contrast to other amplification reactions such as PCR or LCR that require a thermal cycler. TMA can amplify either DNA or RNA, and produces RNA amplicon, in contrast to most other nucleic acid amplification methods that only produce DNA. TMA has very rapid kinetics resulting in a billion fold amplification within 15-30 minutes. Test results are obtained within few hours. Currently used only for respiratory specimens. Sample preparation-releases r-RNA, reverse transcriptase copies the RNA target, RNA polymerase mediated amplification-RNA amplicon, hybridization protection assay detects RNA amplicon. TMA can identify the presence of genetic information unique to *M. tuberculosis* directly from pre-processed clinical specimens. Amplified MTD Test (Gen-Probe, Hologic) detects *M. tuberculosis* ribosomal ribonucleic acid (rRNA) directly and rapidly, with a sensitivity similar to that of culture techniques. Amplicon are then detected through hybridization protection assays (HPA). The nucleic acid amplification test for Gen-Probe's MTD test involves a test kit, lysis of cells and release of the nucleic acid target. The sensitivity of this test is of 96% and its specificity is 100% for *M. tuberculosis* on specimens that are smear-positive for acid-fast bacilli.

## 8- Line probe assay (LPA)

Is hybridization-based test use immobilized DNA probes on nitrocellulose membranes, Colorimetric change indicates hybridization. One reads the bands to determine resistance-associated mutations for RMP and INH. Can be used directly on sputum specimens, results are obtained within 1-2 days (Morgan et al. 2005).

The first commercially available Line Probe assay, the INNO LiPa Mycobacteria (Innogenetics, Ghent, Belgium), uses reverse hybridization technology in which probes are immobilized as parallel lines on a membrane strips as opposed to being in solution as is the case with AccuProbes. Amplified, biotinylated DNA fragments of the 16-23S rRNA spacer region of mycobacterial organisms are incubated with the labeled strips; addition of streptavidin alkaline phosphatase and a chromogenic substrates results in the formation of a precipitate on the membrane where hybridization as occurred. The LiPa assay is able to detect up to 14 different species of mycobacteria.

## 9- Ligase chain reaction (LCR)

Ligase chain reaction (Shetty et al. 2000) is a DNA amplification technique, which is based on the ligation of two adjacent synthetic oligonucleotide primers which uniquely hybridize to one of the strands of the target DNA, amplifies the nucleic acid used as a probe. Two probes are used per each DNA strand and are ligated together to form a single probe. Uses both a DNA polymerase enzyme and a DNA ligase enzyme to drive the reaction, requires Thermocycler. Each cycle results in doubling of target with greater specificity than PCR. The LCX *M. tuberculosis* assay kit (Abbot) is being mainly used for respiratory samples.

## DRUG RESISTANCE TESTING FOR M. TUBERCULOSIS

### 1- Microscopic observation drug susceptibility (MODS)

Microscopic observation drug susceptibility (MODS) (Minion et al. 2010), is a manual liquid culture technique that uses basic laboratory equipment (including an inverted light microscope) and microscopy skills to detect TB bacteria. Microscopic colonies (micro-colonies) of *M. tuberculosis* are observed in the culture media using an inverted microscope, through the bottom of a sealed plastic container. The characteristic pattern of growth of *M. tuberculosis* complex allows ready identification. Concurrent culture of sputum in drug-free and drug-containing media facilitates direct rifampicin and isoniazid DST.



For detection of TB in sputum, MODS has been demonstrated in multi-center studies to be more sensitive and faster than conventional solid culture and automated liquid culture (median time to culture-positivity of the same samples cultured in MODS, automated MB BacT and LJ were 7, 13 and 26 days respectively). Because MODS utilizes direct DST, median MDR detection time is also 7 days. MODS is relatively inexpensive compared to commercial broth-based liquid culturing systems.

Like all liquid and solid culture techniques, MODS is more challenging than smear microscopy. Laboratory technicians may have difficulty distinguishing between the micro-colonies of TB and some nontuberculous mycobacteria (NTM). This could potentially have impact where NTM prevalence is high. MODS is delicate and requires experienced personnel. As with all TB culture systems, biosafety of laboratory workers must be taken into consideration. Further studies are required to adequately validate this technique.

## 2- Rapid broth methods

In order to reduce the long turn-around time, of the agar proportion methods and provide clinicians with timely drug susceptibility results, rapid broth methods were developed for both growth and susceptibility testing of *Mtb*-complex. In the Bactec 460, 7H12 (Venkataraman et al. 1998) liquid medium was supplemented by specific anti tubercular drugs. The agreement between the conventional and the radiometric susceptibility testing assays varied from 96.4-98% with most results obtained within 7 days.

Additionally, unlike the 1% indirect agar method, the radiometric method was conducive to testing of *Mtb*-complex susceptibility against pyrazinamide (PZA), which requires an acidic environment not easily achievable on solid media. The radiometric method was modified by lowering the pH with addition of phosphoric acid at the same time as PZA but after the growing culture had reached exponential phase. This initial study, with limited number of isolates, showed good correlation between PZA susceptibility as measured by the radiometric method and detection of the pyrazinamidase enzyme as described under biochemical testing. The method described by (Isenberg et al. 1991) was further modified and proved useful in facilitating the measurement of PZA susceptibility of *Mtb*-complex by the radiometric method.

The Bactec 460 radiometric method for susceptibility testing was eventually replaced with the fully automated mycobacteria growth indicator tube (MGIT 960, Becton Dickinson, Sparks, MD) (Tortoli et al. 1997), which had been introduced for broth culture of mycobacteria species from clinical specimens without the use of radioactive materials. Each drug-containing MGIT bottle is inoculated with either 0.5 ml of a 1:100 dilution of a MGIT tube positive for 1-2 days, a 1:5 dilution of a MGIT tube positive for 3-5 days or a 0.5 McFarland if the inoculum is prepared from an organism growing on solid media. The control, drug-free MGIT bottle is inoculated with a 1:100 dilution of the inoculum used for the drug-containing MGIT bottle.

The MGIT 960 system automatically interprets the results of the test based on the growth unit (GU). If the GU is greater than 100 for a drug-containing MGIT bottle, the isolate is resistant and if the GU is less than or equal to 100 then the isolate is susceptible. However, for the test to be valid, the GU of the control bottle cannot reach 400 before 4 days or after 13 days, which suggests that the growth was too heavy or too light respectively. The MGIT 960 system (Scarparo et al. 2004) is FDA-approved for susceptibility testing of *Mtb*-complex against the first-line drugs including RIF (2 µg/ml), INH (0.4 µg/ml and 0.1 µg/ml), and EMB (7.5 µg/ml and 2.5 µg/ml), PYZ (100 µg/ml) and streptomycin (STR, 6.0 µg/ml and 2.0 µg/ml). Evaluation of the MGIT performance for the primary tuberculosis drugs showed results that were comparable to the Bactec 460 radiometric methods as well as the agar proportion methods (>90% agreement).

More recently, the MGIT 960 was evaluated for susceptibility testing of second-line drugs including levofloxacin, amikacin, capreomycin and ethionamide with overall agreement of 96% at critical concentrations when compared to the agar proportion method. Similar results were obtained in a study comparing the manual version of the MGIT to the agar method using the second-line drugs ofloxacin, kanamycin, ethionamide, and capreomycin. Another fully automated broth system that is FDA-approved for susceptibility testing of *Mtb*-complex is the Versa-TREK instrument (Ruiz et al. 2000) (TREK Diagnostics, Cleveland, OH). The Versa-TREK (formerly ESP culturesystem II) is FDA-approved for susceptibility testing of *Mtb*-complex against the first-line drugs including RIF (1 µg/ml), INH (0.4 µg/ml and 0.1 µg/ml), ETH (8 µg/ml and 5 µg/ml), and PZA (100 µg/ml). Drug susceptibility for each isolate tested is manually determined by comparing the time to detection of growth between the control bottle and the bottle containing the drug. If the difference is greater than three days or if the bottle remains negative, the isolate is considered susceptible. If the difference is less than or equal to 3 days, then the isolate is considered resistant. However, for the test to be valid, the time to growth in the control bottle has to be between 3 and 10 days following inoculation with 0.5 mL of a 1:10 dilution of a 1.0 McFarland inoculum (Ruiz et al. 2000). Evaluation of the VersaTREK instrument against the agar proportion method or the Bactec 460 for susceptibility testing of *Mtb* against INH, RIF, ETB, and STR showed results similar to the MGIT 960, with agreement > than 95% for all drugs except ETB and STR (agreement between 90-95%) for which the VersaTrek generally called susceptible organisms that were resistant.

## 3- Molecular detection of resistance markers

Several studies have been conducted to develop more rapid and specific methods of detection of multi-drug resistance markers in *Mtb*-complex. These assays are based on the detection of specific mutations in a variety of genes reported to confer resistance to several of the anti-tuberculosis drugs.



#### 4- Line probe assay (1<sup>st</sup> line drugs)

Line probe assay (Ling et al., 2008) (1<sup>st</sup> line drugs) has been manufactured by Hain Lifescience (Morgan et al. 2005; Nyendak et al. 2009) and has been endorsed by WHO in 2008. This kit is capable of detecting *M. tuberculosis* and carrying out drug susceptibility testing (DST) for 1<sup>st</sup> line TB drugs. Line Probe Assay is highly accurate in detecting MDR-TB in a variety of geographical settings (Viveiros et al. 2005; Traore et al. 2000; Rossau et al. 1997) and cost-effective when compared with TB culture and DST. They also demonstrated significant patient benefits, including early targeted treatment of MDR-TB and the potential interruption of transmission.

#### 5- Line probe assay (2<sup>nd</sup> line drugs)

The GenoType MTBDRsl assay is a molecular LPA (Ling et al. 2008) for the rapid determination of genetic mutations associated with resistance to fluoroquinolone, aminoglycosides (kanamycin, amikacin), cyclic peptides (capreomycin), ethambutol, and streptomycin. The test involves DNA extraction from culture isolates or AFB smear-positive respiratory specimens, followed by PCR amplification and reverse hybridization. It provides a visual reading of test results on nitrocellulose strips. Evaluation studies were carried in approximately 250 XDR strains of *M. tuberculosis*, and compared test performance with MGIT DST for 2<sup>nd</sup> line drugs. The evaluation was carried out in three laboratories that have already established LPA and MGIT DST, including the US Centers for Disease Control and Prevention (CDC), University of Cape Town Lung Institute (UCT) in South Africa, and the USNIH-supported International Tuberculosis Research Center (ITRC) in Korea. Subsequently, a study of direct testing at IRTC (150 sputum specimens) and at Hinduja Hospital in Mumbai (170 sputum specimens), provided additional support to UCT for its study that included direct testing of 270 sputum specimens.

Xpert<sup>®</sup> MTB/RIF, has been endorsed by WHO (WHO, 2011), which recommends this machine for diagnosis of pulmonary TB, paediatric TB, extra pulmonary TB and rifampicin resistance (Blakemore et al. 2010; Banada et al. 2010; Chang et al. 2012; Zeka et al. 2011; Hanif et al. 2011). The Xpert MTB/RIF is a self-contained and cartridge-based technological platform that integrates sputum processing, DNA extraction and amplification, TB and MDR-TB diagnosis. It has similar sensitivity to culture, targets *M. tuberculosis* specifically and enables simultaneous detection of rifampicin resistance via the *rpoB* gene, providing results within less than two hours.

#### 6- Reporter phages

Mycobacterial specific phages (Albert et al. 2002; Muzaffar et al. 2002) and reporter genes like luciferase (Cooksey et al. 1993) have been successfully used for detection of growth and for assessing the drug susceptibility to anti-TB drugs. Indication of viability could be either emission of light from organism due to activation of luciferase gene or production of a plaque on an indicator strain of mycobacteria. Results can be obtained in 48 h and such systems are commercially also available (Biotec/Medispan).

#### 7- Genotyping of *M. tuberculosis* isolates

Genotyping or DNA fingerprinting of *M. tuberculosis* refers to procedures developed to identify isolates that are identical in specific parts of genome. The most extensively used method in the last two decades has been the restriction fragment length polymorphism (Park et al. 2000) (RFLP) analysis of the distribution of the insertion sequence IS6110 (Garberi et al. 2011). The more recently developed spoligotyping and 24 locus variable number of tandem repeats (VNTR) techniques are similarly based on genetic polymorphism of additional mycobacterial repetitive sequences. The various DNA fingerprinting methods serve different purposes and have variable characteristics that enable their use in specific applications. They currently support routine contact tracing as well as investigations on person-to-person transmission, early disease outbreaks, identification and laboratory cross contamination and permit determination of whether new cases of TB are due to reinfection or reactivation. In addition, the recognition of different genotype families has facilitated studies on the population structure of *M. tuberculosis* and its dynamic. Due to the fact that VNTR typing combines a more user friendly technique with a significantly shorter turnaround time than RFLP-typing, it is now considered the gold standard.

### SEROLOGICAL TESTS

#### 1- Gamma interferon release assays

One-third of the world's population is estimated to be infected with latent tuberculosis (Diel et al. 2006) (LTBI). This infection leads to active disease in 10% of these individuals during their lifetimes; however, if the infected individuals are immune compromised (e.g., HIV infected), 8 to 10% of them will develop tuberculosis disease within a year. Therefore, it is important to accurately diagnose and treat patients with LTBI and also to predict who among the infected will develop the disease. The currently used tuberculin skin test (Huebner et al. 1993) (TST) is quite inexpensive and has been used worldwide for many years. The TST measures a delayed-type hypersensitivity response to purified protein derivative (PPD), a crude mixture of antigens from the members of the MTBC (and also NTM). Unfortunately, the TST has a low sensitivity (e.g., in patients with either immune suppression or very advanced disease) and a low specificity (e.g., in BCG-vaccinated individuals or in NTM-exposed populations). Also, the administration and reading of the TST require a certain amount of expertise that, when lacking, may result in erroneous interpretations.

The recent introduction of gamma interferon (Mazurek et al. 2005) (IFN- $\gamma$ ) release assays (IGRAs) has provided an alternative test for the diagnosis of LTBI. Currently, commercial assays are available, the Quantiferon-TB assay (Mazurek et al. 2005; Matsumoto and Yamazaki, 2014) (Cellestis Ltd., Carnegie, Victoria, Australia) and the T SPOT-TB assay





(Oxford Immunotec, Oxford, United Kingdom). These tests measure the IFN- $\mu$  release from T cells after stimulation by *M. tuberculosis*-specific antigens via an enzyme-linked immunosorbent assay (ELISA) or an enzyme-linked immunospot (ELISPOT) assay. The first-generation Quantiferon-TB assay is a whole blood test that measures IFN- $\mu$  release to PPD with an ELISA. The Quantiferon-TB Gold test is the enhanced form of the assay, which uses the *M. tuberculosis*-specific ESAT6 and CFP10 antigens instead of PPD. An even newer version of the test is the Quantiferon-TB Gold (Hill et al. 2008) in tube assay, which entails simpler sample preparation and is further enhanced by the addition of the TB 7.7 (p4) antigen. The T SPOT-TB assay, which requires the separation of peripheral blood mononuclear cells, detects IFN- $\mu$  release after exposure to ESAT6 and CFP10 with an ELISPOT assay. A major drawback of either method is that the incubation with the antigens must be initiated within 8 to 16 h following blood collection.

## 2- T-Spot<sup>®</sup>TB assay

The T-Spot<sup>®</sup>TB assay is an in vitro diagnostic test for the detection of effector T cells that respond to stimulation by *M. tuberculosis* antigens and is intended for use as an aid in the diagnosis of tuberculosis (TB) infection. The T-Spot<sup>®</sup>TB assay is a simplified enzyme-linked immunospot (ELISPOT) (Hill et al. 2008) method which enumerates individual TB-specific activated effector T cells.

Peripheral blood mononuclear cells (PBMCs) are separated from a whole blood sample and washed to remove any sources of background interfering signal. The PBMCs are then counted so that a standardised cell number is used in the assay. This ensures that even those who have low T cell titres due to weakened immune systems (the immunocompromised and immunosuppressed) have adequate number of cells added to the microtitre wells. The washing and counting stages as well as the ELISPOT technique provide superior performance for the detection of TB disease and latent TB infection.

## 3- Urinary lipoarabinomannan (antigen) detection (LAM)

Immune based approach consists of directly detecting an *M. tuberculosis* antigen, lipoarabinomannan (Del Prete et al. 1998) (LAM), in urine. LAM is a heat stable glycolipid specific to mycobacteria that is released by metabolically active bacteria, filtered by the kidney, and found in the urine of patients with active TB. LAM was originally detected in serum, but this test was limited by immune complex formation. Recent studies have evaluated commercially available tests that detect LAM in urine by antigen capture ELISA for the diagnosis of tuberculosis. The LAM- ELISA sensitivity ranged from 38% to 50.7% for TB cases, with a specificity range of 87.8% to 89%, as confirmed by smear microscopy, solid culture, and/or liquid culture. The sensitivity was significantly higher for patients with HIV-related TB (62.0%) and for females (66.7%), whereas sensitivity for smear-negative patients was low (28%) for combined HIV positive and negative patients.

The commercially available generation of LAM ELISAs has adequate specificity but suboptimal sensitivity and does not appear to be useful as an independent diagnostic test to confirm or exclude pulmonary TB in either HIV-infected or non infected patients. However, these assays could increase case finding if combined with smear microscopy and/or culture in settings of high HIV prevalence and could be of particular value in diagnosing TB in HIV co infected patients with CD4 cell counts of less than 100 cells/ml. The urine LAM assay also has a reasonably high negative predictive value, suggesting that a negative result could be used as evidence against active TB.

## Competing interests

The authors declare that they have no competing interests.

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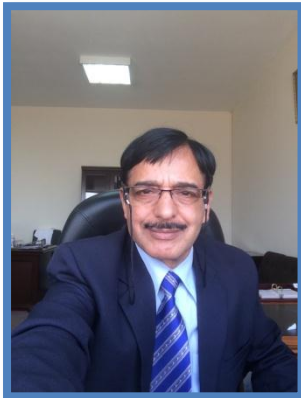
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