

# We are IntechOpen, the world's leading publisher of Open Access books Built by scientists, for scientists

## 4,800

Open access books available

## 122,000

International authors and editors

## 135M

Downloads

Our authors are among the

## 154

Countries delivered to

## TOP 1%

most cited scientists

## 12.2%

Contributors from top 500 universities

**WEB OF SCIENCE™**Selection of our books indexed in the Book Citation Index  
in Web of Science™ Core Collection (BKCI)

Interested in publishing with us?  
Contact [book.department@intechopen.com](mailto:book.department@intechopen.com)

Numbers displayed above are based on latest data collected.

For more information visit [www.intechopen.com](http://www.intechopen.com)

# Clinical Laboratory Diagnostics for *Mycobacterium tuberculosis*

N. Esther Babady<sup>1</sup> and Nancy L. Wengenack<sup>2</sup>

<sup>1</sup>Memorial Sloan-Kettering Cancer Center, New York, New York,

<sup>2</sup>Mayo Clinic, Rochester, Minnesota,

USA

## 1. Introduction

This chapter highlights current state-of-the-art methods for the detection and identification of *Mycobacterium tuberculosis* (*Mtb*) complex in the clinical diagnostic laboratory. Methods discussed include stain and culture which traditionally would have been followed by phenotypic-based identification methods. At this point in time however, molecular methods are considered the gold standard for both the rapid detection of *Mtb* directly from patient specimens as well as for the identification of *Mtb* following growth in culture. There are also instances where speciation of *Mtb* in order to distinguish it from other members of the *Mtb* complex is clinically important and these will be discussed. In addition, this chapter provides an overview of methods used in the clinical laboratory for *Mtb* drug resistance testing and suggests what the future might hold for *Mtb* diagnostics.

## 2. *M. tuberculosis* and biosafety in the clinical laboratory

*M. tuberculosis* presents a risk of laboratory-acquired infection due to its transmission via aerosol routes, ability to withstand common laboratory processing techniques such as heat-fixation or frozen section preparation and a extremely low ID<sub>50</sub> of <10 bacilli. The United States Centers for Disease Control and Prevention (CDC) estimates that laboratory workers are three times more likely than non-laboratory workers to become infected with *Mtb*. Therefore, biological safety organizations have defined a number of safety practices and procedures that must be strictly followed when working with *Mtb* which is classified as a risk group 3 organism. Specimens and cultures of unknown isolates shall be handled as if they contain *Mtb* until proven otherwise. Only non-aerosol generating processes such as accessioning of specimen or reading of acid-fast smears can be done under BSL-2 conditions outside of a BSC. All other specimen-handling including opening/closing of tubes, pipeting and transfer must be done in a BSC. Personnel must exercise caution to avoid aerosol generation. More specifically, smear preparation, culture decontamination and concentration, and culture plating must be done inside a BSC with the possible exception of the centrifugation step. However, centrifugation must be done using aerosol-proof containers which are opened, loaded and closed inside the BSC to reduce the risk of personnel exposure. All laboratory surfaces must be decontaminated with a tuberculocidal reagent before and after working with specimens and cultures. Propagation and

manipulation of *Mtb* complex cultures (e.g., identification and susceptibility testing) requires BSL-3 practices, equipment and facilities. Clinical laboratories without BSL-3 facilities must refer all positive mycobacterial cultures to another laboratory with BSL-3 capabilities for identification and, if necessary, susceptibility testing. Acid-fast smears must not be prepared from positive mycobacterial cultures of unknown identity without BSL-3 facilities. Identification methods (e.g., biochemical analysis, nucleic acid hybridization probes, sequencing, PCR) required initial specimen processing under BSL-3 conditions until any viable mycobacteria have been rendered non-viable by heating and/or lysis via chemical or physical means. Laboratories must verify that their processing methods are effective in rendering *Mtb* nonviable prior to conducting any activities outside of a BSL-3 laboratory.

BSL-3 facilities have highly specialized requirements some of which include restricted laboratory access, self-closing double door entry, directional airflow with a specified number of air exchanges over time, BSCs exhausted to the outside, and posted signage regarding the hazard (in this case *Mtb*). Class II BSCs are one of the most important pieces of equipment in the mycobacteriology laboratory and they must be maintained in good working order at all times. Frequent (at least daily) checks of function by means such as magnehelic gauge monitoring is needed. Regular maintenance and certification programs must be undertaken and documentation of cabinet performance must be maintained by the laboratory. Specimens should be covered before transport and should be transported in well-sealed, leak-proof containers. All biohazard waste should be autoclaved prior to leaving the facility.

BSL-3 personnel safety practices include the use of fluid-resistant, cuffed, solid-front gowns, gloves, eye protection, respiratory protection (N-95 or better fitted respirator or powered air purifying respirator (PAPR)). Each laboratory must perform a risk assessment to define the personal protective equipment, facilities and engineering practices that are appropriate for their institution and that comply with applicable regulations. The risk assessment is the responsibility of the laboratory director but it should be done in collaboration with institutional biosafety officials as this is helpful in making certain that no safety practice has been overlooked. A sample risk assessment is provided in Table 1 but each laboratory must perform their own assessment as situations may differ between laboratories. The laboratory must have a written spill procedure and must review the procedure with lab staff regularly to assure competency. Spill “drills” in which staff physically respond to a simulated spill are highly recommended as they routinely point out any potential gaps in procedures or staff knowledge.

Strict regulations exist in many countries concerning the shipping and transportation of diagnostic specimens that are known to contain or that potentially contain *Mtb* complex and for shipment of known *Mtb* complex isolates. Personnel who package these specimens and isolates must have specialized training that is updated at prescribed intervals. Packaging materials must be leak-proof, able to withstand unpredictable handling throughout the transportation chain and must be properly labeled in order to alert transportation workers of hazards contained within the package. Individuals involved in the shipping of specimens and isolates should be knowledgeable about the regulations within their country and if, sending specimens or isolates internationally, within the destination country.

Procedure	Aerosol Potential	Biosafety Level Required	Personal Protective Equipment Required	Engineering Controls Required	Special Practices or Equipment Required
Reading of smears (AR, Kinyoun, Modified Acid Fast)	Slight	BSL 2	Gown, gloves	May be done on bench top	
Manipulation of mycobacterial cultures for identification (e.g. subculturing)	Significant	BSL 3	Gown, shoe covers, eye protection, gloves and respirator/head cover or PAPR	Work in biological safety cabinet	Use disposable loops; Use racks to prevent tipping/spilling; Work over disinfectant-soaked towel
Susceptibility testing of mycobacteria	Significant	BSL 3 for setup BSL 2 for incubation and reading of closed bottles/plates/tubes	Setup - Gown, shoe covers, eye protection, gloves and respirator/head cover or PAPR	Work in biological safety cabinet to inoculate bottles, tubes or plates with organism	Use extreme care to avoid aerosol generation when inoculating bottles/plates/tubes

Table 1. Sample partial risk assessment – this table is intended as an example of one style of risk assessment that can be developed. Each laboratory must develop a laboratory-specific risk assessment in conjunction with their institutional safety officer(s).

### 3. Stains for mycobacteria

Mycobacteria, including *Mtb* complex, can be rapidly and inexpensively detected directly from pretreated and concentrated respiratory specimens, body fluids, and tissue using acid-fast stains. A Gram stain is not able to reliably detect mycobacteria which can appear as non-stained “ghosts” or as beaded Gram-positive bacilli. Therefore, acid-fast stains, such as the Ziehl-Neelsen stain or the fluorescent auramine-rhodamine stain are recommended for mycobacteria. The acid-fast stain forms a complex between the unique mycolic acids of the mycobacterial cell wall and the dye (e.g., fuchsin). Complex formation makes the mycobacteria resistant to destaining by acid-alcohols providing the basis for the “acid-fast” terminology. Non-acid fast bacteria do not retain the acid-fast dye in the presence of the acid-alcohol decolorizer and are often stained in a subsequent step using a counterstain such as methylene blue. Commonly utilized acid-fast stains contain carbol-fuchsin dye and are the Ziehl-Neelsen stain which utilizes phenol plus heat to aid in dye penetration, and the Kinyoun stain which uses phenol in the absence of heat. The Ziehl-Neelsen stain is considered the more sensitive of the two (Somoskovi et al., 2001). Fluorescent stains such as auramine O are also used alone or in combination with rhodamine B. The fluorescent stains exhibit increased fluorescence upon binding DNA and RNA providing enhanced sensitivity for examining concentrated direct specimens by

staining the bacilli while avoiding non-specific staining of artifacts and background more typical of the non-fluorescent stains.

Mycobacteria appear as long slender rods (1-10 $\mu$ m long x 0.2-0.6 $\mu$ m wide) and are often slightly curved or bent. At least 300 fields should be examined under high power (1000X) when using a carbol-fuchsin stain and light microscopy. The fluorochrome stain can be examined using a lower power (250X) and a minimum of 30 fields should be examined under the lower power (Pfyffer & Palicova, 2011). When positive, an indication of the quantity of acid-fast bacilli present should be provided. Factors which influence the sensitivity of acid-fast smears include the amount of acid-fast bacilli present in the specimen, the experience of the reader, the stain used, the number of fields examined, the type of specimen (e.g., generally respiratory specimens have higher yield than non-respiratory), the patient population being examined, volume of the specimen and smear pre-treatment (direct vs. pre-treated, concentrated). Rigid quality control processes must be followed to prevent cross-contamination and false results. Laboratories should use a positive and negative control slide for each batch of acid-fast smears prepared and should have a second reader confirm positive results and at least 10% of negative slides to reduce the potential for incorrect results. Staining jars or dishes should not be used to prevent potential cross-contamination and care should be taken to avoid the transfer of bacilli via the microscopy oil used for examining the slide. Laboratories must also participate in a proficiency testing programs (e.g., College of American Pathologists) to ensure continued competency.

Acid-fast stains lack sensitivity and a large number of bacilli ( $10^4$ - $10^6$ /mL) are required for a positive stain. Therefore, a positive stain from a respiratory specimen is typically thought to correlate with a higher infectivity potential and patients are routinely placed in airborne isolation rooms until their acid-fast smears convert to negative. Immunocompromised individuals often present with lower bacterial loads making detection by smear difficult (Chegou et al., 2011). Up to 30% of persons (commonly children) are unable to produce sputum for a smear requiring the use of more invasive methods (gastric washing, bronchoalveolar lavage, etc). Smears can be used to follow the response to treatment in smear-positive individuals. A concentration step provides increase sensitivity over direct smear microscopy (Steingart et al., 2006).

Acid-fast stains are also non-specific and the reader cannot determine the species of mycobacteria present in a positive smear. Mycobacteria tend to clump and produce cord-like strands of bacilli and there may be some indication of which species is present based on characteristic cording but this is highly subjective and not recommended as a routine method of determining species (Attorri et al., 2000; Julian et al., 2010).

#### **4. Culturing of *M. tuberculosis***

The growth of *Mtb* in culture is considered the gold standard for identification of a case of tuberculosis. The sensitivity of culture is much better than an acid-fast smear with only 10-100 viable organisms/mL of specimen required for a positive culture. Media for the growth of *Mtb* is the same as that used for other mycobacteria species and generally includes both a solid and a liquid-based medium. Solid media utilized is typically either egg-based such as the Lowenstein-Jensen (L-J) medium or agar-based such as Middlebrook 7H10 medium.

Antimicrobial agents can be added to help with elimination of contaminating organisms which may have a more rapid growth rate than *Mtb* and which may therefore obscure any *Mtb* present on the plate. In general, *Mtb* colonies are seen more rapidly on agar-based medium (10-12 days) as opposed to egg-based medium (18-24 days) (Liu et al., 1973). Care must be taken to protect Middlebrook medium from excessive light and heat which results in breakdown of the medium and release of a formaldehyde byproduct which is toxic to *Mtb* (Miliner et al., 1969). Use of Middlebrook 7H11 medium containing casein is reported to improve the recovery of isoniazid-resistant isolates of *Mtb* (Pfyffer & Palicova, 2011). Broth medium such as Middlebrook 7H9 medium is reported to provide a more rapid recovery of *Mtb* compared with solid medium. There are several commercially-available semi-automated broth culture systems for mycobacteria including *Mtb* complex. The BACTEC 460 radiometric and BACTEC 960 Mycobacterial Growth Indicator Tube (MGIT) fluorimetric systems (Becton, Dickinson, Sparks, MD) and the VersaTREK culture system (TREK Diagnostics Systems, Cleveland, OH) are FDA-cleared in the United States. The BACTEC 460 system is currently being phased out by the manufacturer in favor of the non-radiometric MGIT system. Other culture systems include Septi-Chek biphasic System (Becton, Dickinson,) and the MB/BacT Alert 3D system (bioMérieux, Marcy l'Etoile, France) which has a colorimetric CO<sub>2</sub>-based sensor to detect mycobacterial growth. There are numerous publications in the literature which compare the performance of the commercially-available broth systems but in general, these systems have a sensitivity of 88-93% for the detection of *Mtb* complex (Cruciani et al., 2004).

Cultures for *Mtb* complex should be incubated at 35-37°C in an atmosphere of 5-10% CO<sub>2</sub> for primary cultures on solid medium. Since *Mtb* complex grows slowly in culture, many laboratories choose to examine culture plates for growth twice per week during early stages of growth and then weekly for older cultures. The advantage of semi-automated broth systems such as the MGIT and VersaTREK are that CO<sub>2</sub>-supplementation is not generally required and the cultures are continuously monitored without the need for laboratory technologist intervention unless a culture is flagged by the instrument as positive. After either a solid or broth culture shows growth, the presence of acid-fast bacilli must be confirmed as described below in order to rule out non-mycobacterial contaminants.

## 5. Identification of *M. tuberculosis* from culture isolates

### 5.1 Microscopy

The first step in the examination of organisms growing on either solid media or liquid media is to confirm their identity using various staining methods as discussed in section 3. Depending on the stain used, the identification of *Mycobacterium* bacilli is done using either a light microscope under 100 x oil immersion objective or a fluorescent microscope under 25x or 40x objective. Microscopy however is not specific and cannot differentiate *Mtb* from other *Mycobacterium* and further analysis is required for final identification which can take up to 4-6 weeks.

Recently, the Microscopic Observation Drug Susceptibility (MODS) assay has been reported for the detection of *Mtb* complex in liquid culture through microscopic observation of characteristic cording and this method is characterized as in "late stage development/evaluation" for use in high TB burden settings by the World Health

Organization (WHO) (Caviedes & Moore, 2007; Moore, 2007; WHO, 2008; Ha et al., 2009; Limaye et al., 2010).

## 5.2 Biochemical analysis

Following microscopic and microscopic examination of culture isolates, final identification to the species level of *Mtb* complex is performed using a set of conventional biochemical reactions in combination with growth temperature. Although conventional biochemical assays are relatively inexpensive and simple to perform, they are time-consuming due to required incubation periods of up to 4 weeks, resulting in major delays in identification. Furthermore, with greater than 130 mycobacterial species identified to date, the use of phenotypic methods is limited and biased to identify only the most common species of mycobacteria, underestimating the complexity of the genus and resulting in misidentification of unfamiliar species (Kirschner et al., 1993; Springer et al., 1996). *M. tuberculosis*, as with other members of the *Mtb* complex, is a slow growing mycobacterium, requiring in general 15 days to 40 days to grow in culture. The optimal isolation temperature for the organism is 35-37°C and the organism does not produce pigmentation even following exposure to light (non-chromogen). The most useful biochemical tests used for identification of a nonchromogenic, slow-growing mycobacterium such as *Mtb* complex includes: niacin accumulation, nitrate reduction, pyrazinamidase activity, inhibition of thiophene-2-carboxylic acid hydrazide, urease activity and catalase activity. Other tests that can provide additional information include tellurite reduction, Tween 80 hydrolysis, the arylsulfatase test, iron uptake and NaCl tolerance. In general, a mature growth (2-3 weeks) is required for biochemical tests, they are often performed on a LJ slants and require up to 3 weeks for final read of results.

### 5.2.1 Niacin accumulation

Niacin (nicotinic acid) is produced by all species of mycobacteria and further metabolized to nicotinamide adenine dinucleotide (NAD). However, *Mtb* complex, *M. simiae* and some strains of *M. chelonae*, *M. bovis*, and *M. marinum* do not have the enzyme responsible for metabolizing niacin, resulting in its accumulation in the culture media. A water extract is prepared by adding 1 mL of sterile water to the surface of an LJ slant with a growth of mycobacteria species at least three weeks-old. An aliquot of this extract is then added to a tube containing a niacin strip, incubated for up to 30 minutes with gentle shaking. A positive reaction (presence of niacin) is read as the development of a yellow color. Since species other than *Mtb* complex can accumulate niacin, additional biochemical testing is required for the final identification of *Mtb* complex.

### 5.2.2 Nitrate reduction

*M. tuberculosis* complex contains the enzyme nitroreductase which is able to reduce nitrate ( $\text{NO}_3$ ) into nitrite ( $\text{NO}_2$ ). This reaction is detected in the laboratory by inoculating a nitrate broth with a loopful of a 3-4 weeks old mycobacterial culture and  $\alpha$ -naphthalamine, and sulfanilic acid that will react with the released  $\text{NO}_2$  to produce a red or pink color. A negative result (lack of color) is further confirmed by the addition of zinc dust. If a red color (nitrate reduced) develops following addition of zinc dust, then the original result is confirmed as negative.

### 5.2.3 Pyrazinamidase

The deamination of pyrazinamide into pyrazinoic acid and ammonia by the enzyme pyrazinamidase (PZA) is an essential test to distinguish *Mtb* complex (PZA positive) from *M. bovis* (PZA negative). Other non-tuberculous mycobacteria species including *M. marinum* and *M. avium* complex can also be positive for PZA. The test is performed by inoculating two LJ slants with the mycobacterium species and incubating them for 4 days at 35-37°C in a non-CO<sub>2</sub> incubator. After 4 days, 1% ferric ammonium sulphate is added to one of the tube and incubated at room temperature for 30 minutes. The PZA reaction is positive if a pink band forms on the surface of the LJ slant. If the reaction is negative, the LJ slant is incubated for an additional 4 hours at 2-8 °C and observed for the appearance of the pink band. If the test is still negative after 4 hours, the second tube will be tested on day 7 to finalize the test.

### 5.2.4 Urease

The presence of urease, the enzyme that hydrolyzes urea into ammonia and carbon dioxide, can be detected in mycobacterial species by incubating an actively growing culture to a urea broth for up to 7 days at 35-37°C in a non-CO<sub>2</sub> incubator, with readings done at days 1, 3 and 7. *M. tuberculosis* complex is positive for urease and will produce a dark pink to red color following incubation in urea broth.

### 5.2.5 Inhibition of thiophene-2-carboxylic acid hydrazide (TCH)

*M. tuberculosis* complex can be differentiated from *M. bovis* by its ability to grow in the presence of TCH, a property shared by most mycobacteria species except for *M. bovis*. This test uses quadrant Petri dishes with one of the quadrant containing TCH. A dilute suspension of the mycobacterial growth (10<sup>-3</sup> to 10<sup>-4</sup> in sterile water) is added to each quadrant and the plate incubated for 3 weeks at 35-37°C in a 10 % CO<sub>2</sub> incubator. A resistant organism shows greater than 1% growth in the TCH quadrant when compared to growth in the control quadrant.

### 5.2.6 Arylsulfatase test

Arylsulfatase is an enzyme that hydrolyzes free phenolphthalein from the tri-potassium salt of phenolphthalein disulfite. A suspension of a pure mycobacterium culture in sterile water is incubated with phenolphthalein in oleic acid agar for either 3 days (rapid growers) or 14 days (slow growers) at 35-37°C in a non-CO<sub>2</sub> incubator. The appearance of a pink or red color after addition of sodium bicarbonate (Na<sub>2</sub>CO<sub>3</sub>) indicates a positive reaction. All members of the *Mtb* complex lack the arylsulfatase enzyme and are therefore negative for that reaction (Koneman, 2006; Lee, 2010).

### 5.2.7 Catalase test

Catalase is an enzyme that splits hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) into water (H<sub>2</sub>O) and oxygen (O<sub>2</sub>). Unlike the catalase assay used to identify for other types of bacteria (i.e. *Streptococcus* spp.), the catalase assay for mycobacteria is performed using 30% H<sub>2</sub>O<sub>2</sub> (Superoxol) in 10% Tween-80 and the test performed both at 22-25°C and 68°C. *M. tuberculosis* complex has



catalase activity at 22-25°C but not at 68°C (ie., heat-labile catalase). In addition to determining catalase activity at 68°C, the strength of the catalase reaction is also evaluated to differentiate *Mtb* complex from other mycobacteria. This semiquantitative test is performed by adding Superoxol to a 2-weeks-old culture of mycobacteria growing on a Lowenstein-Jensen slant at 37°C. Five minutes after addition of the Superoxol, the strength of the catalase reaction is determined by measuring the height of the bubbles, characteristic of the catalase reaction, in the tube. If the height of the bubbles is > 45 mm, the reaction is considered high and if the height of the bubbles is < 45 mm, the reaction is considered low. *M. tuberculosis* complex has low catalase activity.

### 5.2.8 Iron uptake

Only a few mycobacteria species are able to take up iron from ferric ammonium citrate and convert it to iron oxide (rust). This biochemical reaction is mainly used to identify *M. fortuitum* which when incubated with 20% ferric ammonium citrate for up to 3 weeks on a Lowenstein-Jensen slant at 28-30°C will turn a dark, rusty brown color. *M. tuberculosis* complex does not take up iron.

### 5.2.9 NaCl tolerance

The ability to grow on media containing 5% NaCl differentiates the slow growing mycobacteria from the rapid growers as only *M. triviale* (a slow grower) can grow on this media and only *M. chelonae* and *M. mucogenicum* (rapid growers) fails to grow in the presence of this salt concentration. The test is performed by inoculating an LJ slant containing 5% NaCl with a 1 MacFarland concentration of a mycobacterial culture and incubating it at 28°C in a 5-10% CO<sub>2</sub> incubator for up to 4 weeks (Witebsky & Kruczak-Filipov, 1996; Lee, 2010). *M. tuberculosis* complex does not grow on LJ slant containing 5% NaCl.

### 5.2.10 Tellurite reduction

Most mycobacterium can reduce potassium tellurite to metallic tellurium in liquid broth within 3 days. The test is performed by inoculating a Middlebrook 7H9 liquid medium containing Tween 80 with a heavy concentration of organisms and incubating at 37°C in a 5-10% CO<sub>2</sub> incubator for up to 7 days. After 7 days, a solution of potassium tellurite is added to the liquid culture and further incubated for 3 days. A positive reaction shows a black precipitate (metallic tellurium) at the bottom of the tube. This test is often used to identify MAC. *M. tuberculosis* complex is negative for tellurite reduction (Witebsky & Kruczak-Filipov, 1996; Lee, 2010).

### 5.2.11 Tween 80 hydrolysis

This assay tests for the presence of the enzyme lipase which can cleave the oleic acid from the detergent Tween 80 (polyoxyethylene sorbitan monooleate). The release of the oleic acid from Tween 80 results in a change of color of the neutral indicator from yellow to red within 5-10 days after incubation of Tween-80 with a mycobacterium at 37°C in a 5-10% CO<sub>2</sub> incubator. *M. tuberculosis* complex is negative for Tween 80 hydrolysis.

The use of conventional methods for the initial identification of *Mtb* complex is not ideal. This section provided a review of the most common tests used traditionally but the combined use of these various assays results in a significant delay in identification of up to one month after growth of the culture in the laboratory. The following sections will cover the more rapid methods currently in use in most mycobacteriology laboratory for identification of *Mtb* complex.

Biochemical Test	Reaction
niacin accumulation	Positive
nitrate reduction	Positive
pyrazinamidase activity	Positive
urease activity	Positive
inhibition of thiophene-2-carboxylic acid hydrazide (TCH)	Positive
arylsulfatase test	Negative
catalase test	Negative (heat-labile)
iron uptake	Negative
NaCl tolerance	Negative
tellurite reduction	Negative
Tween 80 hydrolysis	Negative

Table 2. Biochemical tests for identification of *Mycobacterium tuberculosis* complex from a culture isolate

### 5.3 High-Performance Liquid Chromatography

High-performance liquid chromatography (HPLC) of mycolic acids was first proposed for use as a standard test in mycobacteria species identification by the CDC in 1985. This technique had been widely used by clinical chemistry laboratories for the separation and identification of drug compounds (Butler & Guthertz, 2001). HPLC can be used to differentiate mycobacteria based on differences in their mycolic acid profiles. Mycolic acids are high-molecular weight fatty acids with long carbon side chains present in abundance in the cell wall of mycobacteria and other organisms including *Corynebacterium*, *Dietzia*, *Rhodococcus*, *Nocardia*, *Gordonia*, *Williamsia*, *Skermania*, and *Tsukamurella* species with *Mycobacterium* species containing the longest carbon chain (60-90) (Butler & Guthertz, 2001). Mycolic acids samples are prepared through a series of steps involving saponification of the mycobacteria, organic solvent extraction and derivatization of the mycolic acids to UV-adsorbing *p*-bromophenacyl (PBPA) esters (Durst et al., 1975; Butler et al., 1991). The derivatized mycolic acids solution is then separated on a HPLC instrument and the resulting chromatogram interpreted based on the peak pattern which is specific for each species of mycobacterium (Butler et al., 1991; Butler & Guthertz, 2001).

Although sensitive and specific when compared to biochemical tests and other molecular assays, with agreement ranging from 90-99% depending on the mycobacterial species (Guthertz et al., 1993; Thibert & Lapierre, 1993), HPLC is a technically demanding method which is not easily implemented in routine diagnostic laboratories. This method requires a high level of expertise for recognition of species based on the HPLC chromatogram and therefore only limited to a few reference laboratories including the CDC in Atlanta, GA. A

commercial HPLC assay, the Sherlock Mycobacteria Identification HPLC system (SMIS; MIDI Inc., Newark, DE), was developed to simplify use of HPLC in the clinical laboratories through automated recognition of mycobacterial species based on software that analyzes HPLC peak patterns comparing them to a library containing several *Mycobacterium* species chromatograms (Kellogg et al., 2001; LaBombardi et al., 2006). Of the 370 isolates tested in a multicenter study by SMIS, 327 (88%) were identified to the species level by the SMIS software with 279 (75%) correctly identified (Kellogg et al., 2001). The sensitivity of the SMIS identification could be increased to 98.9% (366/370) by manual calculation of relative peak height ratios and relative retention times and additional biochemical properties. In another study by LaBombardi et al. (LaBombardi et al., 2006), the SIMS correctly identified 61/90 isolates (67.8%) growing on Middlebrook 7H11 plates (BBL, Sparks, MD) and 73/161 (45.3%) isolates growing in VersaTREK Myco bottles (TREK Diagnostic Systems, Cleveland, OH). This performance was increased by used of a modified library to 91% for isolates growing on solid media and 83.2% for isolates recovered from liquid culture. In both studies, no *Mtb* complex isolates were misidentified and the sensitivity ranged from 83-100% (Kellogg et al., 2001; LaBombardi et al., 2006). Of note, although HPLC is a faster and more sensitive technology than conventional biochemical testing, this method cannot be used directly on clinical specimen and is not able to differentiate between members of the *Mtb* complex, except for the *M. bovis* BCG strain (Butler et al., 1991; Floyd et al., 1992).

#### 5.4 Nucleic acid hybridization probes

The introduction of nucleic acid hybridization probes in the clinical laboratory significantly impacted the turn-around time and workload for identification of mycobacteria species. Nucleic acid hybridization probes are single-stranded or double-stranded DNA/RNA fragments, labeled with a radioactive, chemiluminescent or a fluorescent marker, that are complementary to a target DNA or RNA sequence (Wetmur, 1991). In clinical microbiology, nucleic acid hybridization probes often target ribosomal RNA (rRNA) because of their high copy number present in organisms growing in culture. The first nucleic acid hybridization probes from Gen-Probe (San Diego, CA) for rapid identification of *M. avium* complex and *Mtb* complex were labeled with I<sup>125</sup> radioactive isotope. Labeled DNA-RNA complexes were separated from non-hybridized DNA using an adsorption suspension containing hydroxyapatite and the I<sup>125</sup> in the adsorbed labeled complex was counted using a gamma counter. Results were calculated as percentage of input probe hybridized (Drake et al., 1987; Kiehn & Edwards, 1987; Ellner et al., 1988; Musial et al., 1988; Peterson et al., 1989). The introduction of these radioactive probes resulted in a significant decrease in the turn-around time for identification of both *M. avium* complex and *Mtb* complex from weeks to approximately 2 hours with sensitivity and specificity ranging from 83-100% and 99.2-100% respectively (Drake et al., 1987; Kiehn & Edwards, 1987; Ellner et al., 1988; Musial et al., 1988; Peterson et al., 1989).

The I<sup>125</sup> probes were eventually replaced with non-radioactive probes to reduce staff potential for exposure to radioactive materials. Two types of non-isotopic probes were introduced, the synthetic nucleic acid probes (SNAP) (Syngene, Inc., San Diego, CA) and the AccuProbes (Gen-Probe, San Diego, CA). The SNAP probes utilized DNA probes labeled with alkaline-phosphatase and was performed by spotting the isolate to be tested on a nylon membrane and incubating the membrane with the labeled probes, followed by incubation in

a solution of nitroblue tetrazolium chloride, 5-brom-4-chloro-3-indolyphosphate substrates and alkaline phosphatase buffer. A positive reaction was read as the presence of a blue or purple color on the nylon membrane within a few hours (Lim et al., 1991; Woodley et al., 1992). Although 100% sensitive, cross-reactivity was detected with SNAP probes between *Mtb* complex and *M. terrae*, requiring the 68°C catalase test to be performed to differentiate between the two species (Lim et al., 1991; Ford et al., 1993).

AccuProbes are labeled with acridinium ester and hybridization is measured by chemiluminescence following hydrolysis of the label upon addition of H<sub>2</sub>O<sub>2</sub> and NaOH. The chemiluminescence is measured using a luminometer and expressed as RLU (relative light units). Unlike the previous version with the I<sup>125</sup> isotope, no wash steps are necessary as non-hybridized probes are chemically degraded and only acridinium ester-labeled, hybridized probes can produce measurable chemiluminescence (Goto et al., 1991). The AccuProbe for *Mtb* complex can be performed on culture growing from both liquid and solid media and will detect all members of the *Mtb* complex (Gen-Probe, 2011). The sensitivity and specificity of the acridinium ester labeled probes was comparable to that of the radioisotope labeled probes (Goto et al., 1991; Lebrun et al., 1992). Similar to the SNAP probes, detection of some strain of *M. terrae* by *Mtb* complex probes was also observed (Lim et al., 1991; Ford et al., 1993), although increased stringency in the detection method resolved the false-positive detection of *M. terrae* by AccuProbes *Mtb* complex probes.

Although the use of nucleic acid probes has allowed same day identification of *Mtb* complex from culture, the sensitivity of these probes is not high enough for detection of the organisms directly from clinical specimens, which still limits the rapid identification to the time it takes for the organisms to grow in either liquid or solid media. Furthermore, the *Mtb* complex AccuProbes do not distinguish amongst members of the *Mtb* complex.

### 5.5 Line Probe assays

Line Probe assays were developed to expand the range of mycobacterium species identified using nucleic acid probes since those were only available for *Mtb* complex, *M. avium*, *M. intracellulare*, *M. kansasii* and *M. goodii* (Gen-Probe, San Diego, CA). The first commercially available LineProbe 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 (Scarparo et al., 2001; Tortoli et al., 2001). The LiPa assay is able to detect up to 14 different species of mycobacteria (Table 3) and results are interpreted according to a flowchart decision scheme (Tortoli et al., 2001). A multicenter evaluation of LiPa assay conducted in Italy tested 238 mycobacterial organisms from both solid and liquid media as well as two *Nocardia* strains (Tortoli et al., 2001). All 238 mycobacterial strains reacted with the genus specific probes for a sensitivity of 100% and 61 of the 238 strains were identified to the species level. The other 177 strains were outside of the detection range of the LiPa assay. Additional studies using only liquid culture media, MB/BacT Alert 3D (Organon Teknika, Boxtel, The Netherlands), and BACTEC 12B Bottles (BACTEC; Becton Dickinson, Sparks, MD), showed similar sensitivity of 100% for

detection of mycobacterial strains by the genus specific probes as well as correct identification at the species levels and specificity of 100% (Miller et al., 2000; Scarparo et al., 2001).

The LiPa test is a more complex assay than the AccuProbe, requiring highly skilled technologists and has a turn-around time of at least 6 hours, including a PCR amplification step. Furthermore, control of the hybridization temperature is key to preventing formation of non-specific bands. However, in addition to its ability to detect several mycobacterial species compared to AccuProbes, the Inno-LiPa assay has the advantage of being able to detect mixed mycobacterial infections which often results in decreased sensitivity of the AccuProbes (Scarparo et al., 2001). Both assays still have to be performed from organisms growing in culture and are not able to differentiate among the members of the *Mtb* complex.

Another line probe assay, the GenoType Mycobacterium (Hain, Nehren, Germany) was developed and made available in two different kit formats. One kit, the GenoType Mycobacterium CM (Common Mycobacteria) was designed to detect the most frequently isolated mycobacteria species in clinical laboratories while the other kit, the GenoType Mycobacterium AS (Additional Species), was designed for the detection of less frequently encountered mycobacteria species (Makinen et al., 2006; Richter et al., 2006; Russo et al., 2006). Russo and colleagues tested 197 isolates including genera other than mycobacteria previously identified by conventional biochemical tests, HPLC, Inno-LiPa and 16S rRNA sequencing (Russo et al., 2006). The sensitivity of the assay for the mycobacterium genus was 98.9 and 99.4% for the CM and AS kits respectively with a specificity of 100% for the AS kit and 88.9% for the CM kit due to weak cross-reaction of several strains of the genus *Tsukamurella*. The overall sensitivity and specificity for species identification was 97.9% and 92.4% for the CM kit and 99.3% and 99.4% for the AS kit with all members of the *Mtb* complex correctly identified as *Mtb* complex by the CM kit and *Mtb* species by the AS kit. Similar performance were established in other independent studies evaluating the GenoType CM and AS kits, with the sensitivity and specificity for *Mtb* complex members approaching 100% when compared to 16S rRNA sequencing and biochemical testing (Makinen et al., 2006; Richter et al., 2006).

A third kit from Hain LifeScience, the GenoType MTBC DNA strip assay was designed specifically for the differentiation of members of the *Mtb* complex and identification of *M. bovis* BCG. Similar to the LiPa assay, the MTBC DNA strip assay uses reverse hybridization technology on a solid membrane matrix. The DNA probes immobilized on the membranes target polymorphisms in the *gyrB* DNA sequence of the *Mtb* complex and the RD1 deletion of *M. bovis* BCG (Richter et al., 2003). Richter and colleagues (Richter et al., 2003) evaluated the performance of the MTBC assay using well-characterized strains of *Mtb* complex including *Mtb*, *M. bovis* subsp. *bovis*, *M. bovis* subsp. *caprae*, *M. bovis* BCG, *M. africanum* subtype I, *M. africanum* subtype II, *M. canetti*, and *M. microti* as well as clinical isolates of *Mtb* complex identified by conventional methods and other molecular tests (PCR-Restriction fragment length polymorphism). The MTBC assay was able to differentiate all species of the *Mtb* complex except for separating *Mtb* from some strains of *M. africanum* subtype II and *M. canetti* (sensitivity of 94%). A similar study conducted by Neonakis and colleagues showed 100% agreement between conventional methods/AccuProbes and MTBC assay for the identification and differentiation of 120 clinical isolates of the *Mtb* complex (Neonakis et al., 2007).

Species	AccuProbes	Inno-LiPa	GenoType CM	GenoType AS	GenoType MTBC
<i>Mycobacterium spp.</i>		X	X		
<i>M. tuberculosis</i> complex	X	X	X		
<i>M. tuberculosis</i>					X
<i>M. bovis</i> subsp. <i>bovis</i>					X
<i>M. bovis</i> BCG					X
<i>M. bovis</i> subsp. <i>caprae</i>					X
<i>M. africanum</i>					X
<i>M. microti</i>					X
<i>M. kansasii</i>	X	X	X	X	
<i>M. avium</i>	X	X	X		
<i>M. intracellulare</i>	X	X	X		
MAI-X		X			
<i>M. malmonense</i>		X	X		
<i>M. haemophilum</i>		X		X	
<i>M. scrofulaceum</i>		X	X		
<i>M. paratuberculosis</i>		X			
<i>M. silvaticum</i>		X			
<i>M. chelonae</i>		X	X		
<i>M. gastri</i>		X		X	
<i>M. xenopi</i>		X	X		
<i>M. gordonae</i>	X	X	X		
<i>M. abscessus</i>			X		
<i>M. fortuitum</i>			X		
<i>M. marinum</i>			X		
<i>M. ulcerans</i>			X	X	
<i>M. peregrinum</i>			X		
<i>M. simiae</i>				X	
<i>M. mucogenicum</i>				X	
<i>M. goodii</i>				X	
<i>M. celatum</i>				X	
<i>M. smegmatis</i>				X	
<i>M. genavense</i>				X	
<i>M. lentiflavum</i>				X	
<i>M. heckeshornense</i>				X	
<i>M. szulgai</i>				X	
<i>M. phlei</i>				X	
<i>M. asiaticum</i>				X	
<i>M. shimoidei</i>				X	

Table 3. *Mycobacterium* species detected by commercially available probe assays

## 5.6 DNA sequencing

The first report of nucleic acid sequencing for the identification of mycobacteria appeared in the literature in the early 1990s. Rogall et al. (Rogall et al., 1990) described the use of a 1 kb gene fragment targeting the 5' region of the 16S rRNA to detect and differentiate among the various species of mycobacteria, including the *Mtb* complex. In this study, amplified sequences were electrophoresed on a 6% sequencing gel, dried and the gel was exposed to X-rays film for 12 hours. Sequences obtained were then analyzed using a multisequence alignment algorithm from SAGE program (Rogall et al., 1990). This entire process was completed in approximately 2 days. Other nucleic acid targets were analyzed including the *rpoB* gene, encoding the  $\beta$ -subunit of the RNA polymerase, which had the added advantage of detecting rifampin resistance (Kim et al., 1999; Kasai et al., 2000), the 16-23S rDNA internal transcribed spacer (ITS) (Roth et al., 1998), the 32-kDa protein (Soini et al., 1994) and the 65 kDa heat shock protein (Kapur et al., 1995). Each of these targets presented advantages and disadvantages, mainly related to their ability to differentiate closely-related organisms. Today, sequencing of the 16S rDNA has become the gold standard for mycobacteria species identification. The process was eventually automated and commercialized (MicroSeq 500bp and 1500bp 16S rDNA Bacterial Sequencing Kits, Applied Biosystems, Carlsbad, CA), which resulted in standardization of the assay across laboratories. The MicroSeq 16S rDNA bacterial identification assay analyzes a larger portion of the 16S rDNA than the one described earlier by Rogall et al. (Rogall et al., 1990), resulting in increased discriminating power. The introduction of capillary electrophoresis and fluorescent dyes to replace the cumbersome sequencing gels and radioactive labels, the development of genetic analyzer and the National Center for Biotechnology Information (NCBI) tool, BLAST (Basic Local Alignment Sequence Tool), further improved on the use of sequencing for mycobacterial species identification.

DNA sequencing using the MicroSeq system is based on sequencing by capillary electrophoresis and consists of 4 steps: DNA extraction, amplification, sequencing and data analysis. Total genomic DNA extraction is performed simply by lysis of organisms in a chaotropic solution (PrepMan Ultra, Applied Biosystems), followed by heating at 95°C for 10 minutes to kill the organisms. Extraction is followed by 16S rRNA amplification, PCR products clean-up and sequencing PCR and analysis on a genetic analyzer. The sequence obtained is then analyzed against the MicroSeq database library and/or the BLAST database on the NCBI website (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

Studies evaluating the performance of the MicroSeq systems for identification of mycobacteria have repeatedly shown the advantages of using this technique over traditional biochemical tests and with the increase in available and correct sequences in various database, the sensitivity of the assay continues to increase (Patel et al., 2000; Cloud et al., 2002; Hall et al., 2003; Woo et al., 2011). The main limitation of sequencing for identification of mycobacteria as discussed above remains the inability to distinguish several species of mycobacteria based solely on the 16S rRNA including *M. chelonae*, *M. abscessus*, and *M. immunogenum* and the various members of the *Mtb* complex (Hall et al., 2003; Woo et al., 2011). Alternative sequencing targets (eg., *rpoB*) can often be used to distinguish mycobacterial species which cannot be resolved using the 16S target.

### 5.7 PCR methods (conventional and real-time)

The pattern of resistance of pyrazinamide (PZA) is often used to distinguish between *Mtb* and *M. bovis*/*M. bovis* BCG strains (section 5.2.3). Only a few laboratory-developed tests (LDTs) have been designed to differentiate among members of the *Mtb* complex (Parsons et al., 2002; Huard et al., 2003; Pinsky & Banaei, 2008). Pinsky and Banaei (Pinsky & Banaei, 2008) reported a real-time PCR assay, using a series of different primer pairs targeting the RD (region of difference) 9 (present in all *Mtb* complex members), the RD1 (absent in *M. bovis* BCG strains), the RD4 (absent in *M. bovis*). This multiplex real-time PCR uses melt curve analysis from two separate PCRs to identify and distinguish between *Mtb*, *M. bovis*, and *M. bovis* BCG directly from isolates growing in culture. Similarly, although on a conventional PCR format, the RD1 sequence was used as a target for the differentiation of *Mtb*, *M. bovis*, and *M. bovis* BCG from culture isolates (Lee et al., 2010).

### 5.8 Immunoassay methods

There are several commercially-available immunoassay methods which allow the identification of *Mtb* complex from culture by detection of *Mtb*-specific antigens. These tests are rapid requiring only minutes to perform after growth of the organism but literature reports indicate variable performance (Martin et al., 2011; Said et al., 2011; Steingart et al., 2011; Yu et al., 2011)

## 6. Direct identification of *M. tuberculosis* from clinical specimens

### 6.1 Line probe assays

The INNO LiPa Mycobacteria (Innogenetics), described previously in section 5.5, has been evaluated for use directly on clinical specimens without waiting for growth of *Mtb* complex in culture. In a study by Perandin et al. (Perandin et al., 2006), the INNO Lipa Mycobacteria assay was evaluated, with slight modifications, on both pulmonary specimens and extrapulmonary specimens (stools, urines, lymph nodes, gastric fluids and pus). The overall sensitivity and specificity of the test was 79.5% and 84.6% respectively for pulmonary specimens and 71.4% and 100 %, respectively for extrapulmonary specimens. As expected the sensitivity and specificity of the assay was much lower when tested on specimens than culture isolates and the authors suggested this was due to the lower numbers of organisms present in specimens.

The INNO-LiPA Rif TB (Innogenetics) test, designed to detect *Mtb* complex and rifampin susceptibility in culture isolates, has also been tested on clinical specimens in multiple studies (Gamboa et al., 1998; de Oliveira et al., 2003; Johansen et al., 2003; Traore et al., 2006). In one of the largest studies, Traore and colleagues (Traore et al., 2006) evaluated the performance of the INNO-LiPA assay by testing 420 sputum samples from both treated and untreated patients, with 311 smear positive and 109 smear negative specimens. The assay detected *Mtb* complex DNA in 92% of smear positive specimens and 94.5% of smear negative specimens, a higher detection rate than culture which detected *Mtb* complex in 74.3% of all specimens.

An alternative version to the line assay GenoType Mycobacterium (Hain LifeScience), the GenoType Mycobacterium Direct Assay (Hain Lifescience) was designed to detect *Mtb*



complex and *M. avium*, *M. intracellulare*, *M. kansasii*, and *M. malmoeense* directly from clinical specimens. This assay is performed in three parts consisting of an RNA isolation and capture step, followed by an isothermal amplification of the 23S rRNA, and finally a reverse-hybridization of the amplified products on the membrane strips. Evaluation of this assay showed sensitivity and specificity ranging from 80.5-97% to 75-100% respectively when compared to culture (Franco-Alvarez de Luna et al., 2006; Seagar et al., 2008; Neonakis et al., 2009; Kiraz et al., 2010).

In general, the sensitivity of these line assays is lower when tested directly on clinical specimens as compared to their sensitivity when used on culture isolates. However, with a turn-around time of about 2 days compared to at least 6 weeks to obtain growth in culture, these assays, with their high specificity, provide information that is directly useful for clinical management of patients.

## 6.2 FDA-approved PCR methods

The first FDA-approved amplification test for diagnosis of *Mtb* complex was the Amplified Mycobacterium Tuberculosis Direct (MTD) test (Gen-Probe Inc, San Diego, CA). This assay was based on the amplification of *Mtb* complex rRNA followed by detection of the amplified rRNA by hybridization of chemiluminescent acridinium ester-labeled DNA probes. The sensitivity and specificity of the assay in earlier studies testing against N-acetyl-L-cysteine treated respiratory samples (smear positive and smear negative), was 91.9-100% and 97.6-100%, respectively when compared to culture (Abe et al., 1993; Pfyffer et al., 1994; Welch et al., 1995). The MTD test presented several advantages over traditional methods including the use of rRNA, which is present in several copies in *Mtb* complex, rapid turn-around time (day vs. weeks), single tube amplification and detection, although culture is still required for susceptibility testing of the organisms (Abe et al., 1993; Pfyffer et al., 1994). The MTD test is only FDA-approved for respiratory specimens; however, the assay performance in extrapulmonary specimens has also been evaluated by several investigators. Vlaspodler et al. (Vlaspolder et al., 1995) showed variable sensitivity for different specimens with detection in pleural fluids being as low as 20% (with specificity of 96%) and sensitivity and specificity in other specimens, including urines, lymph nodes, CSF, gastric fluids and lung biopsies, of 100% and 95% respectively. Similar results were obtained in other studies that included a greater numbers of non-respiratory specimens, with sensitivities ranging from 93-100% and specificities of 100% for both smear-positive and smear negative specimens (Chedore & Jamieson, 1999; Woods et al., 2001).

A second PCR assay approved by the FDA was the AMPLICOR *Mycobacterium tuberculosis* test (AMPLICOR MTB; Roche Diagnostic Systems, Somerville, N.J.). This assay targeted the 16S rRNA gene with colorimetric detection using probe hybridization (Piersimoni & Scarparo, 2003). In a study by D'Amato and colleagues, the sensitivity and specificity of the Amplicor MTB when compared to culture as the gold standard was: 55.3% and 99.6% from smear negative and 94.1% and 100% from smear positive respiratory specimens (D'Amato et al., 1995). Other studies showed sensitivity and specificity for the assay ranging from 70.4% to 79.5% with specificity greater than 98% when compared to culture and conventional microscopy (Schirm et al., 1995; Bergmann & Woods, 1996; Devallois et al., 1996) and sensitivity generally increasing 75-98 % for AFB positive specimens (Schirm et al., 1995; Bergmann & Woods, 1996). Testing of non-respiratory samples by the Amplicor MTB assay

supported its use in diagnosis of extrapulmonary tuberculosis as the sensitivity and specificity varied from 76%-100% and 99.9-100%, respectively when compared to clinical diagnosis (Shah et al., 1998). These non-respiratory specimens included various tissue biopsies (lung, lymph nodes, liver) and various body fluids (pleural, ascites, CSF, synovial, gastric, pericardial and peritoneal).

A second version of the Amplicor assay, the Cobas Amplicor MTB-PCR (Roche Diagnostics) was developed as a semi-automated assay that combined the amplification, detection and reporting of results (Bodmer et al., 1997; Levidiotou et al., 2003). Evaluation of the new assay showed increased sensitivity over the manual assay with one study testing >1,000 respiratory specimens reporting a sensitivity and specificity of 91.3% and 99.6% (Bodmer et al., 1997) and the other reporting an overall sensitivity and specificity of 82.5% and 99.8% after testing greater than 7,000 specimens (respiratory and non-respiratory)(Levidiotou et al., 2003).

Both the MTD and the Amplicor assays were evaluated on broth samples including BACTECT 12B bottles (Becton Dickinson, Sparks, MD) and the ESP II (Trek Diagnostics, Westlake, OH), with sensitivity of greater than 90% for both assays in both media and specificity of 100% (Hernandez et al., 1997; Smith et al., 1997; Bergmann & Woods, 1999; Desmond & Loretz, 2001).

### 6.3 Laboratory-developed PCR methods

Several targets, including the 65-kD antigen of *Mtb* complex (Pao et al., 1990; Brisson-Noel et al., 1991; Totsch et al., 1994), the protein antigen B (Sjobring et al., 1990), the repetitive sequences IS6110 (Brisson-Noel et al., 1991; Eisenach et al., 1991; Sankar et al., 2010) and IS986 (Abe et al., 1993), have been used over the years for the detection of *Mtb* complex by LDTs. Earlier tests were based on conventional PCR, with a multi-step manual extraction process followed by amplification and detection of the amplified target on polyacrylamide gel. Depending on the target used, the sensitivity and specificity of these conventional PCR assays directly from respiratory specimens ranged from 84.2-100% and 62.6-100% with culture and clinical diagnosis used as a gold standard. However, due to limitations inherent with conventional PCR including high potential for cross-contamination, real-time PCRs, combined with automated extraction, have largely replaced these methods in clinical laboratories.

Real-time PCR is faster than conventional PCR and does not require post-amplification manipulation of the amplified DNA, reducing the potential for cross-contamination. Several real-time PCR LDTs have been developed for diagnosis of pulmonary tuberculosis. In one of the earlier studies by Miller et al (Miller et al., 2002), a real-time PCR assay was developed on the LightCycler platform (Roche Diagnostics, Indianapolis, IN) with primers targeting the internal transcribed spacer region of the mycobacterium genome with specific hybridization probes designed for the detection of *Mtb* complex. The sensitivity and specificity of this assay for smear-positive respiratory specimens was 98.1% and 100% respectively with a turn-around time of less than 5 hours (Miller et al., 2002). Other studies, with different targets, have reported similar results (Shrestha et al., 2003) and many have been designed as multiplex assays to differentiate *Mtb* complex from other non-tuberculosis mycobacteria based on melting curve analysis (Shrestha et al., 2003). Furthermore, these

assays were often shown to be as sensitive and specific as the available commercial assays such as the Cobas Amplicor (Miller et al., 2002; Shrestha et al., 2003).

Both conventional and real-time PCRs have also been developed for the diagnosis of extrapulmonary tuberculosis using both fresh specimens and paraffin-embedded tissues. Some of the fresh specimens that have been evaluated by various PCR include fine needle aspirates and tissue biopsies of lymph nodes, blood, urines, bone marrow aspirates and skin biopsies with variable sensitivities and specificities (Hsiao et al., 2003; Bruijnesteijn Van Coppenraet et al., 2004; Chakravorty & Tyagi, 2005; Ritis et al., 2005; Torrea et al., 2005; Rebollo et al., 2006). Although paraffin-embedded tissues are not optimal samples for PCR, often, they are the only specimens available to rule out tuberculosis. The sensitivity and specificity of PCR assays, conventional and real-time, on paraffin-embedded tissues varies from 64-100% and 73- 100%, respectively (Beqaj et al., 2007; Baba et al., 2008; Nopvichai et al., 2009; Luo et al., 2010).

Unlike PCR assays previously mentioned for differentiation of members of *Mtb* complex, a real-time PCR assay, developed by Halse and colleagues (Halse et al., 2011) at the New York State Public Health Laboratory, showed the ability to differentiate among members of the *Mtb* complex directly from clinical specimens based on the detection of five targets including RD1, RD4, RD9, RD12 and a region external to RD9. The assay was able to detect 155 of 165 clinical specimens (94%) and 708/727 (97%) of positive BACTEC MGIT-960 bottles. Furthermore, this assay was able to distinguish not only *Mtb*, *M. bovis* and *M. bovis* BCG but also *M. africanum*, *M. microti*, and *M. canettii*.

## 7. Drug resistance testing for *M. tuberculosis*

The CDC published recommendations that drug susceptibility testing be performed on the first *Mtb* complex isolate from a each patient and also if a patient is failing therapy (based on clinical evidence or positive culture after three months on therapy) (MMWR, 1993). This recommendation was formulated following a resurgence in cases of tuberculosis from the mid-1980s to the early 1990s of up to 18% with resistance strains present in as high as 33% of cases in New York city (MMWR, 1993). Susceptibility testing is currently performed most commonly by traditional methods either agar-based or broth based methods with resistance defined as growth of greater than 1% of organisms tested against a specific drug (Canetti et al., 1963).

### 7.1 Indirect proportion method

Although patient specimens can be tested directly for drug susceptibility with the advantage of decreased time to results, this method is limited to smear-positive specimens and results may be difficult to interpret if contamination occurs (Woods, 2011). The 1% indirect proportion method is performed on Middlebrook 7H10 agar medium poured in a four-quadrant Petri dish with one quadrant serving as the control quadrant and containing no drug and the other three quadrants containing increasing concentration of the drug being tested. The inoculum is prepared using organisms growing on solid or liquid media adjusted to a 1.0 McFarland and diluted to  $10^{-2}$  and  $10^{-4}$ ; 0.1 mL of each dilution is then added to each quadrant of separate plate and incubated for up to three weeks at  $37 \pm 1^\circ\text{C}$  in an atmosphere of 5 to 10%  $\text{CO}_2$  (CLSI, 2003). The percentage of drug resistance is calculated

by dividing the total colony count in a quadrant containing drug by the total colony count in the control quadrant (at least 50 colonies) and multiplying by 100. If the percentage is greater than 1%, then the organism is resistant and therapy with the drug tested is likely to fail (CLSI, 2008; Woods, 2011). This method is used primarily for susceptibility testing of the first line drugs, isoniazid (INH), rifampin (RIF) and ethambutol (EMB), as well as second line drugs, when resistance to RIF or two of the first-line drug is detected (CLSI, 2008).

## 7.2 Rapid broth methods

In order to circumvent 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. The first system, the BACTEC 460, was based on measurement of radioactive  $^{14}\text{CO}_2$  produced by metabolic breakdown of  $^{14}\text{C}$ -labelled palmitic acid contained in a 7H12 liquid medium in the presence or absence of specific drugs (Middlebrook et al., 1977; Siddiqi et al., 1981; Snider et al., 1981; Laszlo et al., 1983). The agreement between the conventional and the radiometric susceptibility testing assays varied from 96.4-98% with most results obtained within 7 days (Siddiqi et al., 1981; Laszlo et al., 1983). 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. Heifets and Isman modified the radiometric method by lowering the pH with addition of phosphoric acid at the same time as PZA but after the growing culture had reached exponential phase (Heifets & Isman, 1985). 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 (Heifets & Isman, 1985). The method described by Heifets and Isman was further modified and proved useful in facilitating the measurement of PZA susceptibility of *Mtb* complex by the radiometric method (Tarrand et al., 1986; Salfinger & Heifets, 1988).

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), which had been introduced for broth culture of mycobacteria species from clinical specimens without the use of radioactive materials (Chew et al., 1998; Heifets et al., 2000). 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 (Siddiqi, 2010). 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 (Woods, 2011). The MGIT 960 system is FDA-approved for susceptibility testing of *Mtb* complex against the first-line drugs including RIF (2  $\mu\text{g}/\text{ml}$ ), INH (0.4  $\mu\text{g}/\text{ml}$  and 0.1  $\mu\text{g}/\text{ml}$ ), and EMB (7.5  $\mu\text{g}/\text{ml}$  and 2.5  $\mu\text{g}/\text{ml}$ ), PYZ (100  $\mu\text{g}/\text{ml}$ ) and streptomycin (STR, 6.0  $\mu\text{g}/\text{ml}$  and 2.0  $\mu\text{g}/\text{ml}$ ) (CLSI, 2008). 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) (Ardito et al., 2001; Adjers-Koskela & Katila, 2003; Scarparo et al., 2004), except for EMB and STR which had agreement varying from less than 80% to 98% (Adjers-Koskela & Katila, 2003; Scarparo et al., 2004; Hall et al., 2006; Garrigo et al., 2007). 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 (Lin et al., 2009). 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 (Martin et al., 2008). In both studies, the agreement between the two methods for ethionamide was markedly lower (86-88%) than for the other drugs (Martin et al., 2008; Lin et al., 2009).

Another fully automated broth system that is FDA-approved for susceptibility testing of *Mtb* complex is the VersaTREK instrument (TREK Diagnostics, Cleveland, OH). The VersaTREK (formerly ESP culture system 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) (CLSI, 2008). 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 (Bergmann & Woods, 1998; 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, ETH, and STR showed results similar to the MGIT 960, with agreement > than 95% for all drugs except ETH and STR (agreement between 90-95%) for which the VersaTrek generally called susceptible organisms that were resistant (Bergmann & Woods, 1998), although only a few isolates were tested in the study. Similar results were obtained by other investigators, except for STR, which unlike the previous study, had an agreement of 99.7% with the BACTEC 460 method (Ruiz et al., 2000).

### 7.3 Molecular detection of resistance markers

Although conventional methods described above are still the main-stay in detection of drug resistance in most laboratories, 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.

The Genotype MTBDR<sub>plus</sub> (Hain Lifescience) is a commercial line probe assay developed for the detection of INH and RIF resistance in *Mtb* complex isolates and smear-positive specimens (Hillemann et al., 2007). Resistance to INH results from mutations in genes whose products are involved in the activation and binding of INH including the *katG*, the *inhA*, the *ahpC-oxyR*, *ahpC* and *ndh* genes, while resistance to RIF is due mainly to mutation in the *rpoB*

gene, which encodes the  $\beta$ -subunit of the DNA-dependent RNA polymerase, RIF binding target (Zhang & Yew, 2009).

The GenoType MTBDR*plus* assay detects the most common mutations found in the *rpoB*, *katG* and *inhA* genes, an improvement from other line-probe assays such as the INNO-LiPA Rif (Innogenetics) and the GenoType MTBDR (Hain Lifescience), which only target the *rpoB* mutation (INNO-LiPA Rif) or *rpoB* and *katG* mutations (GenoType MTBDR). Evaluation of the GenoType MTBDR*plus* assay by Hillemann et al (Hillemann et al., 2007) on clinical strains and smear-positive sputa revealed a detection rate of 98.7% (74/75) and 96.8% (30/31) of RIF resistance in clinical isolates and sputa specimens respectively. INH resistance was detected in 92% (69/75) and 90% (36/41) of clinical isolates and sputa specimens respectively. Similar detection rate were obtained by Barnard et al (Barnard et al., 2008) who tested 536 consecutive smear-positive sputum specimens with a sensitivity of 98.9% for detection of RIF resistance and 94.2% for the detection of INH resistance when compared to results obtained with conventional methods. Although the assay is limited to the detection of known mutations of RIF and INH, the high concordance rate with conventional methods and the rapid time to results makes the MTBDR*plus* assay a useful test for the management of multi-drug resistance tuberculosis.

Another version of the line probe assay, the GenoType MTBDR*sl* (second-line) (Hain Lifescience), was recently introduced for the detection of mutations in the *gyrA* gene, the 16S rRNA gene and the *embB* gene which confer resistance to fluoroquinolones, aminoglycosides and capreomycin, and ethambutol respectively (Hillemann et al., 2009). This assay shows variable performance characteristics when compared to phenotypic methods or sequencing, ranging from 75.6-90% for fluoroquinolones, 43-100% for aminoglycosides, 71.4-87.5% for capreomycin and suboptimal rate in all studies for ethambutol (38.5-64.2%) (Hillemann et al., 2009; Kiet et al., 2010; Huang et al., 2011; Kontsevaya et al., 2011).

Multiple home-brew PCR assays have been developed for the detection of the most common gene mutations conferring resistance to *Mtb* complex strains. A recent study reports the development of a multiplex real-time PCR assay to detect all known mutations in the *gyrA* gene responsible for conferring resistance to fluoroquinolones (Chakravorty et al., 2011). This assay is based on an asymmetrical PCR using sloppy molecular beacon probes that extends the entire quinolone resistance determining region (QRDR), a region of the *gyrA* gene containing most of the known mutations responsible for fluoroquinolones resistance (Takiff et al., 1994). This assay was 100% sensitive and 100% specific in detecting fluoroquinolones resistance in 92 clinical isolates of *Mtb* complex, when compared to sequencing.

As many as 21 mutations in the *katG* gene can cause decreased activity of INH against *Mtb* complex (Ando et al., 2010). Although several assays have focused on the S315T mutation of *KatG* (Mokrousov et al., 2002; Zhang et al., 2007; Tho et al., 2011), which is known to confer high-level resistance to INH and be present in as many as 90% of resistant *Mtb* isolates in Russia (Marttila et al., 1998; Mokrousov et al., 2002), the study by Ando and colleagues showed that other mutations in the *KatG* gene can cause high-level resistance and those should be included in molecular assays targeting the *katG* gene (Ando et al., 2010).

The utility of the assays is limited since they often target detection of resistance to one class of antimicrobial, depends on the available knowledge of current mutations conferring resistance, and as such, can only be use in conjunction with other assays. Several investigators have focused their efforts in developing assays similar to the MTBDR*plus* assay to include resistant marker to more than one class of antituberculosis drugs and for more than one mutation per gene target (Sekiguchi et al., 2007; Zhang et al., 2007; Ong et al., 2010; Pholwat et al., 2011).

Other molecular assays that have been developed for the detection of resistance markers have included locked nucleic acid probes (van Doorn et al., 2008) and multiplex PCR amplicon conformation analysis (Cheng et al., 2004), pyrosequencing (Marttila et al., 2009; Garza-Gonzalez et al., 2010; Halse et al., 2010), oligonucleotide microarray (Caoili et al., 2006), and mass spectrometry (Wang et al., 2011).

## 8. Future directions

Clinical diagnostics for *Mtb* continue to evolve (Wilson, 2011) and in some cases, the future may be at our fingertips (Van Rie et al., 2010). For example, the GeneXpert MTB/RIF PCR assay (Cepheid, Sunnyvale, CA) allows for the automated, direct detection of *Mtb* complex in respiratory specimens and it has been endorsed by the WHO for use in low-income countries (WHO, 2008). The Xpert assay provides excellent sensitivity and specificity from direct specimens while providing a same day turn-around time for results (Helb et al., 2010; Boehme et al., 2011; Marlowe et al., 2011; Rachow et al., 2011; Scott et al., 2011). In addition, the assay provides immediate information on RIF resistance and has recently been successfully evaluated using non-respiratory specimens (Ioannidis et al., 2011; Miller et al., 2011).

Older technologies utilized in new ways are also making inroads in *Mtb* diagnostics. The use of mass spectrometry for the identification of *Mtb* complex from culture isolates may replace current standards such as biochemical analysis, nucleic acid hybridization probes, and DNA sequencing due to the ability of mass spectrometry to rapidly and accurately identify *Mtb* in a cost-effective manner while minimizing technologist hands-on time and effort (Saleeb et al., 2011). Although still in its infancy in the diagnostic microbiology laboratory, this technology may also have utility in predicting drug resistance patterns and evaluating epidemiologic groups (Bouakaze et al., 2011; Massire et al., 2011; Schurch et al., 2011).

Finally, new technologies such as next generation sequencing are still largely utilized for research purposes in microbiology but some authors have suggested that there may come a time when application of this powerful technology will find a niche in the diagnostic microbiology laboratory (Ansorge, 2009; Rogers & Bruce, 2010; Engelthaler et al., 2011; Pallen & Loman, 2011).

## 9. References

- Abe, C., K. Hirano, et al. (1993). "Detection of Mycobacterium tuberculosis in clinical specimens by polymerase chain reaction and Gen-Probe Amplified Mycobacterium Tuberculosis Direct Test." *J Clin Microbiol* 31(12): 3270-3274.

- Adjers-Koskela, K. & M. L. Katila (2003). "Susceptibility testing with the manual mycobacteria growth indicator tube (MGIT) and the MGIT 960 system provides rapid and reliable verification of multidrug-resistant tuberculosis." *J Clin Microbiol* 41(3): 1235-1239.
- Ando, H., Y. Kondo, et al. (2010). "Identification of katG mutations associated with high-level isoniazid resistance in *Mycobacterium tuberculosis*." *Antimicrob Agents Chemother* 54(5): 1793-1799.
- Ansorge, W. J. (2009). "Next-generation DNA sequencing techniques." *New biotechnology* 25(4): 195-203.
- Ardito, F., B. Posteraro, et al. (2001). "Evaluation of BACTEC Mycobacteria Growth Indicator Tube (MGIT 960) automated system for drug susceptibility testing of *Mycobacterium tuberculosis*." *J Clin Microbiol* 39(12): 4440-4444.
- Attorri, S., S. Dunbar, et al. (2000). "Assessment of morphology for rapid presumptive identification of *Mycobacterium tuberculosis* and *Mycobacterium kansasii*." *Journal of clinical microbiology* 38(4): 1426-1429.
- Baba, K., S. Pathak, et al. (2008). "Real-time quantitative PCR in the diagnosis of tuberculosis in formalin-fixed paraffin-embedded pleural tissue in patients from a high HIV endemic area." *Diagn Mol Pathol* 17(2): 112-117.
- Barnard, M., H. Albert, et al. (2008). "Rapid molecular screening for multidrug-resistant tuberculosis in a high-volume public health laboratory in South Africa." *Am J Respir Crit Care Med* 177(7): 787-792.
- Beqaj, S. H., R. Flesher, et al. (2007). "Use of the real-time PCR assay in conjunction with MagNA Pure for the detection of mycobacterial DNA from fixed specimens." *Diagn Mol Pathol* 16(3): 169-173.
- Bergmann, J. S. & G. L. Woods (1996). "Clinical evaluation of the Roche AMPLICOR PCR *Mycobacterium tuberculosis* test for detection of *M. tuberculosis* in respiratory specimens." *J Clin Microbiol* 34(5): 1083-1085.
- Bergmann, J. S. & G. L. Woods (1998). "Evaluation of the ESP culture system II for testing susceptibilities of *Mycobacterium tuberculosis* isolates to four primary antituberculous drugs." *J Clin Microbiol* 36(10): 2940-2943.
- Bergmann, J. S. & G. L. Woods (1999). "Enhanced Amplified Mycobacterium Tuberculosis Direct Test for detection of *Mycobacterium tuberculosis* complex in positive BACTEC 12B broth cultures of respiratory specimens." *J Clin Microbiol* 37(6): 2099-2101.
- Bodmer, T., A. Gurtner, et al. (1997). "Evaluation of the COBAS AMPLICOR MTB system." *J Clin Microbiol* 35(6): 1604-1605.
- Boehme, C. C., M. P. Nicol, et al. (2011). "Feasibility, diagnostic accuracy, and effectiveness of decentralised use of the Xpert MTB/RIF test for diagnosis of tuberculosis and multidrug resistance: a multicentre implementation study." *Lancet* 377(9776): 1495-1505.
- Bouakaze, C., C. Keyser, et al. (2011). "Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry-Based Single Nucleotide Polymorphism Genotyping Assay Using iPLEX Gold Technology for Identification of *Mycobacterium tuberculosis* Complex Species and Lineages." *J Clin Microbiol* 49(9): 3292-3299.
- Brisson-Noel, A., C. Aznar, et al. (1991). "Diagnosis of tuberculosis by DNA amplification in clinical practice evaluation." *Lancet* 338(8763): 364-366.



- Bruijnesteijn Van Coppenraet, E. S., J. A. Lindeboom, et al. (2004). "Real-time PCR assay using fine-needle aspirates and tissue biopsy specimens for rapid diagnosis of mycobacterial lymphadenitis in children." *J Clin Microbiol* 42(6): 2644-2650.
- Butler, W. R. & L. S. Guthertz (2001). "Mycolic acid analysis by high-performance liquid chromatography for identification of Mycobacterium species." *Clin Microbiol Rev* 14(4): 704-726.
- Butler, W. R., K. C. Jost, Jr., et al. (1991). "Identification of mycobacteria by high-performance liquid chromatography." *J Clin Microbiol* 29(11): 2468-2472.
- Canetti, G., S. Froman, et al. (1963). "Mycobacteria: Laboratory Methods for Testing Drug Sensitivity and Resistance." *Bull World Health Organ* 29: 565-578.
- Caoili, J. C., A. Mayorova, et al. (2006). "Evaluation of the TB-Biochip oligonucleotide microarray system for rapid detection of rifampin resistance in Mycobacterium tuberculosis." *J Clin Microbiol* 44(7): 2378-2381.
- Caviedes, L. & D. A. Moore (2007). "Introducing MODS: a low-cost, low-tech tool for high-performance detection of tuberculosis and multidrug resistant tuberculosis." *Indian J Med Microbiol* 25(2): 87-88.
- Chakravorty, S., B. Aladegbami, et al. (2011). "Rapid detection of fluoroquinolone-resistant and heteroresistant Mycobacterium tuberculosis by use of sloppy molecular beacons and dual melting-temperature codes in a real-time PCR assay." *J Clin Microbiol* 49(3): 932-940.
- Chakravorty, S. & J. S. Tyagi (2005). "Novel multipurpose methodology for detection of mycobacteria in pulmonary and extrapulmonary specimens by smear microscopy, culture, and PCR." *J Clin Microbiol* 43(6): 2697-2702.
- Chedore, P. & F. B. Jamieson (1999). "Routine use of the Gen-Probe MTD2 amplification test for detection of Mycobacterium tuberculosis in clinical specimens in a large public health mycobacteriology laboratory." *Diagn Microbiol Infect Dis* 35(3): 185-191.
- Chegou, N. N., K. G. Hoek, et al. (2011). "Tuberculosis assays: past, present and future." *Expert review of anti-infective therapy* 9(4): 457-469.
- Cheng, A. F., W. W. Yew, et al. (2004). "Multiplex PCR amplicon conformation analysis for rapid detection of gyrA mutations in fluoroquinolone-resistant Mycobacterium tuberculosis clinical isolates." *Antimicrob Agents Chemother* 48(2): 596-601.
- Chew, W. K., R. M. Lasaitis, et al. (1998). "Clinical evaluation of the Mycobacteria Growth Indicator Tube (MGIT) compared with radiometric (Bactec) and solid media for isolation of Mycobacterium species." *J Med Microbiol* 47(9): 821-827.
- CLSI (2008). *Susceptibility Testing of Mycobacteria, Nocardiae, and Other Aerobic Actinomycetes; M24-A2, Approved Standard*. Wayne, PA, Clinical and Laboratory Standards Institute.
- Cloud, J. L., H. Neal, et al. (2002). "Identification of Mycobacterium spp. by using a commercial 16S ribosomal DNA sequencing kit and additional sequencing libraries." *J Clin Microbiol* 40(2): 400-406.
- Cruciani, M., C. Scarparo, et al. (2004). "Meta-analysis of BACTEC MGIT 960 and BACTEC 460 TB, with or without solid media, for detection of mycobacteria." *J Clin Microbiol* 42(5): 2321-2325.
- D'Amato, R. F., A. A. Wallman, et al. (1995). "Rapid diagnosis of pulmonary tuberculosis by using Roche AMPLICOR Mycobacterium tuberculosis PCR test." *J Clin Microbiol* 33(7): 1832-1834.

- de Oliveira, M. M., A. da Silva Rocha, et al. (2003). "Rapid detection of resistance against rifampicin in isolates of *Mycobacterium tuberculosis* from Brazilian patients using a reverse-phase hybridization assay." *J Microbiol Methods* 53(3): 335-342.
- Desmond, E. P. & K. Loretz (2001). "Use of the Gen-Probe amplified mycobacterium tuberculosis direct test for early detection of *Mycobacterium tuberculosis* in BACTEC 12B medium." *J Clin Microbiol* 39(5): 1993-1995.
- Devallois, A., E. Legrand, et al. (1996). "Evaluation of Amplicor MTB test as adjunct to smears and culture for direct detection of *Mycobacterium tuberculosis* in the French Caribbean." *J Clin Microbiol* 34(5): 1065-1068.
- Drake, T. A., J. A. Hindler, et al. (1987). "Rapid identification of *Mycobacterium avium* complex in culture using DNA probes." *J Clin Microbiol* 25(8): 1442-1445.
- Durst, H. D., M. Milano, et al. (1975). "Phenacyl esters of fatty acids via crown ether catalysts for enhanced ultraviolet detection in liquid chromatography." *Anal Chem* 47(11): 1797-1801.
- Eisenach, K. D., M. D. Sifford, et al. (1991). "Detection of *Mycobacterium tuberculosis* in sputum samples using a polymerase chain reaction." *Am Rev Respir Dis* 144(5): 1160-1163.
- Ellner, P. D., T. E. Kiehn, et al. (1988). "Rapid detection and identification of pathogenic mycobacteria by combining radiometric and nucleic acid probe methods." *J Clin Microbiol* 26(7): 1349-1352.
- Engelthaler, D. M., T. Chiller, et al. (2011). "Next-generation sequencing of *Coccidioides immitis* isolated during cluster investigation." *Emerg Infect Dis* 17(2): 227-232.
- Floyd, M. M., V. A. Silcox, et al. (1992). "Separation of *Mycobacterium bovis* BCG from *Mycobacterium tuberculosis* and *Mycobacterium bovis* by using high-performance liquid chromatography of mycolic acids." *J Clin Microbiol* 30(5): 1327-1330.
- Ford, E. G., S. J. Snead, et al. (1993). "Strains of *Mycobacterium terrae* complex which react with DNA probes for *M. tuberculosis* complex." *J Clin Microbiol* 31(10): 2805-2806.
- Franco-Alvarez de Luna, F., P. Ruiz, et al. (2006). "Evaluation of the GenoType Mycobacteria Direct assay for detection of *Mycobacterium tuberculosis* complex and four atypical mycobacterial species in clinical samples." *J Clin Microbiol* 44(8): 3025-3027.
- Gamboa, F., P. J. Cardona, et al. (1998). "Evaluation of a commercial probe assay for detection of rifampin resistance in *Mycobacterium tuberculosis* directly from respiratory and nonrespiratory clinical samples." *Eur J Clin Microbiol Infect Dis* 17(3): 189-192.
- Garrigo, M., L. M. Aragon, et al. (2007). "Multicenter laboratory evaluation of the MB/BacT *Mycobacterium* detection system and the BACTEC MGIT-960 system in comparison with the BACTEC 460TB system for susceptibility testing of *Mycobacterium tuberculosis*." *J Clin Microbiol* 45(6): 1766-1770.
- Garza-Gonzalez, E., G. M. Gonzalez, et al. (2010). "A pyrosequencing method for molecular monitoring of regions in the *inhA*, *ahpC* and *rpoB* genes of *Mycobacterium tuberculosis*." *Clin Microbiol Infect* 16(6): 607-612.
- Gen-Probe (2011). AccuProbe: *Mycobacterium Tuberculosis* Complex Culture Identification Test.
- Goto, M., S. Oka, et al. (1991). "Evaluation of acridinium-ester-labeled DNA probes for identification of *Mycobacterium tuberculosis* and *Mycobacterium avium*-

- Mycobacterium intracellulare complex in culture." *J Clin Microbiol* 29(11): 2473-2476.
- Guthertz, L. S., S. D. Lim, et al. (1993). "Curvilinear-gradient high-performance liquid chromatography for identification of mycobacteria." *J Clin Microbiol* 31(7): 1876-1881.
- Ha, D. T., N. T. Lan, et al. (2009). "Microscopic observation drug susceptibility assay (MODS) for early diagnosis of tuberculosis in children." *PloS one* 4(12): e8341.
- Hall, L., K. A. Doerr, et al. (2006). "Verification of antimicrobial susceptibility testing of Mycobacterium tuberculosis." *J Clin Microbiol* 44(5): 1921.
- Hall, L., K. A. Doerr, et al. (2003). "Evaluation of the MicroSeq system for identification of mycobacteria by 16S ribosomal DNA sequencing and its integration into a routine clinical mycobacteriology laboratory." *J Clin Microbiol* 41(4): 1447-1453.
- Halse, T. A., J. Edwards, et al. (2010). "Combined real-time PCR and rpoB gene pyrosequencing for rapid identification of Mycobacterium tuberculosis and determination of rifampin resistance directly in clinical specimens." *J Clin Microbiol* 48(4): 1182-1188.
- Halse, T. A., V. E. Escuyer, et al. (2011). "Evaluation of a single-tube multiplex real-time PCR for differentiation of members of the Mycobacterium tuberculosis complex in clinical specimens." *J Clin Microbiol* 49(7): 2562-2567.
- Heifets, L., T. Linder, et al. (2000). "Two liquid medium systems, mycobacteria growth indicator tube and MB redox tube, for Mycobacterium tuberculosis isolation from sputum specimens." *J Clin Microbiol* 38(3): 1227-1230.
- Heifets, L. B. & M. D. Iseman (1985). "Radiometric method for testing susceptibility of mycobacteria to pyrazinamide in 7H12 broth." *J Clin Microbiol* 21(2): 200-204.
- Helb, D., M. Jones, et al. (2010). "Rapid detection of Mycobacterium tuberculosis and rifampin resistance by use of on-demand, near-patient technology." *J Clin Microbiol* 48(1): 229-237.
- Hernandez, A., J. S. Bergmann, et al. (1997). "AMPLICOR MTB polymerase chain reaction test for identification of Mycobacterium tuberculosis in positive Difco ESP II broth cultures." *Diagn Microbiol Infect Dis* 27(1-2): 17-20.
- Hillemann, D., S. Rusch-Gerdes, et al. (2007). "Evaluation of the GenoType MTBDRplus assay for rifampin and isoniazid susceptibility testing of Mycobacterium tuberculosis strains and clinical specimens." *J Clin Microbiol* 45(8): 2635-2640.
- Hillemann, D., S. Rusch-Gerdes, et al. (2009). "Feasibility of the GenoType MTBDRsl assay for fluoroquinolone, amikacin-capreomycin, and ethambutol resistance testing of Mycobacterium tuberculosis strains and clinical specimens." *J Clin Microbiol* 47(6): 1767-1772.
- Hsiao, P. F., C. Y. Tzen, et al. (2003). "Polymerase chain reaction based detection of Mycobacterium tuberculosis in tissues showing granulomatous inflammation without demonstrable acid-fast bacilli." *Int J Dermatol* 42(4): 281-286.
- Huang, W. L., T. L. Chi, et al. (2011). "Performance assessment of the GenoType MTBDRsl test and DNA sequencing for detection of second-line and ethambutol drug resistance among patients infected with multidrug-resistant Mycobacterium tuberculosis." *J Clin Microbiol* 49(7): 2502-2508.

- Huard, R. C., L. C. Lazzarini, et al. (2003). "PCR-based method to differentiate the subspecies of the *Mycobacterium tuberculosis* complex on the basis of genomic deletions." *J Clin Microbiol* 41(4): 1637-1650.
- Ioannidis, P., D. Papaventsis, et al. (2011). "Cepheid GeneXpert MTB/RIF assay for *Mycobacterium tuberculosis* detection and rifampin resistance identification in patients with substantial clinical indications of tuberculosis and smear-negative microscopy results." *J Clin Microbiol* 49(8): 3068-3070.
- Johansen, I. S., B. Lundgren, et al. (2003). "Direct detection of multidrug-resistant *Mycobacterium tuberculosis* in clinical specimens in low- and high-incidence countries by line probe assay." *J Clin Microbiol* 41(9): 4454-4456.
- Julian, E., M. Roldan, et al. (2010). "Microscopic cords, a virulence-related characteristic of *Mycobacterium tuberculosis*, are also present in nonpathogenic mycobacteria." *J Bacteriol* 192(7): 1751-1760.
- Kapur, V., L. L. Li, et al. (1995). "Rapid *Mycobacterium* species assignment and unambiguous identification of mutations associated with antimicrobial resistance in *Mycobacterium tuberculosis* by automated DNA sequencing." *Arch Pathol Lab Med* 119(2): 131-138.
- Kasai, H., T. Ezaki, et al. (2000). "Differentiation of phylogenetically related slowly growing mycobacteria by their *gyrB* sequences." *J Clin Microbiol* 38(1): 301-308.
- Kellogg, J. A., D. A. Bankert, et al. (2001). "Application of the Sherlock Mycobacteria Identification System using high-performance liquid chromatography in a clinical laboratory." *J Clin Microbiol* 39(3): 964-970.
- Kiehn, T. E. & F. F. Edwards (1987). "Rapid identification using a specific DNA probe of *Mycobacterium avium* complex from patients with acquired immunodeficiency syndrome." *J Clin Microbiol* 25(8): 1551-1552.
- Kiet, V. S., N. T. Lan, et al. (2010). "Evaluation of the MTBDRsl test for detection of second-line-drug resistance in *Mycobacterium tuberculosis*." *J Clin Microbiol* 48(8): 2934-2939.
- Kim, B. J., S. H. Lee, et al. (1999). "Identification of mycobacterial species by comparative sequence analysis of the RNA polymerase gene (*rpoB*)." *J Clin Microbiol* 37(6): 1714-1720.
- Kiraz, N., I. Saglik, et al. (2010). "Evaluation of the GenoType Mycobacteria Direct assay for direct detection of the *Mycobacterium tuberculosis* complex obtained from sputum samples." *J Med Microbiol* 59(Pt 8): 930-934.
- Kirschner, P., B. Springer, et al. (1993). "Genotypic identification of mycobacteria by nucleic acid sequence determination: report of a 2-year experience in a clinical laboratory." *J Clin Microbiol* 31(11): 2882-2889.
- Koneman, E. W. (2006). Identification of Mycobacteria using Conventional Methods. *In Color Atlas and Textbook of Diagnostic Microbiology*. E. W. Koneman. Philadelphia, Lippincott: 1085-1090.
- Kontsevaya, I., S. Mironova, et al. (2011). "Evaluation of Two Molecular Assays for Rapid Detection of *Mycobacterium tuberculosis* Resistance to Fluoroquinolones in High-Tuberculosis and -Multidrug-Resistance Settings." *J Clin Microbiol* 49(8): 2832-2837.
- LaBombardi, V. J., R. Katariwala, et al. (2006). "The identification of mycobacteria from solid media and directly from VersaTREK Myco bottles using the Sherlock Mycobacteria Identification HPLC system." *Clin Microbiol Infect* 12(5): 478-481.

- Laszlo, A., P. Gill, et al. (1983). "Conventional and radiometric drug susceptibility testing of *Mycobacterium tuberculosis* complex." *J Clin Microbiol* 18(6): 1335-1339.
- Lebrun, L., F. Espinasse, et al. (1992). "Evaluation of nonradioactive DNA probes for identification of mycobacteria." *J Clin Microbiol* 30(9): 2476-2478.
- Lee, H. R., S. Y. Kim, et al. (2010). "Novel multiplex PCR using dual-priming oligonucleotides for detection and discrimination of the *Mycobacterium tuberculosis* complex and *M. bovis* BCG." *J Clin Microbiol* 48(12): 4612-4614.
- Lee, L. V. (2010). Conventional Biochemicals. *Clinical Microbiology Procedures Handbook*. L. S. Garcia. Washington, D.C, American Society for Microbiology. 2: 7.6.1.1.-7.6.3.3.
- Levidiotou, S., G. Vrioni, et al. (2003). "Four-year experience of use of the Cobas Amplicor system for rapid detection of *Mycobacterium tuberculosis* complex in respiratory and nonrespiratory specimens in Greece." *Eur J Clin Microbiol Infect Dis* 22(6): 349-356.
- Lim, S. D., J. Todd, et al. (1991). "Genotypic identification of pathogenic *Mycobacterium* species by using a nonradioactive oligonucleotide probe." *J Clin Microbiol* 29(6): 1276-1278.
- Limaye, K., S. Kanade, et al. (2010). "Utility of Microscopic Observation of Drug Susceptibility (MODS) assay for *Mycobacterium tuberculosis* in resource constrained settings." *The Indian journal of tuberculosis* 57(4): 207-212.
- Lin, S. Y., E. Desmond, et al. (2009). "Multicenter evaluation of Bactec MGIT 960 system for second-line drug susceptibility testing of *Mycobacterium tuberculosis* complex." *J Clin Microbiol* 47(11): 3630-3634.
- Liu, P. I., D. H. McGregor, et al. (1973). "Comparison of three culture media for isolation of *Mycobacterium tuberculosis*: a 6-year study." *Appl Microbiol* 26(6): 880-883.
- Luo, R. F., M. D. Scahill, et al. (2010). "Comparison of single-copy and multicopy real-time PCR targets for detection of *Mycobacterium tuberculosis* in paraffin-embedded tissue." *J Clin Microbiol* 48(7): 2569-2570.
- Makinen, J., M. Marjamaki, et al. (2006). "Evaluation of a novel strip test, GenoType *Mycobacterium* CM/AS, for species identification of mycobacterial cultures." *Clin Microbiol Infect* 12(5): 481-483.
- Marlowe, E. M., S. M. Novak-Weekley, et al. (2011). "Evaluation of the Cepheid Xpert MTB/RIF assay for direct detection of *Mycobacterium tuberculosis* complex in respiratory specimens." *J Clin Microbiol* 49(4): 1621-1623.
- Martin, A., D. Bombeeck, et al. (2011). "Evaluation of the BD MGIT TBc Identification Test (TBc ID), a rapid chromatographic immunoassay for the detection of *Mycobacterium tuberculosis* complex from liquid culture." *J Microbiol Meth* 84(2): 255-257.
- Martin, A., A. von Groll, et al. (2008). "Rapid detection of *Mycobacterium tuberculosis* resistance to second-line drugs by use of the manual mycobacterium growth indicator tube system." *J Clin Microbiol* 46(12): 3952-3956.
- Marttila, H. J., J. Makinen, et al. (2009). "Prospective evaluation of pyrosequencing for the rapid detection of isoniazid and rifampin resistance in clinical *Mycobacterium tuberculosis* isolates." *Eur J Clin Microbiol Infect Dis* 28(1): 33-38.
- Marttila, H. J., H. Soini, et al. (1998). "A Ser315Thr substitution in KatG is predominant in genetically heterogeneous multidrug-resistant *Mycobacterium tuberculosis* isolates

- originating from the St. Petersburg area in Russia." *Antimicrob Agents Chemother* 42(9): 2443-2445.
- Massire, C., C. A. Ivy, et al. (2011). "Simultaneous identification of mycobacterial isolates to the species level and determination of tuberculosis drug resistance by PCR followed by electrospray ionization mass spectrometry." *J Clin Microbiol* 49(3): 908-917.
- Middlebrook, G., Z. Reggiardo, et al. (1977). "Automatable radiometric detection of growth of *Mycobacterium tuberculosis* in selective media." *Am Rev Respir Dis* 115(6): 1066-1069.
- Miliner, R. A., K. D. Stottmeier, et al. (1969). "Formaldehyde: a photothermal activated toxic substance produced in Middlebrook 7H10 medium." *Am Rev Respir Dis* 99(4): 603-607.
- Miller, M. B., E. B. Popowitch, et al. (2011). "Performance of Xpert MTB/RIF RUO Assay and IS6110 Real-Time PCR for *Mycobacterium tuberculosis* Detection in Clinical Samples." *J Clin Microbiol*.
- Miller, N., T. Cleary, et al. (2002). "Rapid and specific detection of *Mycobacterium tuberculosis* from acid-fast bacillus smear-positive respiratory specimens and BacT/ALERT MP culture bottles by using fluorogenic probes and real-time PCR." *J Clin Microbiol* 40(11): 4143-4147.
- Miller, N., S. Infante, et al. (2000). "Evaluation of the LiPA MYCOBACTERIA assay for identification of mycobacterial species from BACTEC 12B bottles." *J Clin Microbiol* 38(5): 1915-1919.
- MMWR (1993). "Initial therapy for tuberculosis in the era of multidrug resistance. Recommendations of the Advisory Council for the Elimination of Tuberculosis." *MMWR Recomm Rep* 42(RR-7): 1-8.
- Mokrousov, I., O. Narvskaya, et al. (2002). "High prevalence of KatG Ser315Thr substitution among isoniazid-resistant *Mycobacterium tuberculosis* clinical isolates from northwestern Russia, 1996 to 2001." *Antimicrob Agents Chemother* 46(5): 1417-1424.
- Mokrousov, I., T. Otten, et al. (2002). "Detection of isoniazid-resistant *Mycobacterium tuberculosis* strains by a multiplex allele-specific PCR assay targeting katG codon 315 variation." *J Clin Microbiol* 40(7): 2509-2512.
- Moore, D. A. (2007). "Future prospects for the MODS assay in multidrug-resistant tuberculosis diagnosis." *Future Microbiol* 2(2): 97-101.
- Musial, C. E., L. S. Tice, et al. (1988). "Identification of mycobacteria from culture by using the Gen-Probe Rapid Diagnostic System for *Mycobacterium avium* complex and *Mycobacterium tuberculosis* complex." *J Clin Microbiol* 26(10): 2120-2123.
- Neonakis, I. K., Z. Gitti, et al. (2009). "Evaluation of GenoType mycobacteria direct assay in comparison with Gen-Probe *Mycobacterium tuberculosis* amplified direct test and GenoType MTBDRplus for direct detection of *Mycobacterium tuberculosis* complex in clinical samples." *J Clin Microbiol* 47(8): 2601-2603.
- Neonakis, I. K., Z. Gitti, et al. (2007). "Evaluation of the GenoType MTBC assay for differentiating 120 clinical *Mycobacterium tuberculosis* complex isolates." *Eur J Clin Microbiol Infect Dis* 26(2): 151-152.
- Nopvichai, C., A. Sanpavat, et al. (2009). "PCR detection of *Mycobacterium tuberculosis* in necrotising non-granulomatous lymphadenitis using formalin-fixed paraffin-embedded tissue: a study in Thai patients." *J Clin Pathol* 62(9): 812-815.

- Ong, D. C., W. C. Yam, et al. (2010). "Rapid detection of rifampicin- and isoniazid-resistant *Mycobacterium tuberculosis* by high-resolution melting analysis." *J Clin Microbiol* 48(4): 1047-1054.
- Pallen, M. J. & N. J. Loman (2011). "Are diagnostic and public health bacteriology ready to become branches of genomic medicine?" *Genome Med* 3(8): 53.
- Pao, C. C., T. S. Yen, et al. (1990). "Detection and identification of *Mycobacterium tuberculosis* by DNA amplification." *J Clin Microbiol* 28(9): 1877-1880.
- Parsons, L. M., R. Brosch, et al. (2002). "Rapid and simple approach for identification of *Mycobacterium tuberculosis* complex isolates by PCR-based genomic deletion analysis." *J Clin Microbiol* 40(7): 2339-2345.
- Patel, J. B., D. G. Leonard, et al. (2000). "Sequence-based identification of *Mycobacterium* species using the MicroSeq 500 16S rDNA bacterial identification system." *J Clin Microbiol* 38(1): 246-251.
- Perandin, F., G. Pinsi, et al. (2006). "Evaluation of INNO-LiPA assay for direct detection of mycobacteria in pulmonary and extrapulmonary specimens." *New Microbiol* 29(2): 133-138.
- Peterson, E. M., R. Lu, et al. (1989). "Direct identification of *Mycobacterium tuberculosis*, *Mycobacterium avium*, and *Mycobacterium intracellulare* from amplified primary cultures in BACTEC media using DNA probes." *J Clin Microbiol* 27(7): 1543-1547.
- Pfyffer, G. E., P. Kissling, et al. (1994). "Direct detection of *Mycobacterium tuberculosis* complex in respiratory specimens by a target-amplified test system." *J Clin Microbiol* 32(4): 918-923.
- Pfyffer, G. E. & F. Palicova (2011). *Mycobacterium: General Characteristics, Laboratory Detection, and Staining Procedures. Manual of Clinical Microbiology, 10th edition.* J. C. Versalovic, K.C.; Funke, G.; Jorgensen, J.H.; Landry, M.L.; and Warnock, D.W. Washington, DC, ASM Press: 472-502.
- Pholwat, S., S. Heysell, et al. (2011). "Rapid first- and second-line drug susceptibility assay for *Mycobacterium tuberculosis* isolates by use of quantitative PCR." *J Clin Microbiol* 49(1): 69-75.
- Piersimoni, C. & C. Scarparo (2003). "Relevance of commercial amplification methods for direct detection of *Mycobacterium tuberculosis* complex in clinical samples." *J Clin Microbiol* 41(12): 5355-5365.
- Pinsky, B. A. & N. Banaei (2008). "Multiplex real-time PCR assay for rapid identification of *Mycobacterium tuberculosis* complex members to the species level." *J Clin Microbiol* 46(7): 2241-2246.
- Rachow, A., A. Zumla, et al. (2011). "Rapid and accurate detection of *Mycobacterium tuberculosis* in sputum samples by Cepheid Xpert MTB/RIF assay--a clinical validation study." *PloS one* 6(6): e20458.
- Rebollo, M. J., R. San Juan Garrido, et al. (2006). "Blood and urine samples as useful sources for the direct detection of tuberculosis by polymerase chain reaction." *Diagn Microbiol Infect Dis* 56(2): 141-146.
- Richter, E., S. Rusch-Gerdes, et al. (2006). "Evaluation of the GenoType *Mycobacterium* Assay for identification of mycobacterial species from cultures." *J Clin Microbiol* 44(5): 1769-1775.

- Richter, E., M. Weizenegger, et al. (2003). "Evaluation of genotype MTBC assay for differentiation of clinical *Mycobacterium tuberculosis* complex isolates." *J Clin Microbiol* 41(6): 2672-2675.
- Ritis, K., S. Giaglis, et al. (2005). "Diagnostic usefulness of bone marrow aspiration material for the amplification of IS6110 insertion element in extrapulmonary tuberculosis: comparison of two PCR techniques." *Int J Tuberc Lung Dis* 9(4): 455-460.
- Rogall, T., T. Flohr, et al. (1990). "Differentiation of *Mycobacterium* species by direct sequencing of amplified DNA." *J Gen Microbiol* 136(9): 1915-1920.
- Rogers, G. B. & K. D. Bruce (2010). "Next-generation sequencing in the analysis of human microbiota: essential considerations for clinical application." *Mol Diagn Ther* 14(6): 343-350.
- Roth, A., M. Fischer, et al. (1998). "Differentiation of phylogenetically related slowly growing mycobacteria based on 16S-23S rRNA gene internal transcribed spacer sequences." *J Clin Microbiol* 36(1): 139-147.
- Ruiz, P., F. J. Zerolo, et al. (2000). "Comparison of susceptibility testing of *Mycobacterium tuberculosis* using the ESP culture system II with that using the BACTEC method." *J Clin Microbiol* 38(12): 4663-4664.
- Russo, C., E. Tortoli, et al. (2006). "Evaluation of the new GenoType *Mycobacterium* assay for identification of mycobacterial species." *J Clin Microbiol* 44(2): 334-339.
- Said, H. M., N. Ismail, et al. (2011). "Evaluation of TBc identification immunochromatographic assay for rapid identification of *Mycobacterium tuberculosis* complex in samples from broth cultures." *J Clin Microbiol* 49(5): 1939-1942.
- Saleeb, P. G., S. K. Drake, et al. (2011). "Identification of mycobacteria in solid-culture media by matrix-assisted laser desorption ionization-time of flight mass spectrometry." *J Clin Microbiol* 49(5): 1790-1794.
- Salfinger, M. & L. B. Heifets (1988). "Determination of pyrazinamide MICs for *Mycobacterium tuberculosis* at different pHs by the radiometric method." *Antimicrob Agents Chemother* 32(7): 1002-1004.
- Sankar, S., B. Balakrishnan, et al. (2010). "Comparative evaluation of nested PCR and conventional smear methods for the detection of *Mycobacterium tuberculosis* in sputum samples." *Mol Diagn Ther* 14(4): 223-227.
- Scarparo, C., P. Piccoli, et al. (2001). "Direct identification of mycobacteria from MB/BacT alert 3D bottles: comparative evaluation of two commercial probe assays." *J Clin Microbiol* 39(9): 3222-3227.
- Scarparo, C., P. Ricordi, et al. (2004). "Evaluation of the fully automated BACTEC MGIT 960 system for testing susceptibility of *Mycobacterium tuberculosis* to pyrazinamide, streptomycin, isoniazid, rifampin, and ethambutol and comparison with the radiometric BACTEC 460TB method." *J Clin Microbiol* 42(3): 1109-1114.
- Schirm, J., L. A. Oostendorp, et al. (1995). "Comparison of Amplicor, in-house PCR, and conventional culture for detection of *Mycobacterium tuberculosis* in clinical samples." *J Clin Microbiol* 33(12): 3221-3224.
- Schurch, A. C., K. Kremer, et al. (2011). "Mutations in the regulatory network underlie the recent clonal expansion of a dominant subclone of the *Mycobacterium tuberculosis* Beijing genotype." *Infect Genet Evol: journal of molecular epidemiology and evolutionary genetics in infectious diseases* 11(3): 587-597.

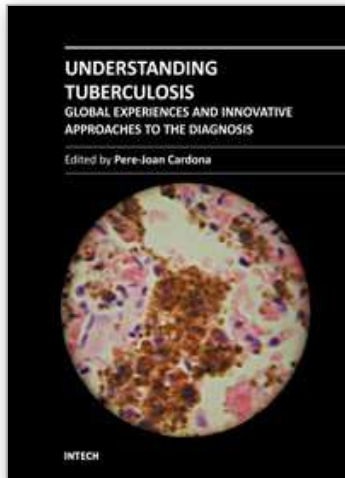


- Scott, L. E., K. McCarthy, et al. (2011). "Comparison of Xpert MTB/RIF with other nucleic acid technologies for diagnosing pulmonary tuberculosis in a high HIV prevalence setting: a prospective study." *PLoS medicine* 8(7): e1001061.
- Seagar, A. L., C. Prendergast, et al. (2008). "Evaluation of the GenoType Mycobacteria Direct assay for the simultaneous detection of the Mycobacterium tuberculosis complex and four atypical mycobacterial species in smear-positive respiratory specimens." *J Med Microbiol* 57(Pt 5): 605-611.
- Sekiguchi, J., T. Miyoshi-Akiyama, et al. (2007). "Detection of multidrug resistance in Mycobacterium tuberculosis." *J Clin Microbiol* 45(1): 179-192.
- Shah, S., A. Miller, et al. (1998). "Rapid diagnosis of tuberculosis in various biopsy and body fluid specimens by the AMPLICOR Mycobacterium tuberculosis polymerase chain reaction test." *Chest* 113(5): 1190-1194.
- Shrestha, N. K., M. J. Tuohy, et al. (2003). "Detection and differentiation of Mycobacterium tuberculosis and nontuberculous mycobacterial isolates by real-time PCR." *J Clin Microbiol* 41(11): 5121-5126.
- Siddiqi, S. (2010). BACTEC MGIT 960 SIRE-Nonradiometric Susceptibility Testing for Mycobacterium tuberculosis. *Clinical Microbiology Procedures Handbook*. L. S. Garcia. Washington, D.C, American Society for Microbiology. 2: 7.8.5.1-7.8.5.5.
- Siddiqi, S. H., J. P. Libonati, et al. (1981). "Evaluation of rapid radiometric method for drug susceptibility testing of Mycobacterium tuberculosis." *J Clin Microbiol* 13(5): 908-912.
- Sjoberg, U., M. Mecklenburg, et al. (1990). "Polymerase chain reaction for detection of Mycobacterium tuberculosis." *J Clin Microbiol* 28(10): 2200-2204.
- Smith, M. B., J. S. Bergmann, et al. (1997). "Detection of Mycobacterium tuberculosis in BACTEC 12B broth cultures by the Roche Amplicor PCR assay." *J Clin Microbiol* 35(4): 900-902.
- Snider, D. E., Jr., R. C. Good, et al. (1981). "Rapid drug-susceptibility testing of Mycobacterium tuberculosis." *Am Rev Respir Dis* 123(4 Pt 1): 402-406.
- Soini, H., E. C. Bottger, et al. (1994). "Identification of mycobacteria by PCR-based sequence determination of the 32-kilodalton protein gene." *J Clin Microbiol* 32(12): 2944-2947.
- Somoskovi, A., J. E. Hotaling, et al. (2001). "Lessons from a proficiency testing event for acid-fast microscopy." *Chest* 120(1): 250-257.
- Springer, B., L. Stockman, et al. (1996). "Two-laboratory collaborative study on identification of mycobacteria: molecular versus phenotypic methods." *J Clin Microbiol* 34(2): 296-303.
- Steingart, K. R., L. L. Flores, et al. (2011). "Commercial serological tests for the diagnosis of active pulmonary and extrapulmonary tuberculosis: an updated systematic review and meta-analysis." *PLoS medicine* 8(8): e1001062.
- Steingart, K. R., M. Henry, et al. (2006). "Fluorescence versus conventional sputum smear microscopy for tuberculosis: a systematic review." *The Lancet Infect Dis* 6(9): 570-581.
- Takiff, H. E., L. Salazar, et al. (1994). "Cloning and nucleotide sequence of Mycobacterium tuberculosis gyrA and gyrB genes and detection of quinolone resistance mutations." *Antimicrob Agents Chemother* 38(4): 773-780.

- Tarrand, J. J., A. D. Spicer, et al. (1986). "Evaluation of a radiometric method for pyrazinamide susceptibility testing of *Mycobacterium tuberculosis*." *Antimicrob Agents Chemother* 30(6): 852-855.
- Thibert, L. & S. Lapierre (1993). "Routine application of high-performance liquid chromatography for identification of mycobacteria." *J Clin Microbiol* 31(7): 1759-1763.
- Tho, D. Q., N. T. Lan, et al. (2011). "Multiplex allele-specific polymerase chain reaction for detection of isoniazid resistance in *Mycobacterium tuberculosis*." *Int J Tuberc Lung Dis* 15(6): 799-803.
- Torrea, G., P. Van de Perre, et al. (2005). "PCR-based detection of the *Mycobacterium tuberculosis* complex in urine of HIV-infected and uninfected pulmonary and extrapulmonary tuberculosis patients in Burkina Faso." *J Med Microbiol* 54(Pt 1): 39-44.
- Tortoli, E., A. Nanetti, et al. (2001). "Performance assessment of new multiplex probe assay for identification of mycobacteria." *J Clin Microbiol* 39(3): 1079-1084.
- Totsch, M., K. W. Schmid, et al. (1994). "Rapid detection of mycobacterial DNA in clinical samples by multiplex PCR." *Diagn Mol Pathol* 3(4): 260-264.
- Traore, H., A. van Deun, et al. (2006). "Direct detection of *Mycobacterium tuberculosis* complex DNA and rifampin resistance in clinical specimens from tuberculosis patients by line probe assay." *J Clin Microbiol* 44(12): 4384-4388.
- van Doorn, H. R., D. D. An, et al. (2008). "Fluoroquinolone resistance detection in *Mycobacterium tuberculosis* with locked nucleic acid probe real-time PCR." *Int J Tuberc Lung Dis* 12(7): 736-742.
- Van Rie, A., L. Page-Shipp, et al. (2010). "Xpert((R)) MTB/RIF for point-of-care diagnosis of TB in high-HIV burden, resource-limited countries: hype or hope?" *Expert review of molecular diagnostics* 10(7): 937-946.
- Vlasplolder, F., P. Singer, et al. (1995). "Diagnostic value of an amplification method (Gen-Probe) compared with that of culture for diagnosis of tuberculosis." *J Clin Microbiol* 33(10): 2699-2703.
- Wang, F., C. Massire, et al. (2011). "Molecular characterization of drug-resistant *Mycobacterium tuberculosis* isolates circulating in China by multilocus PCR and electrospray ionization mass spectrometry." *J Clin Microbiol* 49(7): 2719-2721.
- Welch, K., G. Brown, et al. (1995). "Performance of the Gen-Probe amplified *Mycobacterium tuberculosis* direct test in a laboratory that infrequently isolates *Mycobacterium tuberculosis*." *Diagn Microbiol Infect Dis* 22(3): 297-299.
- Wetmur, J. G. (1991). "DNA probes: applications of the principles of nucleic acid hybridization." *Crit Rev Biochem Mol Biol* 26(3-4): 227-259.
- WHO (2008). *New Laboratory Diagnostic Tools for Tuberculosis Control*. Geneva, Stop TB Partnership and World Health Organization.
- Wilson, M. L. (2011). "Recent advances in the laboratory detection of *Mycobacterium tuberculosis* complex and drug resistance." *Clin Infect Dis: an official publication of the Infectious Diseases Society of America* 52(11): 1350-1355.
- Witebsky, F. G. & P. Kruczak-Filipov (1996). "Identification of mycobacteria by conventional methods." *Clin Lab Med* 16(3): 569-601.

- Woo, P. C., J. L. Teng, et al. (2011). "Automated identification of medically important bacteria by 16S rRNA gene sequencing using a novel comprehensive database, 16SpathDB." *J Clin Microbiol* 49(5): 1799-1809.
- Woodley, C. L., M. M. Floyd, et al. (1992). "Evaluation of Syngene DNA-DNA probe assays for the identification of the Mycobacterium tuberculosis complex and the Mycobacterium avium complex." *Diagn Microbiol Infect Dis* 15(8): 657-662.
- Woods, G. L., J. S. Bergmann, et al. (2001). "Clinical Evaluation of the Gen-Probe amplified mycobacterium tuberculosis direct test for rapid detection of Mycobacterium tuberculosis in select nonrespiratory specimens." *J Clin Microbiol* 39(2): 747-749.
- Woods, G. L. L., S-Y. G., Desmond, E.P. (2011). Susceptibility Test Methods: Mycobacteria, Nocardia, and Other Actinomycetes. *Manual of Clinical Microbiology*. J. C. Versalovic, K.C.; Funke, G.; Jorgensen, J.H.; Landry, M.L.; and Warnock, D.W. Washington, D.C., ASM Press. 1: 1215-1238.
- Yu, M. C., H. Y. Chen, et al. (2011). "Evaluation of the rapid MGIT TBc identification test for culture confirmation of Mycobacterium tuberculosis complex strain detection." *J Clin Microbiol* 49(3): 802-807.
- Zhang, S. L., J. G. Shen, et al. (2007). "A novel genotypic test for rapid detection of multidrug-resistant Mycobacterium tuberculosis isolates by a multiplex probe array." *J Appl Microbiol* 103(4): 1262-1271.
- Zhang, Y. & W. W. Yew (2009). "Mechanisms of drug resistance in Mycobacterium tuberculosis." *Int J Tuberc Lung Dis* 13(11): 1320-1330.

IntechOpen



## **Understanding Tuberculosis - Global Experiences and Innovative Approaches to the Diagnosis**

Edited by Dr. Pere-Joan Cardona

ISBN 978-953-307-938-7

Hard cover, 552 pages

**Publisher** InTech

**Published online** 15, February, 2012

**Published in print edition** February, 2012

Mycobacterium tuberculosis is a disease that is transmitted through aerosol. This is the reason why it is estimated that a third of humankind is already infected by Mycobacterium tuberculosis. The vast majority of the infected do not know about their status. Mycobacterium tuberculosis is a silent pathogen, causing no symptomatology at all during the infection. In addition, infected people cannot cause further infections. Unfortunately, an estimated 10 per cent of the infected population has the probability to develop the disease, making it very difficult to eradicate. Once in this stage, the bacilli can be transmitted to other persons and the development of clinical symptoms is very progressive. Therefore the diagnosis, especially the discrimination between infection and disease, is a real challenge. In this book, we present the experience of worldwide specialists on the diagnosis, along with its lights and shadows.

### **How to reference**

In order to correctly reference this scholarly work, feel free to copy and paste the following:

N. Esther Babady and Nancy L. Wengenack (2012). Clinical Laboratory Diagnostics for Mycobacterium tuberculosis, Understanding Tuberculosis - Global Experiences and Innovative Approaches to the Diagnosis, Dr. Pere-Joan Cardona (Ed.), ISBN: 978-953-307-938-7, InTech, Available from:  
<http://www.intechopen.com/books/understanding-tuberculosis-global-experiences-and-innovative-approaches-to-the-diagnosis/clinical-laboratory-diagnostics-for-mycobacterium-tuberculosis>

**INTECH**  
open science | open minds

### **InTech Europe**

University Campus STeP Ri  
Slavka Krautzeka 83/A  
51000 Rijeka, Croatia  
Phone: +385 (51) 770 447  
Fax: +385 (51) 686 166  
[www.intechopen.com](http://www.intechopen.com)

### **InTech China**

Unit 405, Office Block, Hotel Equatorial Shanghai  
No.65, Yan An Road (West), Shanghai, 200040, China  
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元  
Phone: +86-21-62489820  
Fax: +86-21-62489821

© 2012 The Author(s). Licensee IntechOpen. This is an open access article distributed under the terms of the [Creative Commons Attribution 3.0 License](#), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

IntechOpen

IntechOpen