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## Biochemical Isolation and Identification of Mycobacteria

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### 1. Introduction

The identification of the species that comprise the *Mycobacterium* genus is one of the best documented examples of scientific development and technology. Approximately 150 species are currently recognized that, besides comprising a genus because of structural and biochemical similarities, exhibit a great variety of characteristics that permit the establishment of differential patterns in order to identify and characterize them, or to establish clusters or complexes. At the present time it is insufficient for the performance of tests that would determine if a given specie belongs to the *Mycobacterium* genus. There is clearly a pressing need to establish its membership in a group or complex, and to determine its specie and genetic characterization so as to permit a comparison of characteristics such as frequency and distribution at a global level.

Within this bacterial genus appear species that, for their public health impact, have been extensively studied; this is in contrast with those that have been recently identified, or of limited frequency, and of which only general characteristics are known.

These species share conventional microbiological methodologies that are based on the classification made by Dr. Runyon in designating them as photochromogens, scotochromogens, and non-chromogens, depending on their capacity, or inability, to synthesize pigments with or without light stimulus. Other characteristics, such as the speed of growth, have been used in differentiation through a wide variety of biochemical tests. The development of molecular biology contributes to the identification and characterization of species in only one test, thus requiring less time, and promotes the process of biosecurity as it does not necessitate a live microorganism beyond the initial steps of each experimental protocol. The advance of the science is evidenced through the adoption of recent methodologies that quantify volatile compounds through the odors of a patient or from certain clinical specimens; the use of nanoparticles and mass spectrometry appear to be the most promising developments for the very near future.

In conclusion, the analysis and documentation demonstrate that we do not yet possess the ideal methodology for the identification of mycobacteria. The most promising outlook for the identification of the species that comprise the *Mycobacterium* genus can be found in the recognition of its biochemical characteristics; the use of conventional methodologies; an

appreciation of the techniques of molecular biology in its contribution to science and the mitigation of human suffering, while being reasonable in its use; and an unceasing advancement toward the development and implementation of new methodologies produced through scientific, technical, and social advancement.

## 2. Phenotypic identification

The phenotypic identification of mycobacteria is based on the characteristics of the culture and biochemical features, and comprises a large variety of tests, some of which are conducted in order to locate the microorganisms into two large groups:

1. Species that comprise the *Mycobacterium tuberculosis* complex: to date, it has been reported that 9 species are included in this complex and, as the species are closely-related genetically, occasionally the phenotypic methodologies are unable to discriminate among some of the species, being able to determine only membership in the *M. tuberculosis* complex (Brosch et al 2002).

*M. tuberculosis*, as a causal agent of tuberculosis, is the species with the greatest world-wide distribution. The other species that comprise this complex are: *M. bovis*, *M. africanum*, *Bacille Calmette- Guérin* (BCG) vaccine strain, *M. microti*, *M. cannetti*, *M. caprae*, *M. pinnipedii*, and *M. mungi*.

The following tests and phenotypic observations identify this complex: velocity of growth; colony morphology; pigment production; niacin test; nitrate reduction; catalase activity; formation of the cord factor; urease test; pyrazinamidase test; growth in the presence of p-nitrobenzoic acid; and, growth in the presence of hydrazide of thiophene-2-carboxylic acid.

2. Non-Tuberculous Mycobacteria (NTM): reports indicate that about 140 species have been documented, with similar phenotypic characteristics; a large number of biochemical tests, in different culture media, are needed for identification and observation of the growth of the microorganism; various molecules are added to the culture to highlight the differential characteristics among the species that comprise the *Mycobacterium* genus. These tests should be conducted in conjunction with tests that identify the *M. tuberculosis* complex in order to guarantee an adequate analysis of identification.
3. *M. leprae*: is a non-cultivable species.

The fundamentals of each test used in the identification of mycobacteria are described as follows:

### 2.1 Characteristics deduced from the direct observation of a culture medium, or growth inhibition, and microscopic observation

#### 2.1.1 Velocity of growth

Mycobacteria are grouped according to velocity of growth as either slow or rapid growth. mycobacteria that develop colonies, visible by eyesight, in a culture medium in less than 7 days are classified as rapid growth; those requiring more than 7 days in which to form visible colonies are designated as slow growth. The rRNA comprises 80% of the RNA of mycobacteria and one unit of its DNA. The RNA produced depends upon the number of *rrn*

operons and the efficiency of their transcription. One or two *rrn* operons are found in mycobacteria, regulated by P1 and P2 promoters. The *rrnA* operon, located in the *murA* gene, is found in all species, including *M. tuberculosis* and *M. leprae*; but, in some species, like those of rapid growth, an additional *rrnB* operon is found, located in the *tyrS* gene (Arnvig et al. 2005, Verma et al 1999).

### 2.1.2 Colony morphology

Mycobacteria in culture generally appear as two types of colonies: rough or smooth, and with a shiny or opaque aspect (Tasso et al 2003).

### 2.1.3 Pigment

Some species of mycobacteria, classified as photochromogens, produce carotenoid pigment in the presence of light; the group designated as scotochromogenic develops yellow colonies independent of light stimulus; the non-chromogenic group does not produce pigment; their colonies appear with shades of pale yellow or cream, in the presence of light or in darkness, and the color does not intensify when exposed to light (Bernardelli et al 2007, Belén et al 2007).

### 2.1.4 Growth in presence of P-nitrobenzoic acid

This test contributes to the differentiation of species of the *M. tuberculosis* complex. P-nitrobenzoic acid inhibits the growth of: *M. tuberculosis*, *M. bovis*, *M. africanum*, and *M. microti* (Palomino et al 2007).

### 2.1.5 Growth in the presence of hydrazide of thiophene-thiophene-2-carboxilic acid

Permits the differentiation of *M. tuberculosis* that grows in the presence of the compound as opposed to other species such as *M. bovis*; it is sensitive to the compound when added to the Lowenstein Jensen (LJ) medium. Some species of NMT are positive for this test.

### 2.1.6 Growth in McConkey agar without violet crystal

Mycobacteria such as *M. fortuitum*, *M. chelonae*, and *M. abscessus* have the ability to grow in McConkey agar without violet crystal, as opposed to other mycobacteria such as *M. tuberculosis* and *M. bovis* that cannot grow in this medium.

### 2.1.7 Growth in 5% sodium chloride

Species of mycobacteria such as *M. fortuitum* show the capacity to grow in a LJ culture medium to which has been added 5% sodium chloride.

### 2.1.8 Growth in the presence of hydroxylamine

This test plays a very important role in determining the difference in capacity of growth of species such as *M. bovis* in LJ media supplemented with a concentration of 250 mg of hydroxylamine. NTM does not have this capability.

### 2.1.9 Growth in Sauton picric medium and Sauton agar with 0.2% picric acid

This test is fundamental to differentiate mycobacteria of slow and rapid growth. Mycobacteria of rapid growth, with the exception of *M. chelonae*, have the capacity to grow in this medium. Among mycobacteria of slow growth, *M. simie* is unable to grow in this medium.

### 2.1.10 Cord formation in acid-alcohol resistant bacilli

This involves demonstrating the capacity to form cords from aggregates of acid-alcohol resistant bacilli in which the longitudinal axis of the bacteria is parallel to the longitudinal axis of the cord (Tasso et al 2003). This characteristic is attributed to glycolipid trehalose 6,6-dimycolate or cord factor, which is composed of molecules of mycolic acids (Palomino et al 2007). The presence of the cord in Ziehl Neelsen (ZN) coloration from rough, non-chromogenic colonies is indicative of the presence of *M. tuberculosis*; on the contrary, the absence of a cord, or the uniform distribution of bacilli, is consistent with NTM (Tasso et al 2003).

## 2.2 Biochemical test of identification

### 2.2.1 Niacin test

Niacin is part of the energy metabolism of mycobacteria in redox reactions. All mycobacteria produce niacin, but *M. tuberculosis* accumulates it as a result of the major activity of Nicotinamide Adenine Dinucleotide and the inability to process the resulting Niacine (Palomino et al 2007, Cardoso et al 2004)). The test demonstrates the presence of cyanogen chloride formed through the reaction of chloramine T and potassium thiocyanate in the presence of citric acid. The cyanogen chloride breaks the pyridine ring of niacin, forming the aldehyde gamma-carboxyglutamate that binds with the aromatic amine producing a yellow color.

### 2.2.2 Nitrate reduction test

Nitrate reductase, an enzyme capable of reducing nitrates to nitrites, appears in the cellular membranes of mycobacteria; the bacteria can utilize this enzyme as a source of nitrogen (Palomino et al 2007). The test detects the presence of nitrate reductase in a medium that contains sodium nitrate. The enzyme reduces nitrate to nitrite that appears through the addition of sulfalinamide and dihydrochloride -N- naphthyl ethylenediamine, forming a complex of diazonium chloride with a fuchsia color ( Bernardelli et al 2007).

### 2.2.3 Catalase test

This is an antioxidant enzyme responsible for eliminating molecules of hydrogen peroxide from the cells that are produced during respiration. The reaction results in the release of water and free oxygen (Palomino et al 2007). Two classes of catalase, thermolabile and thermostable, appear in mycobacteria. In *M. tuberculosis* and *M. bovis* the enzymatic activity is inhibited at 68°C; this contrasts with the remaining species of mycobacteria that maintain enzymatic activity following the increase in temperature (Organización Panamericana de la Salud 2008). Oxygen in the form of bubbles, caused by the activity of the enzyme in a

solution of perhydrol 30% and Tween 80 at 10%, appears during the test. The height of the column of bubbles can be measured, thus quantifying the enzymatic activity (Bernardelli et al 2007).

#### 2.2.4 Urease test

The urease enzyme is coded by the *ureABC* genes and is able to hydrolyze urea, forming two molecules of ammonium; this is used by the mycobacteria in the process of biosynthesis (Palomino et al 2007). The determination is made by using a medium containing red phenol; production of ammonium results in alkalization of the medium with a change in color to fuchsia.

#### 2.2.5 Pyrazinamidase test

This is an intracellular enzyme codified by the *pncA* gene which is able to hydrolyze pyrazinamide (PZA) in pyrazinoic acid. Some strains present a mutation in the *pncA* gene that generates resistance to PZA, the principal mechanism of resistance in *M. tuberculosis* to this drug. The method of transport of PZA into *M. tuberculosis* is by passive diffusion, where it is converted into pyrazinoic acid through the action of the pyrazinamidase enzyme. Its usefulness as a test of identification is based on the differentiation of *M. tuberculosis* (positive pyrazinamidase) from other species of the *M. tuberculosis* complex (negative pyrazinamidase), with the exception of *M. canetti* which is also positive (Palomino et al 2007, Zhang et al 2003).

#### 2.2.6 Acid phosphatase

The acid phosphatase of some mycobacteria separates the free phenolphthalein from phenolphthalein diphosphate using as a substrate of the reaction the magnesium salt thymolphthalein monophosphate. The appearance of a red color is positive for acid phosphatase. The *M. tuberculosis* species is negative in this test.

#### 2.2.7 Hydrolysis of polyoxyethylene mono-oleate

This shows the capability of some species of mycobacteria to separate the oleic acid which is esterified in polyoxyethylene monoleate; it is better known commercially and in the laboratory environment as "Tween 80".

#### 2.2.8 Arylsulfatase test

The test is based on the capability of some species of mycobacteria, through the activity of arylsulfatase which acts on sulfate esters, to release its aryl radical. To confirm the activity of the enzyme a color development system should be attached, using phenolphthalein disulfate potassium in the culture medium, that is hydrolyzed by the enzyme producing free phenolphthalein; in the presence of an alkali, a red color appears when the test is positive. Examples of species positive for this test, after three days, are: *M. fortuitum* and *M. abscessus*; *M. gastri* presents a positive reaction only after two weeks.



### 2.2.9 Other phenotypic test

Tellurite reduction, oxygen preference, utilization of carbon sources, iron uptake, *B* galactosidase. (See figure 1).

## 3. Molecular identification of mycobacteria

The amazing development of biological molecular methods, in the last years, for identification of mycobacteria were implemented for clinical and research uses. Different molecular approaches developed in research laboratories became speedily in diagnostic test. The reference molecular method for identification of mycobacteria is the determination of sequences of 16S ribosomal DNA, due to this molecule is highly conserved. (Kirschner et al 1993). The 16S-23S internal transcribed spacer (ITS) sequencing is a supplement to 16S rRNA gene sequencing for identification of closely related species (Roth et al 1998). Others examples of DNA sequencing assays are, *gyrB*, and *rpoB* sequences. The DNA probes used in conjunction with culture methods was employed in many countries for identification of mycobacteria, examples of this methodologies are, AccuProbe, Line probe Assays, INNO LiPA Mycobacteria, GenoType Mycobacterium, and GenoType MTBC. Currently, the most reported methodology for mycobacterial identification is the polymerase chain reaction (PCR) restriction-enzyme analysis, this methods is based on the amplification of a 441.bp fragment of the *hsp65* gene by PCR and discriminate between all mycobacterial species (*M. tuberculosis* complex species presents the same patrons) (Telenty 1993, Castro et al 2007, 2010, Torres et al 2010).

Different molecular typing methods developed and implemented as a result of the accelerated development of molecular biology have been useful in various contexts of public health and scientific research, among which we can cite: the monitoring of individual clinical isolates of species belonging to the *M. tuberculosis* complex; studies of the transmission of tuberculosis (TB) in urban areas with low, medium, and high rates of transmission; analysis of recent cases of transmission, or TB cases caused by reactivation, reinfection, or mixed diseases; establishment of relationships between remote geographical areas; the influence of transcontinental movements of large numbers of tourists; determination of the geographical origin of isolates; epidemiological analysis of TB outbreaks in closed areas such as prisons, child-care centers, geriatric homes, and indigenous communities; the establishment of the epidemiological nexus of cases that are the product of contacts with a specific patient; and the identification of the index case through a combination of molecular typing techniques.

As typing techniques, both the spoligotyping and Mycobacterial Interspersed Repetitive Units (MIRU) methodologies are the most commonly used tools; they provide a high degree of reproducibility of results and every day are reported in more publications that attest to their versatility and wide use throughout the world; but it cannot be denied that other methodologies exist, which are described below, that have made great contributions in typing processes.

### 3.1 Typing methods

The *M. tuberculosis* genome is markedly homogeneous with 99.9% similarity and identity to the 16s rRNA sequence, with *M. africanum* and *M. mungi* being species genetically related to

the *M. tuberculosis* complex and presenting few mutations (Niobe-Eyangoh et al 2004, Brosh et al 2002). This characteristic has been employed in the development of methodologies that permit the differentiation of species of this complex. As an example of the contribution of this characteristic in the differential identification of *M. tuberculosis* isolates, the following techniques were initially employed: determination of unusual resistance to drugs; serotyping; multilocus enzyme electrophoresis; biochemical heterogeneity, and typing through the use of phages, with the last being the standard technique in use until the end of the 1980's, but presenting problems such as the low number of phage types identified and the labor intensity associated with this experimental protocol. (Jones et al 1975, 1978, Kalndtri et al 2005, Diaz et al 2003). The genome of the species that comprise the *M. tuberculosis* complex presents occasional recombinations caused by mobile fragments or sequences of DNA known as transposons; these are unstable elements potentially capable of causing rearrangements through transposition, deletion, inversion, and duplication, since they are insertion sequences. These mobile insertion elements are responsible for directing the generation of genetic polymorphisms which are commonly employed for discriminating among different isolates. In the studies of *M. tuberculosis* molecular epidemiology the most frequently used insertion sequence is *IS6110*, which is found exclusively in members of the *M. tuberculosis* complex, presenting between 0 – 25 copies which differ in position and number. The study of this element soon became an epidemiological milestone and a methodology of great clinical contribution (Coros et al 2008, Mostrom et al 2002).

In this context the clinical isolates of the *M. tuberculosis* complex that present the same genetic pattern are defined as groupings and it is believed that these cases could have been caused by a recent infection, being part of the same chain of transmission, as opposed to cases of family origin, nosocomial, reinfections, or reactivations. More recently, with genomic sequencing of various members of the *M. tuberculosis* complex, techniques have been employed such as: Sequencing of single nucleotide polymorphism (SNP) spoligotyping, Fluorescent amplified fragment length polymorphism (FAFLP), and, among others. (National TB Controllers Association 2004, Centers for Diseases Control and Prevention (CDC) 2006 and 2007, Caminero et al 2001, Shamputa et al 2007, Small et al 1994, Kamerbeek et al 1997, Kassama et al 2006, Gibson et al 2008, Kremer et al 2005, Orjuela et al 2010, Hernandez et al 2010).

Currently, the methods of molecular typing of members of the *M. tuberculosis* complex can be grouped as (Mostrom 2002, Narayabab et al 2004, Parra et al 2003, and Diaz 2003):

### 3.1.1 Genomic methods for DNA studies

Restriction Fragment Length Polymorphism *IS6110* (RFLP *IS6110*)  
Polymorphic GC rich sequence (PGRS)  
Analysis and pulsed-field gel electrophoresis (PFGE)

### 3.1.2 Methods based on the amplification of specific sequences of DNA using the PCR

Spoligotyping, Fast ligation mediated PCR (Flip), Double-Repetitive Element (DRE-PCR), MIRU, Ligation Mediated PCR (LM-PCR), Fluorescent Amplified Fragment Length Polymorphism (FAFLP), and Single Nucleotide Polymorphism (SNP). (Steinlein et al 2001,



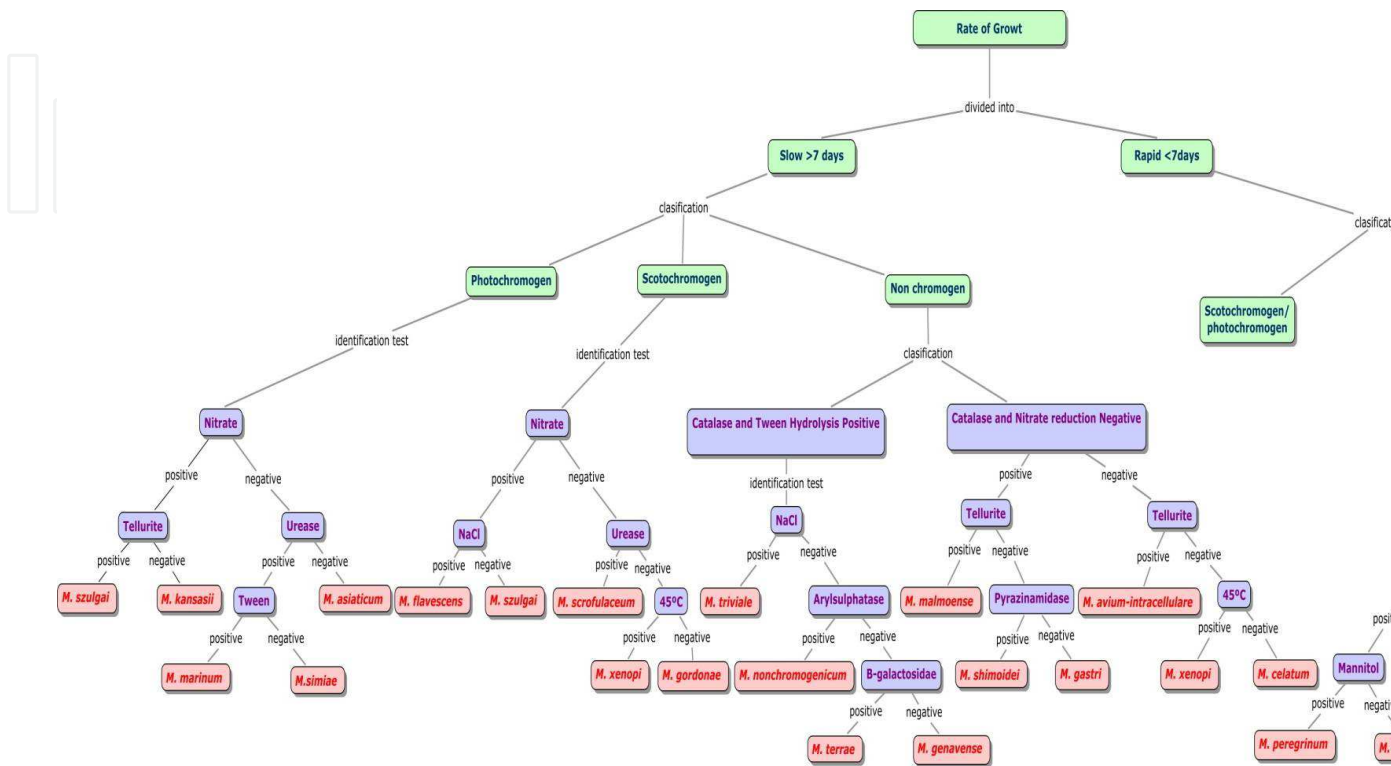


Fig. 1. Flowchart for phenotypic identification of mycobacteria.

Burgos et al 2004, Varela et al 2005, Reisig et al 2005, Kremer et al 2005, Kasama et al 2006, Ashworth et al 2008, Rozo et al 2010, Collins 2011, Li et al 2011, Blaschitz et al 2011).

### 3.1.3 Typing methods based on genomic DNA

#### 3.1.3.1 RFLP IS6110

Given the current knowledge of the *M. tuberculosis* genome H37Rv, the use of *IS6110* was, until recently, the most frequently reported method for discriminating among bacteriological clinical isolates of members of the *M. tuberculosis* complex; a reduction in its frequency of use is now observed given new methods such as those based on PCR. The RFLP *IS6110* technique has certain disadvantages such as: being labor-intensive; slow; requiring a large quantity of good-quality DNA (1-2 µg); limited ability to discriminate isolates with less than 6 copies of *IS6110* (< 6 bands in the RFLP pattern); and difficulty in comparing results obtained in different laboratories since an international database does not yet exist due to results being reported in patterns and not codes. This molecular marker possesses stability of approximately one to three years in patterns of RFLP *IS6110*, which is related to the number of copies present, since a larger number of copies imply a greater possibility of transposition, especially among isolates of 8 to 12 copies. In general, studies show a high stability in patterns of RFLP *IS6110* (Van et al 1991, Gordon et al 1999, Diel et al 2002, Mostrom et al 2002, Gopaul 2006).

#### 3.1.3.2 PGRS

This method has been of great usefulness in typing clinical isolates of members of the *M. tuberculosis* complex that present a low number of copies of the insertion sequence *IS6110*. These repetitive sequences are present in multiple copies through the mycobacterial genome; approximately 26 to 30 copies of these repetitive sequences are found per chromosome in members of the *M. tuberculosis* complex. It has a discriminating power very similar to that of RFLP, requiring DNA of good quality extracted from cultures of approximately 15 days growth in order to conduct the experimental protocol; several weeks are needed in order to obtain the pattern of each bacterial isolate in electrophoresis gel similar to that obtained through analysis of the *IS6110* sequence. It has a low discrimination in isolates that possess multiple copies of this genetic marker. (Burgos et al 2004, Rozo et al 2010).

#### 3.1.3.3 PFGE

This method possesses a high power of discrimination, requiring large concentrations of DNA with purity and quality; for its implementation it is necessary to make various adaptations in the experimental protocol according to the unique requirements of each laboratory. The introduction of PFGE has had great impact in the study and investigation of the mycobacterial genome, above all in the creation of genomic maps of closely related species such as those that comprise the *M. tuberculosis* complex, thus allowing for the establishment of differences through the identification of multiple rearrangements and of the nonrandom location of insertion elements (Wolfgang et al 1998).

### 3.1.4 Typing methods based on PCR

#### 3.1.4.1 Spoligotyping

The direct repetitive regions of the “Direct Repeat” sequence in members of the *M. tuberculosis* complex are composed of multiple direct variant repetitive sequences, each of which is comprised of direct repetitions of approximately 36 pairs of bases separated by unique spacer sequences of 35 to 41 pb, generating a large polymorphism which can be used in molecular epidemiological studies for the differentiation of species of the *M. tuberculosis* complex. The methodology of spoligotyping is based on PCR which is targeted on a small DR sequence sandwiched within a spacer region; a total of 94 sequences have been identified, of which 43 are generally used in the genotyping of isolates. All of these can be simultaneously amplified using a single primer set. The presence or absence of these sequences is determined by hybridization with a set of 43 oligonucleotides derived from *M. tuberculosis* H37Rv. This technique has been shown to be useful in the typing of clinical isolates of species of the *M. tuberculosis* complex, especially in those with less than 6 copies of *IS6110*, and in different clinical samples (Bauer 1999, Doroudchi et al 2000, Parra et al 2003).

#### 3.1.4.2 Flip

A rapid method with high reproducibility and great power of discrimination, based on the study of the *IS6110* sequence. In comparison with the RFLP *IS6110* methodology, this technique requires small quantities of DNA (1ng) or crude cell lysates. This methodology has limited discriminating power in isolates with less than six copies of the *IS6110* sequence; it gives results in less time than LM-PCR (Reisig et al 2005).

#### 3.1.4.3 DRE - PCR technique

A methodology with great power of discrimination that consists in the amplification of DNA segments located between the *IS6110* sequence or the polymorphic region rich in guanine cytosine (PGRS). The DRE-PCR method is based on the number of copies and the distance between the repetitive sequences of *IS6110* and PGRS; these distances vary among the different clinical isolates analyzed, and the variations allow a differentiation with respect to the size and number of the amplified fragments of DNA, producing a single pattern band for the different strains of *M. tuberculosis*. It is a method that can be completed using the primary culture of the microorganism and the results can be interpreted after eight hours of work. One of the limitations of this methodology has been the poor power of resolution of the bands procured (Mostron et al 2002).

#### 3.1.4.4 MIRU

A typing methodology based on the variable number tandem repeat (VNTR) or MIRU, with a power of resolution similar to that produced by RFLP *IS6110*, has been useful for typing of members of the *M. tuberculosis* complex. The MIRU are short elements of DNA (40 to 100 pb) found in tandem repetitions and dispersed in intergenic regions of the genome within members of the complex. Given its discriminating power, among the 41 sequences of MIRU that have been described, only 12 or 16 of them are frequently reported as genetic markers. The clinical isolates that are characterized by this genetic marker are designated with a 12-digit code corresponding to the number of repetitions in each MIRU locus, thereby forming the basis of a system that facilitates global comparison among laboratories (Supply et al 2000, 2001, Evans et al 2004, Narayanan et al 2004, Gibson et al 2005).

#### 3.1.4.5 LM-PCR

This is a highly reproducible technique that uses a primer directed toward a specific region of insertion sequence *IS6110*, and a second that is targeted to a linker ligated to restricted genomic DNA. This methodology requires small quantities of DNA and has a high power of discrimination (Prodhon et al 1997, Kremer et al 2005).

#### 3.1.4.6 FAFLP

The FAFLP is a complementary technique for the typing of *M. tuberculosis*, principally in clinical isolates with less than 6 copies of *IS6110*. It has the limitation of requiring the extraction of DNA from the microorganism in growth; subsequently, this is digested with *EcoRI* and *MseI* restriction enzymes, although some studies report using *BamHI* and *MspI* (Mortimer 2001, Sims et al 2002).

#### 3.1.4.7 SNP

This methodology is based on the premise that MicroRNAs (miRNA) are thought to play important roles in the pathogenesis of diseases. SNPs within miRNAs can change their characteristics via altering their target selection and/or expression, resulting in functional and/or phenotypic changes. This methodology has produced results similar to those obtained from RFLP *IS6110* and spoligotyping. This technique generates a binary code that permits the creation of a comparative database of different studies (Li et al 2011).

#### 3.1.4.8 Other methodologies

Other methodologies have been developed for the identification and study of mycobacteria such as “in house” PCR (Puerto et al 2007), but statistics have not been reported that would permit an extensive evaluation of these methodologies. There is a large variation among the reported studies which precludes any conclusions regarding their contributions.

### 4. Recent promising technologies for the detection of *M. tuberculosis*

#### 4.1 Urinary antigen detection

This method is based on the direct detection of lipoarabinomannan (LAM) in urine, when the mycobacteria are metabolically active; accordingly, this glycolipid is found in the urine of patients with active tuberculosis. This antigen can be detected through an ELISA test and the determination presents high ranges of sensitivity (from 38 to 51%) and of specificity, close to 89% (Daley et al 2009, Lawn et al 2009, Mutetwa et al 2009, Reither et al 2009, Deng et al 2011).

#### 4.2 Volatile markers

Through chromatographic techniques, various molecules or organic compounds have been identified in human clinical samples with the objective of showing their potential use as markers in the surveillance of illnesses. The volatile organic compounds (VOCs) patterns identified in the *M. tuberculosis* specie through sputum samples allow, through the use of an electronic nose based 14-sensor conducting polymer array, the performance of in vitro and in situ studies. Reports indicate that this methodology identifies 100% of positive *M. tuberculosis* cultures from among others and were able to discriminate between sputum

containing either *M. tuberculosis* alone, or a mixed infection. The technique is commonly referred to as “breath testing,” with the great advantage of not being an invasive procedure; in a small study it distinguished between patients with a positive sputum culture from those with a negative sputum culture (Pavlou et al 2000, 2004, Buszewski et al 2007, Phillips et al 2007).

#### 4.3 Bead-based methods

This method is based on the application of monoclonal antibodies to nano magnetic beads in order to identify the bacillus. Microsciences Medtech Ltd. (London, United Kingdom) developed a kit of beads coated with a chemical ligand that binds to mycobacteria present in the sputum of patients; the linkage is evident after coloring the preparation and observing it through a fluorescent microscope. Alternative diagnostic techniques have been developed using nanoparticles coupled to PCR, while other methodologies employ monoclonal anti-BCG antibodies. This methodology can be used for detection and identification of *M. tuberculosis* (Kluge et al 2008, Lee et al 2009).

#### 4.4 Simplified smart flow cytometry (S-FC)

Following the use by many researchers of flow cytometry to count CD4 T cells, the S-FC has been recently proposed for arriving at a rapid diagnosis of active TB among Human Immunodeficiency Virus (HIV) negative patients and those with the TB/HIV coinfection. The S-FC is considered to be of great use when a sputum sample cannot be obtained, or among patients with negative sputum, with the recommendation that it be combined with microbiological procedures (Castiblanco et al 2006, Breen et al 2007, Streitz et al 2007, Janossy et al 2008).

#### 4.5 Broad nucleic acid amplification-mass spectrometry

The integration of advances in various technologies such as the technique of analysis and separation of masses, bioinformatics, and ionization techniques linked to mass spectrometry, has facilitated their application in the identification and characterization of pathogens. The successful identification of *M. tuberculosis* complex through the pattern of its mycolic acids has been achieved through the use of Electrospray ionization-tandem mass spectrometry (ESI-MS). Other similar methodologies developed by Abbott Laboratories include the IBIS T5000 which is based upon the amplification of genome sequences of the pathogen linked to mass spectrometry (Ecker et al 2008, 2009, Ho et al 2010, Eshoo et al 2010, Grant et al 2010).

### 5. Detection of drug susceptibility testing

The increase in TB cases has been accompanied by a growth in the number of patients with isolates of resistant *M. tuberculosis*, thereby confirming TB as a grave, world-wide public health problem. The situation becomes even more complex with the association of *M. tuberculosis* resistant bacilli with HIV, making control more difficult (Bentwich et al 2000, Castiblanco et al 2006, Lemus et al 2004, and World Health Organization (WHO) 2009).

Multidrug-resistant TB (MDR TB), defined as a combined resistance to two first-line medications, isoniazid (INH) and rifampicin (RIF), attained a global level of 500,000 cases in



the year 2007 and is expected to continue to increase. According to WHO, 85% of these cases are concentrated in 27 countries. The clinical significance of resistance has been defined as the in vitro growth of the microorganism in the presence of the critical concentration of the drug used in treatment, which should be equal or superior to 1% of the growth of the microorganism in the absence of the drug. The critical concentration of a drug is the concentration that inhibits the growth of most wild type within a population without affecting the growth of resistant cells that might be present (Kent et al 1985, Woods et al 2007).

The problem of *M. tuberculosis* resistance appears when the resistant mutants that naturally occur in the microbial population are selected as result of inadequate administration of treatment (irregular administration of dosages, poor absorption, or an inadequate treatment plan) (Parson et al 2004).

When a diagnosis is made of a type of resistance to first-line medications such as INH, RIF, ethambutol (EMB), streptomycin (SM), and PZA, the WHO recommends treatment plans that should be adjusted based upon the pattern of resistance identified in the microorganism and each patient's particular state of health. Second-line medications include: aminoglycosides, cycloserine, capreomycin, fluoroquinolones, and para aminosalicylic acid. These treatment plans are prolonged in comparison with those of the first-line, with increased costs and toxicity, resulting in reduced patient compliance with treatment. When second-line treatment plans are not adequately administered, extensively drug-resistant TB (TB XDR) will probably appear, which is defined as TB MDR with greater resistance to any fluoroquinolone and to at least one of the three injectable medications (amikacin, kanamycin, or capreomycin) (Zumia et al 2001, Gillespie 2002, Raviglione et al 2007, 2004, 2003, 2006, 2007, 2008).

The treatment of TB, compared with that used to cure other infectious diseases, has some differences due to the particular physiological characteristics of *M. tuberculosis*, such as generation time and the capacity to enter into long periods of latency with minimal metabolic activity. In the process that arises from the interaction between the bacteria and the host's defenses, populations of bacilli are identified that are differentiated based upon location, metabolic activity, or velocity of multiplication. Characteristics, therefore, vary depending upon location. For example, when located within pulmonary cavities, they actively multiply; as opposed to being situated in the interior of macrophages where reduced pH and oxygen induce a physiology approximating a state of latency; or, if found within the site of necrosis, they are occasionally able to replicate. Due to the characteristics of the bacillus and the interaction with the host, various effective drugs are used, as appropriate, in the treatment of TB for each of the bacterial populations. Another fundamental aspect in the selection and study of drugs used in the treatment of TB involves the study of the cell structure of the microorganism, in which the most studied has been the bacterial cell wall complex, being hydrophobic, with little permeability, thereby precluding the entry of many therapeutic alternatives (Wayne 1974, Gillespie 2002, Coll 2003, Parson et al 2004, De Rossi et al 2006).

INH, RIF, SM, EMB and PZA are first-line medications for their efficacy in bactericidal or bacteriostatic activity, having less adverse effects than other antimycobacterial medications, as opposed to second-line drugs such as aminoglycosides, polypeptides, fluoroquinolones, ethionamide, cycloserine, and para aminosalicylic acid.

Some characteristics of first- and second-line medications used in the treatment of TB are described below in table 1:



*M. tuberculosis* acquires resistance to anti-TB agents through mutations in the genome due to such causes as previous exposure to some drug or rearrangements in restricted regions of the DNA of the bacillus; this generally produces resistance of the microorganism to only one medication. No plasmids or transposable elements are involved in this process. (Musser 1995, Quros et al 2001).

Drug		Nature of compound	Administration way	Mechanism of action	Daily dose	Microbial activity	Susceptible population	Pharmacological interactions	Side effects	7H10 solid medium
First line	INH	Synthetic	Oral	Inhibition of mycolic acid synthesis	5mg/kg	Bactericidal	Active and dormant bacilli	Fenitoina	Hepatotoxic, peripheral neuropathy, seizures	0.2; 1.0
	RIF	Semisynthetic	Oral	Inhibition of transcription by interacting with RNA polymerase	10mg/kg	Bactericidal	All population	Inhibe anticonceptivos orales, Quinidina.	Hepatotoxic, gastric intolerance, hypersensitivity	1.0
	EMB	Synthetic	Oral	Affects cell wall biosynthesis	20mg/kg	Bacteriostatic	Active bacilli	ND	Optic neuritis, alopecia, hypersensitivity	5.0; 10.0
	PZA	Synthetic	Oral	Affects the membrane potential for bioaccumulation	25mg/kg	Bactericidal	Persistent and dormant bacilli	ND	Arthritis, dermatitis, hepatotoxicity, gastrointestinal disorders	NA
	SM	Natural ( <i>Streptomyces griseus</i> )	Parenteral	Blocking RNA translation	15mg/kg	Bactericidal	Active bacilli	Neuromuscular blocking drugs, cross resistance with capreomycin	Nephrotoxicity, ototoxicity	2; 10
Second line	Amikacin	Semisynthetic	Parenteral	Inhibition of protein synthesis	15mg/kg	Bactericidal	Active bacilli	Neuromuscular blocking drugs	Lesion of cranial pair VIII, nephrotoxicity	4.0
	Capreomycin	Natural ( <i>Streptomyces capreolus</i> )	Parenteral	Inhibition of protein synthesis	15-30mg/kg until 1g	Bacteriostatic	Active bacilli	Neuromuscular blocking drugs	Lesion of cranial pair VIII, nephrotoxicity	10.0
	Ciprofloxacin	Synthetic molecule derived of carbonic acid	Oral and parenteral	Direct inhibition of DNA gyrase	150mg/kg until 12g	Bactericidal	Active bacilli	Cross-resistance with other quinolones	Gastrointestinal disorder; CNS; hypersensitivity	2.0
	Ethionamide	Synthetic	Oral	Inhibition of mycolic acid synthesis	15-30mg/kg until 1g	Bacteriostatic	Active and dormant bacilli	ND	Gastrointestinal disorder, hepatotoxic	5.0
	Kanamycin	Natural ( <i>Streptomyces kanamyceticus</i> )	Parenteral	Inhibition of protein synthesis	15-30mg/kg until 1g	Bactericidal	Active bacilli	Neuromuscular blocking drugs	Lesion of cranial pair VIII, nephrotoxicity	5.0
	Levofloxacin	Synthetic molecule derived of carbonic acid	Oral and parenteral	Direct inhibition of DNA gyrase	500mg	Bactericidal	Active and dormant bacilli	Cross-resistance with other quinolones	Tendinitis, neurotoxicity, intestinal disorders, rash, photosensitivity.	2.0
	Cycloserine	Natural ( <i>Streptomyces garryphalus</i> )	Oral	Inhibition of essential pyridoxal 5 phosphate (PLP)-dependent enzymes.	10-20mg/kg until 1g	Bacteriostatic and bactericidal	Active and dormant bacilli	Alcohol	Neurotoxicity, psychosis, seizures, depression	25-30
	Ofloxacin	Synthetic molecule derived of carbonic acid	Oral	Direct inhibition of DNA gyrase	600-800mg	Bactericidal	Active bacilli	Cross-resistance with other quinolones	Gastrointestinal disorder; CNS; hypersensitivity	2.0
	Para-aminosalicylic acid	Synthetic molecule derive from salicylic acid	Oral	Antagonist in the synthesis of folate from all mycobacteria	200mg/kg	Bactericidal	Active bacilli	ND	Gastrointestinal disorder; hepatotoxic	2.0

References: Svensson et al 1982,Pfyffer et al 1999, Caminero 2003, Coll 2003, Cohenet al 2003, ZH et al 2007, Boldú et al 2007, Loether et al 2010,Cremades et al 2011, Pholwat et al 2011, pholwat et al 2011

Table 1.

Determining the probability that *M. tuberculosis* will develop spontaneous resistance to two drugs used in treatment can be established through the sum of probabilities; for example, a probability exists of  $1 \times 10^6$ - $1 \times 10^8$  of a combined resistance to INH and RIF, being equal to a total probability of  $1 \times 10^{14}$ . In the process of the development of TB, the total number of possible bacilli in the patient, including cases of chronic cavitory disease, seldom results in this quantity of bacilli ( $10^{14}$ ). Consequently, the natural development of bacilli resistant to two medications is infrequent; therefore, it is assumed that in the majority of cases, this type of resistance is due to previous exposure to the medications (Long 2000).

Some data suggest that about 17% of new TB cases in a zone of high incidence evidence multiple infections, causing discordance and difficulty in the interpretation of tests and in patient management. The expansion of isolates of resistant *M. tuberculosis* is a concern and creates urgency in the implementation of effective diagnostic measures and control, focusing hopes on the development of a new vaccine, diagnostic methods, and effective alternative therapies (Zumia et al 2001, Richardson et al 2002, Gandhi et al 2006, Palomino et al 2007, WHO 2006, Raizada et al 2009).

## 5.1 Diagnostic methods of susceptibility

A great variety of methodologies exist for determining the susceptibility of *M. tuberculosis* to medications, among which include standard tests showing the growth of bacillus, such as the method of multiple proportions and BACTEC MIGHT 960. Other methods of great impact, and experiencing growing implementation at a global level, are those based on the amplification of nucleic acids through PCR, designated as genotypic methods (Wilson M. et al 2011). Some of these include microarrays of DNA, and solid- phase hybridization, among others. The aforementioned methodologies possess excellent percentages of sensitivity and specificity; however, they vary in the time required to obtain results, cost, and technical complexity, thus complicating their large-scale use, especially in countries with limited resources that employ conventional methods. Rapid phenotypic techniques have recently been endorsed by WHO, and they constitute good solutions for a fast and timely diagnosis of susceptibility compared with standard methodologies, and with the cost and infrastructure required for molecular techniques (Canetti et al 1963, 1969, Siddiqi et al 1981, Telenti et al 1993, Abate et al 1998, Palomino et al 2002, 2005, Mitchison 2005, Lakshmi et al 2006).

### 5.1.1 Phenotypic methods

Phenotypic methods are extensively used and a wide variety of studies are available that establish their principal advantages and disadvantages. They begin with the cultivation of isolates of *M. tuberculosis* obtained from clinical samples processed in an egg- or liquid-based culture medium, some of which can include antibiotics, which are used for direct determination (observation of the microorganism) or indirect (for example, through color reactions) of the growth of the microorganism and subsequent identification of the resistance of the microorganism through its growth in the presence of anti-TB drugs.

#### 5.1.1.1 Mycobacteria Growth indicator Tube (MGIT BACTEC)

Although various conventional phenotypic methods have been employed in the study of the susceptibility of *M. tuberculosis*, the most frequently reported, using solid media, are: the

multiple proportions method; the ratio of resistance and absolute concentrations; and the standard, automated radiometric BACTEC method that, as opposed to the others, uses a culture in liquid medium and the tube growth indicator method. Modifications to the BACTEC system have been adopted in order to avoid the use of radioactive material; these have consisted of using the principle of detecting oxygen consumption and recording the emission of fluorescent signals as a product of the reaction in each test, which is detected and controlled from the interior of the equipment coupled to software algorithms. The system interprets as resistant all cultures with growth equal or greater than that of the tube used as a control in the test (Pffyer et al 1997, 1999, Heifets et al 2000, Lin et al 2009, Yu et al 2011).

#### **5.1.1.2 The Trek Diagnostic System Inc**

This method measures the growth of mycobacteria through changes of pressure that occur in the space between the liquid medium and the cap of the tube where the reaction occurs. Results are obtained in approximately three days (Woods et al 2007).

#### **5.1.1.3 The MB/BacT Alert instrument (bioMérieux)**

This system emits results from an isolate of *M. tuberculosis* when the bottle that contains the microorganism and the drug is positive before the control tube. The result is obtained through measurement of carbon dioxide released to the medium, detected by a sensor located at the bottom of the bottle used in the test (Siddiqi et al 2006, Woods et al 2007, Lin et al 2009).

#### **5.1.1.4 The multiple proportions method**

This method determines the number of mutants resistant to each drug, establishing a relation between the numbers of bacilli that grow in a solid medium with antibiotic as compared with the number of bacilli that grow in a medium without antibiotic. The methodology used with solid medium evaluates the susceptibility of *M. tuberculosis* to first- and second-line medications. Frequently, the method employed uses the LJ culture medium and results are obtained in 21 days, or before, if there are indications of growth (Cannetti et al 1963, 1969, Heifets 2000, WHO 2004, Fisher 2002, Clinical and Laboratory Standards Institute 2003, Woods et al 2007, Pfyffer 1997, 2007).

Currently, BACTEC MGIT (version 960 or 320) and the multiple proportions method are considered as “gold standard” for the diagnosis of TB susceptibility to first- and second-line medications (Pino et al 1998).

As mentioned previously, phenotypic methods of relatively recent implementation now exist, and are noted for having succeeded in establishing a group of non-conventional phenotypic techniques that have been postulated as good diagnostic alternatives for the susceptibility of *M. tuberculosis* depending upon their performance given the particular situations in which they are used.

#### **5.1.1.5 The diagnosis of TB and drug resistance with mycobacteriophages**

This group of methodologies offers rapid results, low cost, and uses only viable cells, thereby differing from molecular methodologies. Two types of tests that use

mycobacteriophages have been documented: the phage amplified biological assay (phaB) and the luciferase reported phages (LRPs). Both methodologies study the capacity of a clinical isolate of *M. tuberculosis* to harbor an infectious mycobacteriophage and to permit its replication (Albert 2001, Butt et al 2004, Pai et al 2005, and Kalantri et al 2005).

#### 5.1.1.5.1 The PhaB

The PhaB determines the protection and amplification of a phage by mycobacteria in clinical specimens (Eltringham et al 1999). The methodology consists of:

1. Adding phages to the decontaminated clinical sample in order to infect the bacilli present in the samples;
2. Adding a viricidal solution (ammonium ferrous sulfates) that destroys the non-infective phages.
3. Initiating a lytic cycle of the bacilli for the replication of phages; this phase terminates with the release of the phages to the Medium.
4. Released phages added to a cell sensor (*M. smegmatis*) to produce plaques; the formation of plaques occurs if the sample is positive (Albert et al 2004).

#### 5.1.1.5.2 LRPs

The LRPs are phages harboring the firefly luciferase (*lux*) gene, which produces visible light when expressed in the presence of luciferin and cellular ATP (Hazbon 2004). The LRPs behave as molecular vectors of the *lux* gene that encodes for firefly luciferase in the interior of *M. tuberculosis*. Once this genetic information has been incorporated within the bacterial chromosome, it is expressed, and there is availability of ATP and luciferin, photon emissions are produced that are detected by a photographic film in a luminometer. The registering of light emitted indicates the viability of the bacilli (Riska et al 1997, 1998, 1999, Carriere et al 1997, 2003, Bardarov et al 2003).

#### 5.1.1.6 The E-Test

The E-Test determines the pattern of susceptibility of *M. tuberculosis* through growth inhibition halos around strips impregnated with known concentrations of different drugs. Solid medium is used in Middlebrook 7H10 agar plates; the microorganism to be studied should be viable and concentrated in the solution that will be used for inoculation of the medium (Heifets et al 1999, Djiba et al 2004).

#### 5.1.1.7 The colorimetric methods

These methodologies are simple and coupled to oxidation-reduction reactions that employ different indicators. Alamar blue salt is used in the case of REMA and MABA, and bromide 3 (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium, or MTT in the case of TEMA. Fundamentally, the result is obtained through observing a change of color, proportional to the number of viable mycobacteria in the test. TEMA was implemented in 1983 for the study of cellular proliferation and was subsequently utilized to evaluate the viability of microorganisms such as *Staphylococcus aureus* and *Listeria monocytogenes*; it has been extensively used in the study of *M. tuberculosis* resistance. The resulting color in this methodology changes from yellow to purple from the production of insoluble crystals of formazan that can be quantified by spectrophotometry, or a qualitative interpretation can be made by observing the change of color. The methodology provides results within 7 to 9 days

in the model proposed by Abate and Mishana for the rapid detection of RIF resistance. The advances of this methodology have included the study of clinical samples applied directly to the test, reporting good values of sensitivity and specificity, close to 100%, for sputum samples. This test permits the determination of the susceptibility of *M. tuberculosis* to first- and second-line drugs in establishing the diagnosis of MDR and XDR TB (Mossmann 1983, Abate et al 1998, Palomino et al 1999, 2002, Morcillo et al 2004, WoldeMeskel et al 2005, Montoro et al 2005, Palomino et al 2007, CDC 2007). The REMA test employs resazurin as an indicator, with an increase in the production of NADPH/NADP, FADH/FAD, FMNH/FMN, and NADH/NAD during multiplication of the bacilli to be studied. The viability of the microorganism is evidenced by the change from blue to pink in the indicator color. This test shows values of sensitivity and specificity exceeding 90% in the evaluation of drugs such as RIF and INH, as well as for second-line drugs. Some reports indicate difficulty in the interpretation of results due to intermediate tones in the change of indicator color (BioSource International, O'Brien et al 2000, Maeda et al 2001, Palomino et al 2002, Martin et al 2003, Montoro et al 2005, Rivoire et al 2007).

#### **5.1.1.8 Microscopic-observation drug-susceptibility (MODS)**

The MODS are used in various countries with TB MDR problems and permit the rapid observation of the growth of *M. tuberculosis* in culture media with the different first- and second-line drugs employed in the treatment of TB. One of those methods is MODS, that uses an inverted microscope to view the growth of the microorganism. Other methods include a thin layer of agar (TLA) and the HSTB agar method, that use special, transparent, solid culture media, permitting the early observation of the growth of the microorganism with the aid of a conventional microscope (Caviedes et al 2000, Heifets et al 2003, Mejia et al 2004, Robledo et al 2008).

#### **5.1.1.9 The Nitrate Reductase Assay (NRA) or Griess Method**

The NRA is based upon the characteristic expressed by mycobacteria to reduce nitrate to nitrite, is a rapid method used in the TB control programs of various countries for the evaluation of the susceptibility of *M. tuberculosis*; this is coupled to processes that permit observation of color change. The test employs the LJ solid medium (Angeby et al 2002, lemus et al 2006, Palomino et al 2007).

### **5.1.2 Genotypic methods**

The genotypic molecular methods for the diagnosis of the susceptibility of *M. tuberculosis* to drugs used in the treatment of TB are based on genetic determinants of resistance, requiring for its development the amplification of specific segments of DNA, and the identification of point mutations in the amplified products, thereby permitting the detection of resistance to medications (Cockerill et al 1999, Telenti et al 1993, Palomino 2005, Fluit et al 2011). Compared to phenotypic methods, these methodologies have the advantage of not requiring the previous growth of the microorganism, with results being obtained in a short time (24 to 48 hours). The following is a description of some methods:

#### **5.1.2.1 DNA sequencing**

This methodology is the best and most accurate method for the detection of previously known mutations and for the identification of new mutations. It is the "gold standard"



method. It is very effective for detecting mutations to RIF in *M. tuberculosis* by amplification of a single target of study in contrast to studies of various genes involved in resistance.

#### 5.1.2.2 DNA microarrays

This methodology is based on hybridization and allow for the simultaneous analysis of a large quantity of gene segments involved in resistance. The segments amplified by PCR are marked with a fluorescent substance and hybridized with probes attached to a solid phase. Consistent results have been reported for this methodology in the study of resistance to RIF (*rpoB* gene) and genes implicated in resistance to other drugs such as: *KatG*, *inhA*, *rpsL*, and *gyrA*. The wide-scale implementation of this methodology has been limited due to the required technical expertise of the operator and the sophisticated equipment. The QIAplex test (Qiagen) system has been recently developed which detects 24 mutations in the *katG*, *inhA*, *rpoB*, *rrs*, *rpsL*, and *embB* genes (Gegia et al 2008).

#### 5.1.2.3 The Line Probes Assay (LPA)

This method is employed to identify *M. tuberculosis* and determine resistance to RIF, identifying mutations in a specific region of the *rpoB* gene through amplification of this segment using PCR-based reverse hybridization, and using biotin in the test. These labeled products of PCR hybridize with immobilized probes coupling to a color development system that permits the subsequent analysis of resulting color patterns. This test detects four of the most frequent mutations in the *rpoB* gene arguing that 75% of the clinical isolates resistant to RIF have one of these four mutations. The method can be developed from clinical samples with results obtained in 48 hours or less (De Beenhouwer et al 1995, Somoskovi et al 2003, Ramaswamy et al 1998, WHO 2007, Ando et al 2011).

#### 5.1.2.4 Single-strand conformation polymorphism (SSCP)

This methodology detects specific mutations in the region of DNA under study, through the difference in patterns of movement of polyacrylamide gels. The region of the gene involved in resistance should be amplified by PCR and then denatured until two completely separated DNA strands are obtained. These denatured strands migrate by electrophoresis permitting the detection of mutated sequences by the migration pattern, as compared with that which has been established for the wild strain. This methodology has been employed in studies of resistance to RIF; it has not been practical for the investigation of other drugs as it requires the study of various genes involved in resistance to those medications (Kim et al 1997).

#### 5.1.2.5 Real - time PCR or molecular beacons

This methodology is reported as the most rapid methodology among those most frequently used. This technique includes the use of a probe that is designed in the form of a loop that possesses a sequence that is complementary with the sequence of the gene under study, and with the appearance of a fluorescent moiety in one of its extremities. When the probe locates the complementary sequence, hybridization occurs with the emission of fluorescence; the signal is detected and monitored by the associated system. This methodology is highly sensitive and specific; only one change in the sequence under study prevents hybridization, allowing the identification of point mutations. This is a closed system; therefore, minimizing the possibilities of amplification of contaminants. For the study of resistance to RIF, a set of



five beacons has been designed, which includes the study of the entire *rpoB* region in only one test. Results are provided in three hours using this methodology, detecting a minimum of two bacilli present in the sample. The test includes the study of genes such as *katG*, *inhA*, *oxyR-ahpC*, and *kasA* (Cockeril 1999, Fluid et al 2001, Piatek et al 1998, 2000, Torres et al 2000, De Viedman 2003).

#### 5.1.2.6 Fluorescence resonance energy transfer (FRET) probes

The FRET is a widely-studied, well-established methodology referred to as “light cycler probes.” It is highly specific in identifying mutations in real-time PCR tests. The test permits the detection of RIF and INH resistance in less than two hours with the advantage of facilitating the study of large gene segments involved in resistance (Garcia et al 2002, Torres et al 2003).

#### 5.1.2.7 Other methodologies

Other methodologies have been developed for the study of resistance to drugs used in the treatment of TB such as “in house” PCR, but statistics have not been reported that would permit an extensive evaluation of these methodologies. There is a large variation among the reported studies which precludes any conclusions regarding their contributions.

#### 5.1.2.8 Amplification refractory mutation system (ARMS)

This methodology uses a set of primers, one of which should hybridize with the mutation site; in the absence of this, there is not a place for the amplification of the gene involved in resistance; therefore, the band of the expected product is not observed in the electrophoresis gel. This methodology is used for the study of mutations in the *rpoB*, *katG*, and *embB* genes with good results (Mokrousov et al 2002, Fan et al 2003).

#### 5.1.2.9 Branch migration inhibition (BMI)

The BMI requires two reactions from PCR, one using DNA from a known strain, and the other using DNA which is the object of study, using a primer labeled with digoxigenin or biotin. This test has been used to study resistance to RIF and PZA; a disadvantage is the identification of silent mutations that conflict with findings reported by phenotypic methods (Lishanski et al 2000, Lyiu et al 2000).

## 6. Conclusion

The implications of the processes in which mycobacteria participate are diverse and documented but there is a lack of knowledge concerning many of their characteristics. Health, environment, industry, and research are involved with these mycobacterial species in order to advance or improve their processes, verify their findings, or confirm their suspicions. In each of these fields the principal objectives are the detection, identification, and characterization of the species of the *Mycobacterium* genus; only the knowledge of its biological characteristics together with the availability of methods for its study can help us to achieve a complete documentation. The disciplined, periodic and timely revision of the biology, tendencies, innovations, and discoveries regarding this microbial genus should prove to be the best allies of academics, researchers and the general community in addressing any situation that involves mycobacteria in its processes.

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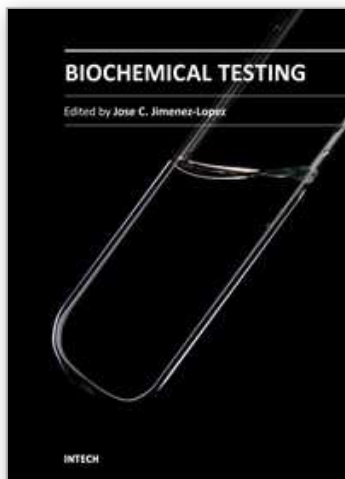
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Biochemical testing necessitates the determination of different parameters, and the identification of the main biological chemical compounds, by using molecular and biochemical tools. The purpose of this book is to introduce a variety of methods and tools to isolate and identify unknown bacteria through biochemical and molecular differences, based on characteristic gene sequences. Furthermore, molecular tools involving DNA sequencing, and biochemical tools based in enzymatic reactions and proteins reactivity, will serve to identify genetically modified organisms in agriculture, as well as for food preservation and healthcare, and improvement through natural products utilization, vaccination and prophylactic treatments, and drugs testing in medical trials.

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