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Conventional and Molecular Diagnosis of Drug-Sensitive and Drug-Resistant Pulmonary Tuberculosis

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Abstract

Tuberculosis is a transmissible disease, which is primarily caused by the bacteria *Mycobacterium tuberculosis* and by other *Mycobacterium* species, forming the *Mycobacterium tuberculosis* complex. Until the end of the 20th Century, most cases of pulmonary tuberculosis were considered curable. Nevertheless, the rising of tuberculosis resistant to first- and second-line anti-tuberculous drugs is threatening the world's tuberculosis control programs. Due to this fact, the World Health Organization and other public health institutions recommended applying the conventional methods, affordable by low-income countries, to diagnose tuberculosis and to develop faster and more sensitive and specific methods to identify *M. tuberculosis* and determine their condition of anti-tuberculous drug resistance or drug sensitivity. In this chapter, we mention the most used conventional and molecular methods designed to identify *M. tuberculosis* and to determine their drug sensitivity or drug resistance. We also briefly describe the fundamentals of methods and its advantages and limitations.

Keywords: TB diagnosis, conventional and molecular methods, resistance detection

1. Introduction

Tuberculosis (TB) is a transmissible disease, which is mainly caused by the bacteria *Mycobacterium tuberculosis* (MTB) [1] and, in a minor grade, by other *Mycobacterium* species, which form the *Mycobacterium tuberculosis* complex (MTBC), where *M. canettii*, *M. tuberculosis*, *M. africanum*, *M. microti*, *M. pinnipedii*, *M. caprae*, *M. bovis* and *M. bovis Bacillus Calmette-Guérin* (BCG) are included [2, 3]. Until the last third of the 20th Century, most cases of active pulmonary tuberculosis (PTB)

were considered curable, while the patient stringently followed an appropriate treatment. Otherwise, eventually, patients could no longer respond to their treatment. Due to this circumstance, in 1993, the World Health Organization (WHO) recommended a global TB control strategy, called directly observed treatment, short-course (DOTS) [4]. According to the WHO, DOTS is *“the most cost-effective way to stop the spread of TB in communities with a high incidence is by curing it.”* This asseveration is correct if, and only if, the MTB strain is sensitive to the first-line anti-TB drugs (isoniazid, rifampin, ethambutol and pyrazinamide). The TB epidemiological problem arose when drug-resistant (DR) MTB strains appeared in the world scenario. Consequently, DOTS, in the cases of DR-TB, was no longer effective in a considerable number of TB patients, especially when the causal agents were resistant to rifampin and isoniazid (the most effective anti-TB drugs). This kind of DR-MTB strains is called multidrug-resistant MTB (MDR-MTB). Faced with that situation, the WHO recommended to all government countries applying the DOTS-plus strategy. The DOTS-plus regimen includes two or more drugs to which the isolate is susceptible, including one drug given parenterally for 6 months or more to patients having MDR-TB [5]. Over the years, the TB epidemiological situation has turned even worst. In 2006, the WHO declared a new modality of DR-TB as an emergency. This modality of TB was called extensive drug-resistant TB strains (XDR-TB), which was considered virtually untreatable with conventional drugs, 11 years ago. XDR-TB is an MDR-TB, which is also resistant to three or more second-line drugs [6]. Nevertheless, even for these extreme cases of DR-TB, there is a hope to find a cure, considering that, lastly, new drugs are under development [6]. Therefore, it is mandatory to identify the causal agent of TB and to determine if MTB bacteria are sensitive to first-line anti-tubercular drugs or resistant; and, in the second case, it is also necessary to define the drug resistance profile of each particular clinical MTB isolate.

In spite of the scientific advances—described below—to identify and characterize MTB isolates, a great challenge is facing, mainly by the developing countries, to train and hire appropriate personnel, as well as to implement adequate diagnosis laboratories. Many countries have joined the efforts of the WHO and other agencies to improve identification and treatment of cases of pulmonary TB, like the Stop Tuberculosis Partnership [7]. However, it is essential to increase the efforts of all involved, scientists, medical professionals, governments, altruist institutions and the general population to contain and control TB. The presence of XDR-TB is an uncontrolled threat, which could effectively become a pandemic with catastrophic consequences.

In this chapter, we describe those conventional and modern methods more often used to diagnose, isolate characterize and determine drug resistance profiles of MTB isolates and discuss the advantages and limitations of each of them.

2. Conventional TB diagnosis methods

2.1. Clinical diagnosis of active TB

A person is suspicious of having pulmonary tuberculosis (PTB) if shows at least 2- to 3-week duration of cough and had or has been in contact with PTB-infectious individuals [8].

Nevertheless, an MTB infection is usually asymptomatic in people who are well nourished and free of complications that compromise their immune system. This form of TB is known as latent TB infection (LTBI), which means that they do not have active TB disease, but could develop it in the future (a process known as TB reactivation). It is estimated that one in three people in the world has LTBI, and that, among these individuals, 5–10% present a risk of TB reactivation; and most of them will develop TB within the first 5 years after their MTB infection. Considerably, the risk of reactivation increases when predisposing factors are present. LTBI eventually progresses to active TB, when the infected individual experiences any condition which compromises his general health state, as HIV/AIDS or malnutrition [9]. On the other hand, PTB is considered, if the patient presents the following signs and symptoms: a frequent cough, usually throwing sputum (phlegm), chest pain, weakness, fever and evening or night sweats. In advanced cases, secretions are often accompanied by blood (hemoptysis), loss of weight, a pale skin and bright and sunken eyes [10]. The PTB diagnostic is reinforced by a positive Mantoux test (also known as tuberculin skin test [see below]). The presence of lung lesions (caverns), observed by a chest X-ray, denotes an advanced PTB [11, 12].

2.2. Mantoux test

The Mantoux test is performed to determine the sensitivity to tuberculin of each patient. This test consists of inoculating on the forearm, a small amount (5.0 units of tuberculin in 0.1 mL) intradermal (between the skin layers) of a protein extract of the mycobacteria dissolved in glycerol. This extract is known as PPD (purified protein derivative). Determine the diameter of the skin induration 45–72h after injection (thickened and hard skin), the halo (circle) of erythema (reddening of the skin) should not be taken into account. An intradermal reaction is considered positive when the induration diameter is 5.0–15.0 mm or greater. A positive test indicates that the person was in contact with MTB, but does not necessarily indicate that person is ill. A consideration of TB illness or not should be given if risk factors and medical history suggest this condition. For example, a person allergic to tuberculin or one who received the BCG vaccine against TB will give a positive reaction to tuberculin without being sick. These people are false positives. In contrast, an individual may have a Mantoux negative reaction and an PTB, if he also has AIDS (having his immune system compromised). Thus, this individual is anergic and a false negative [13].

2.3. Chest X-ray

Chest radiographs should be used to rule out the possibility of PTB in a person who has a positive reaction to a tuberculin skin test and no symptoms of the disease. An anteroposterior chest X-ray is one of the most important tests to be performed in a patient having TB or suspected TB. Chest X-ray findings include parenchymal infiltrates, hilar adenopathy, cavitation, nodules and pleural effusion [14]. Infiltrates or consolidations and cavities are often seen at the lung upper lobes, with or without mediastinal or hilar lymphadenopathy. However, lesions may appear anywhere in the lungs. In HIV/AIDS suffers or other immunosuppressed persons, any abnormality may indicate TB. In contrast, the chest X-ray may even appear entirely normal. On the other hand, longstanding healed TB lesions are usually presented as

pulmonary nodules in their hilar area or upper lobes, with or without fibrotic scars, volume loss and bronchiectasis (enlargement of parts of the airways within the lung). Furthermore, pleural scarring may be present. Nodules and fibrotic scars may contain slowly multiplying tubercle bacilli with the potential for future progression to active TB. Persons showing these lesions, if they have a positive tuberculin skin test reaction, should be considered high-priority candidates for the treatment of latent infection, regardless of age. Conversely, calcified nodular lesions (calcified granuloma) represent a very low risk for future progression to active TB [15].

2.4. Classical laboratory methods to identify PTB

Classical laboratory methods to identify MTB are used worldwide due to these are very useful and reliable.

TB-diagnostic methods are the detection of mycobacteria, by acid-fast bacilli (AFB) staining and fluorescence microscopy and isolation and cultivation of mycobacteria, allow establishing a most specific diagnostic of PTB than only above clinical diagnostic methods. Both identifying, isolating and characterizing MTB in a patient sputum along with a PTB clinical picture are necessary because to all signs and symptoms of PTB, described above, may be confused with other diseases, such as coccidiomycosis [16]. In other words, it is essential, in first instance, a clinical diagnostic of PTB and confirm or discard it by conventional laboratory procedures [11, 12].

3. Detection of AFB

3.1. Ziehl-Neelsen staining (ZN)

Mycobacteria are not well stained by Gram's method. Therefore, alternative methods were developed long time ago. The most commonly used technique to identify AFB is ZN. Notwithstanding, this method is not 100% specific or sensitive for MTB, since some bacteria species, like those of the *Nocardia* genus, are also AFB [17]. In addition, there are more than 120 species of mycobacteria, which are not causal agents of PTB and that also are AFB. On the contrary, some mycobacteria produce atypical pulmonary symptoms that are not MTB [18].

The ZN staining consists of dying, discoloring, counterstain and observing. The culture in suspension, or a liquid biological sample, is deposited onto a slide and dried and fixed with a flux of heat air. The slide is submerged into a phenol-carbol fuchsin solution; then, this smear is heated to enable the dye that penetrates the waxy mycobacterial cell wall and bind mycolic acids. Once the preparation was dyed, this is rinsed with tap water, and an acid decolorizing solution (1% solution of hydrochloric acid in isopropyl alcohol or methanol) is applied to remove the red dye from any non-AFB cell or material. Only AFBs (such as mycobacteria) will retain the phenol-carbol fuchsin dye, because of the protection provided by a waxy lipid layer, characteristic of AFB. After discoloration of the sputum smear, counterstaining is performed with malachite green or methylene blue, which will stain non-AFB material that was not able to retain the first dye. After that, a contrast between the red AFB, of the non-AFB material green or blue color will be observed with a microscope. The above procedure may vary due to structural differences between mycobacteria genera. For instance, *M. ulcerans* is strongly AFB,

whereas others, like *M. leprae*, are weakly AFB. *M. ulcerans* is decolorized with 3% ethanol, whereas it is needed 0.5–1% sulfuric acid to decolorize *M. leprae* [19]; in addition, the staining and discoloration times can also change from one mycobacterium genera to another.

The ZN is considered as the gold standard. This technique requires highly trained personnel in AFB staining and detection. According to the WHO [20], the AFB detection time and training of specialized personnel need to be improved, and so make TB diagnoses and start treatments for patients in a more opportune manner [20].

3.2. Fluorescence microscopy

An alternative for ZN staining is fluorescence microscopy (FM), which is currently used in many laboratories with available equipment. FM is faster than ZN, because FM allows visualize MTB bacilli in an easier manner than ZN. An FM study lasts 1.0 min and ZN requires 4.0 min. In addition, FM has been shown to be at least 10% more sensitive than classical light microscopy [21]. In high-volume laboratories, rapid laboratory turnaround times can prove to be crucial in the diagnosis of presumptive mycobacterial disease. In such settings, test characteristics such as staining time and background fluorescence contribute to overall laboratory efficiency in reporting results [22]. Also, FM is a semi-automated method and ZN must be carried out manually and to observe AFB directly under the microscope, by an experimented operator. On the other hand, ZN method is more affordable for low-resource institutions than FM, since fluorescence microscopes are noticeably more expensive than light-field microscopes, which are used to perform a sputum inspection.

Auramine and rhodamine are the main stains used in FM to detect mycobacteria in biological samples. These dyes are non-specific fluorochromes that bind mycolic acids of the mycobacterial wall. Once the dye has penetrated into the MTB wall, the mycolic acid-dye complex resists discoloration by alcohol-acid solutions. Counterstain helps to prevent fluorescence other than AFB, thus making the test more specific by reducing the possibility of artifacts. Counterstain is done with potassium permanganate. Once the staining is finished, AFBs are observed under an epifluorescence microscope. Under the UV light, AFBs gleam in yellow or bright orange over a dark background. The AFB-screening examination of smears is performed with a fluorescent microscope equipped with a 20X or 40X objective and a 100X oil immersion objective to observe the morphology of fluorescing organisms [23].

Nowadays, there are available new systems for identifying mycobacteria in clinical samples, which is based on light-emitting diodes (LEDs). This LED system costs considerably less than the original system and deserves to be evaluated in developing countries [24]. One of these methods describes an adaptation of a standard fluorescent microscope for illumination using a 'Royal Blue' Luxeon™ LED and demonstrates that this form of lighting is suitable for detection of auramine O-stained *Mycobacterium* spp. The authors claim that their method is of low cost, low power consumption, safety and that the reliability of LEDs makes them an attractive alternative for mercury vapor lamps [25].

In conclusion, in spite of the usefulness of AFB stain, using the ZN staining or FM, without a mycobacteria culture, AFB visualization has a poor negative predictive value. An AFB culture should be performed along with an AFB stain; this has a much higher negative predictive value.

4. Isolation and culture of MTB

4.1. Conventional procedure

All following procedures mandatorily are carried out in laboratories of level III microbiological contention and must be performed by trained personnel. A TB diagnostic must include an identification of MTB in appropriate biological samples. This is achieved by isolating the infectious agents and observing their colonies, developed in a solid culture medium (usually Lowenstein-Jensen's). Typically, PTB forms white, opaque, cauliflower-shaped colonies. Using a few of these colonies, the biological and biochemical characteristics of the isolates must be determined to identify MTB [26]. It must be kept in mind that a definitive TB diagnostic must be integrated by the correspondent clinical signs and symptoms and the bacteriological findings [27].

A standard laboratory procedure to identify MTB consists of decontaminate and liquefy freshly obtained sputum samples. One of the most used methods to liquefy and decontaminate sputum is Petroff's method, which has been modified several times [28]. One of the most important characteristics of method consists of the destruction, by sodium hydroxide, of many of the rapidly growing micro-organisms, which are contaminating to biological samples. After this decontamination, the viable bacteria are concentrated by centrifugation. The sediment is inoculated in a culture medium and incubated at 37°C in a 5% CO₂ atmosphere until typical MTB colonies are observed. The most used method to isolate MTB from sputum or other body fluids, like urine or cerebrospinal fluid, is the Lowenstein-Jensen solid medium, which usual composition is malachite green, glycerol, asparagine, hen's eggs and a salt solution (composed by potassium dihydro phosphate, magnesium sulfate and sodium citrate). The medium is aliquoted, put into glass screw-tapped bottom flat tubes and coagulated in an oven by heat. The sedimented bacteria are inoculated on the surface of a Lowenstein-Jensen medium slant. MTB is characterized by a slow growth, being its doubling time 10–20 h. It is required to incubate the sputum preparations for 5–8 weeks before typical colonies appear. Usually, the slow growth of mycobacteria in vitro is an obstacle to start an adequate treatment for TB opportunely. Other essential bacteriological characteristics of MTB are as follows: unlike other mycobacteria, MTB colonies are white (they do not produce pigments in the presence or absence of light) [29]. MTB is niacin- and catalase-positive and reduces nitrates to nitrites [30]. AFB detection by microscopy, plus culture, isolation of mycobacteria and their laboratory biochemical characterization are the gold standard for TB diagnosis. Even though the processes above have a low sensitivity (between 60 and 80%) [30], the generalized use of the theme is vital for preventing TB spreading worldwide, with an emphasis in DR-TB and XDR-TB [2].

4.2. Automated procedure

At a date, a totally automated system is available. This system is the BD BACTEC™ MGIT™ Automated Mycobacterial Detection System 960. MGIT means Mycobacteria Growth Indicator Tubes (MGIT). The BD BACTEC™ MGIT™ system offers several advantages over other tests and systems, among which can be emphasized an easy operation, an automated and a continuous quality checks, saving hours of work of the personnel, as well as of the equipment. By

showing positivity as it happens, time is optimized for patient care. It also uses barcode technology to facilitate data entry and processing, and patient and sample tracking.

The fundamentals of BD BACTEC™ MGIT™ system is as follows: at bottom of each MGIT unit is placed an oxygen-quenched fluorochrome (tris 4,7-diphenyl-1, 10-phenanthroline ruthenium chloride pentahydrate) embedded in silicone. As mycobacteria grow into the culture tube: the free oxygen is utilized and replaced by carbon dioxide. Because of depletion of free oxygen, into the culture tube, the fluorochrome is no longer inhibited, resulting in fluorescence within the MGIT tube: under a source of UV light a fluorochrome is stimulated and emits visible light. The fluorescence is quantified by the instrument, as the intensity of fluorescence is directly proportional to the extent of oxygen depletion and, therefore, to the concentration of viable mycobacteria.

Procedure. Each MGIT unit contains 7.0 ml sterile modified Middlebrook 7H9 broth base, which must be added with MGIT PANTA—an antibiotic mixture designed for suppress contamination—OADC (oleic acid, albumin, dextrose and catalase) or, preferably, MGIT 960 growth supplement. According to the manufacturer, MGIT growth supplement is essential for growth of mycobacteria belonging to MTBC. MGIT tubes are incubated, inside the instrument, and monitored for increasing fluorescence every 60 min. Under the above conditions, a typical MTB culture reaches a concentration of mycobacteria equivalent to 10^5 – 10^6 colony forming units (CFU) per mL. On the other hand, when a preparation remains non-fluorescent after 6 weeks (42 days), it is considered as negative. The presence of contaminating bacteria can be detected, because these microorganisms generally produce a heavy turbidity [31].

5. Determination of MTB drug resistance

5.1. Conventional methods

Once MTB isolates are identified, it is necessary to determine their drug resistance profile. Conventional drug-resistant tests (DRTs) are first used to evaluate resistance to streptomycin, isoniazid, rifampin, ethambutol and pyrazinamide. This information is crucial for the treating physician knows if DOTS will be effective to cure a patient having TB. Nevertheless, if a particular isolate shows resistance to rifampin, isoniazid or both drugs, the resistance profile to second-line drugs [injectable aminoglycosides (amikacin or kanamycin), capreomycin and fluoroquinolones] must be determined [32]. This information will permit that the physician decides if the patient should be treated with a combination of first- and second-line drugs, following the current recommendations of the WHO for MD-TB. Three of the most used DRTs are the classical agar proportion method, the BACTEC 960 fluorometric method and microplate Alamar blue assay (MABA).

5.2. Agar proportion method

The method of proportion using Middlebrook 7H10 agar has been considered the “gold standard” method for several decades. This method allows separate the resistant bacilli from

the susceptible bacilli and establishes their proportions in the culture (% of resistant bacilli and % of sensible bacilli to a particular drug). This information is obtained by culturing the isolated strain in duplicate. One slant or plate has only Middlebrook 7H10 agar, and the duplicate is cultured with the same medium, added with the anti-tuberculous drug to be evaluated. Then, the percentage of colony forming units (CFU) present in the treated culture is calculated with respect to the total number of CFU found in the untreated culture [33].

5.3. Fluorometric method: BACTEC 960 system

The BACTEC MGIT 960 SIRE (streptomycin, isoniazid, rifampin and ethambutol) and PZA (pyrazinamide) susceptibility test for MTB are used extensively in the world to identify the presence of mycobacteria in biological samples and to determine the sensitivity to first-line drugs [34]. The drug susceptibility test, using this system, is based on the same principle than the aforementioned BACTEC 960 system for mycobacteria detection, and the same instrument is used, but following the particularities that request this drug-sensitivity system. The procedure is as follows: two MGIT units are needed to perform the mycobacteria DRTs for each first-line anti-MTB drug. Each tube is inoculated with the test mycobacteria culture. One MGIT unit is added with a known concentration of one of the test anti-MTB drugs, and the other one is a non-treated growth control, which is inoculated and incubated under the same conditions than the test culture. Mycobacteria growth is monitored by the BACTEC 960 instrument, which automatically interprets if a particular culture is susceptible or resistant to the drug of interest. In other words, when the control culture reaches a mycobacteria concentration equivalent to 10^5 – 10^6 CFU, in about 7–8 days, the growth in the test tube is compared with the control. If the test drug is active against the isolated mycobacteria, its fluorescence will be significantly lower than controls.

5.4. Microplate Alamar Blue Assay (MABA)

MABA is a micromethod, which takes advantage of the dichromatism of resazurin: when the medium is reduced, resazurin changes irreversibly from blue to an intense pink and also intensely fluoresces under UV light. In addition, resazurin is soluble in water. These remarkable properties made possible that Alamar blueTM or resazurin has been widely used to determine cell viability and growth in MTB and many other cells and microorganisms [35]. MABA has been specifically applied to determine drug resistance of MTB [36] in synthetic, semisynthetic [37] and natural products [38]. MABA is also used to determine the minimal inhibitory concentrations (MIC) of isoniazid, rifampin, streptomycin and ethambutol in MTB. In general, the procedure followed with MABA consists of using bacterial suspensions having approximately 6×10^6 (CFU)/mL. The product to be evaluated for its anti-MTB activity is previously sterilized—by filtration or dissolved in 100% dimethyl sulfoxide (DMSO)—and then diluted in fresh Middlebrook 7H9 broth supplemented with an oleic acid dextrose catalase supplement (OADC). Then, 200 μ L of this mycobacterial suspension are put into the first well of 96-well microplates. From this well, a twofold diluted series is formed, using the remaining wells, placed in the same lane than the first.

The MIC of the evaluated product is equivalent to its concentration being in the first pink-colored well [38].

5.5. Tools for molecular diagnosis and drug resistance determination

During the last decades, the number of tests to identify MTB has been growing rapidly. Today, the molecular diagnostic tools are faster, more sensitive and more specific than the conventional tools. Nevertheless, the conventional microbiological methods remain being the gold standards. Furthermore, the isolation, culture, identification of MTB and determination of first- and second-line anti-TB drug profile have not been replaced, with advantage, by any molecular tool. In other words, currently, molecular diagnosis tools are only complimentary for traditional diagnosis methods.

5.6. Molecular diagnosis tools

Currently, many molecular tools are available. In general, these methods analyze MTB-DNA or -RNA by polymerase chain reaction (PCR)-amplification, with very few exceptions. There are three basic molecular procedures for amplifying MTB nucleic acids: PCR final point, RT-PCR and quantitative or real-time PCR (qPCR). In addition, multiple variants have been described for the above nucleic acid amplification methods (see below). In general, the above methods detect polymorphisms, mutations or deletions to discriminate between MTB and other bacteria species and different MTB clusters. In addition, molecular methods offer the possibility of rapidly and accurately identifying MTB, genotyping and determining resistance to rifampin or rifampin and isoniazid [30, 39, 40].

5.7. Main genetic markers to identify MTB

The genetic markers currently used to identify and classify MTB are as follows: the IS6110 insertion element, the gene encoding the 65-kDa heat shock protein, named hsp65, a polymorphic guanine-cytosine-rich sequence (PGRS-RFLP), mycobacterial interspersed repetitive units-variable number tandem repeat (MIRU-VNTR), direct repeats (DRs) or clustered regularly interspersed short palindromic repeats (CRISPR), single nucleotide polymorphisms (SNPs) and large sequence polymorphisms (LSPs) [41]. Nevertheless, not all the above markers are used in identifying MTB with clinical purposes, but in epidemiological and basic studies. Thus, in the following paragraphs, we discuss only the markers and methods useful to identify MTB with TB-diagnosis purposes, although some of them are also using to perform epidemiological studies.

IS6110 considered as an ideal target to identify MTB and classify clinical isolates in clusters, according to their random fragment length polymorphism (RFLP [see below]). IS6110 is randomly distributed throughout the MTB genome and its number of copies ranges from 0 to 26 [42]. A limitation of RFLP-based detection of IS6110 is the inability of this method to discriminate strains with less than six copies of IS6110. This situation is especially important in India. Narayanan et al. in 2002 reported that 41% of clinical isolates contained a single copy of IS6110 [43]. In addition, Sankar et al. specifically pointed IS6110 as a molecular target that is not

sensitive enough to encompass all TB cases, especially PTB. In fact, it is well known that some MTB strains lack the element IS6110 [44]. However, a group of investigators reported up to 94.1% MTB in clinical isolates using IS6110 as the molecular target and nested PCR—see below—as the method of choice. Until relatively recently, IS6110 was considered exclusive to the MTBC. However, Coros et al. [45] reported that *M. smegmatis* also has the IS6110 element. This latter finding could negatively affect the specificity of IS6110.

Gene hsp65. The second most widely used molecular target is hsp65. This gene encodes for a heat shock protein of 65 kda, and the MTB genome contains a single copy of that gene. Variations in the primary sequence of hsp65 serve to identify species of rapidly and slowly growing mycobacteria [46]. The limitation of this target for the molecular diagnosis of TB is precise that the MTB genome has a single copy of the hsp65 gene.

Direct repeats. The direct repeat (DR) region in MTBC strains (do not confuse DR region) with drug resistance, see above) is a fragment of DNA located in a hot spot for the integration of IS elements. This region is composed of multiple repeat sequences of direct variants (DVRS). Each DR replicate [47] is composed of 36 bp repetitive sequences separated by a non-repetitive sequence of 35–41 bp in size called spacers [48]. Generally, each spacer is found only once in the DR region. During the MTB evolution, some spacers could be deleted from the MTB-genome, due to a high mutation rate in the DR region [49]. Genome DR region of MTBC strains is extensively used for genotyping mycobacteria, due to DR spoligotyping-based patterns allow distinguishing MTB from *M. bovis* and other members of the MTBC [47].

6. Methods to identify MTB in biological samples

6.1. Method to determine RFLP

This method is an exception for others based on nucleic acid amplification. Therefore, it is necessary purifying a considerably greater quantity of DNA from MTB isolates than the DNA quantity needed for those methods based on nucleic acids amplification. RFLP consists in isolating, propagating MTB, purifying the DNA, cutting up the DNA with a specific restriction enzyme, separating the fragments by gel-electrophoresis, hybridizing the DNA fragments with an IS6110 labeled probe and visualizing the RFLP pattern and analyzing it [50].

6.2. Methods to identify MTB by amplifying its genomic DNA

6.2.1. Polymerase chain reaction (PCR)

PCR provides a very useful tool for diagnosing TB. The endpoint PCR has two clinical applications: the identification of MTB and the determination of drug resistance of the clinical isolates.

6.2.2. End-point PCR

A typical analysis of end-point PCR consists in a separation of the PCR product by gel-electrophoresis and its visualization and certification of the expected PCR product. Originally,

end-point PCR was described by Kary Banks Mullis, who received the Nobel Prize in chemistry in 1993, for his invention of this procedure. The process, which Mullis conceptualized in 1983, is hailed as one of the monumental scientific techniques of the twentieth century [51]. PCR allows obtaining millions of DNA or RNA —see below—sequences from few original copies of the sequences of interest, called amplicons [2]. The success of molecular detection of MTB depends on the quality of the biological samples, the methods of extraction of the nucleic acids and the amplification and detection techniques. In addition, it is of fundamental importance to select the appropriate amplicons and to design and synthesize the complementary oligonucleotides (primers) to generate genuine DNA copies of the amplicons [52].

MTB identification by PCR can be done starting from sputum samples, MTB isolates or MTB laboratory strains (as H37Rv). In general, the PCR procedure, starting from sputum, is as follows: the sputum sample is decontaminated and concentrated usually using Petroff's method and centrifugation (see above). Mycobacteria are washed with a saline pH buffer and lysed by incubating them with a solution of lysozyme, proteinase K and sodium dodecyl sulfate (SDS). The DNA is extracted with chloroform-cetyl trimethyl ammonium bromide (chloroform-CTAB) and precipitated with isopropanol. The amplification of specific regions of DNA needs a very pure and non-degraded DNA, a heat-resistant DNA polymerase (usually Taq polymerase, which is obtained from the thermophile bacteria *Thermus aquaticus*), a mixture of the four deoxyribonucleoside triphosphates [deoxyadenosine triphosphate (dATP), deoxyguanosine triphosphate (dGTP), deoxycytidine triphosphate (dCTP) and deoxythymidine triphosphate (dTTP)], a pH buffer solution, salts of Mg^{2+} or Mn^{2+} and K^+ and a pair of DNA primers, which sequences are complementary to the 3' ends of the two DNA strands, embracing the region to be amplified. Typically, PCR consists of a series of 20–40 cycles. Each cycle consist of three steps: (1) denaturation of DNA at $>90^{\circ}C$ (separation of the two strands of DNA), (2) annealing (hybridization of primers with each DNA strand) and (3) extension (synthesis of the two new DNA strands) [53].

The analysis of DNA fragments produced by PCR consists of separate them according to their size (number of base-pairs [bp]). DNA band separation is achieved by applying an electrophoresis run in gels of agarose. After the electrophoresis is complete, DNA appears ordered in the gel as bands, from the heaviest (top) to the lightest (bottom). DNA bands in the gel must be stained to make them visible. DNA may be visualized using ethidium bromide, which, when intercalated into DNA, fluoresces under ultraviolet light. This stain must be managed carefully, using adequate protective equipment, because ethidium bromide is carcinogenic. A good alternative is using fluorescent-specific DNA stains, like GelStar[®] and SYBR[®] Green. Both strains are very sensitive and offer the advantage, over ethidium bromide, of being non-carcinogenic. In addition, it is not necessary washing the gels after staining for increasing the specificity of analyses. Therefore, both stains are excellent for detecting nucleic acids in agarose gels. GelStar is used to detect both single-stranded and double-stranded DNA and RNA, whereas SYBR Green is preferred for visualizing bands of double-stranded DNA in gel electrophoresis [54]. Then, the PCR product is sized, with the aid of a DNA molecular weight ladder, which must be placed in the same gel of experimental DNA, before running the electrophoresis. Usually, the first lane at left of the gel is chosen to put the molecular weight ladder. This DNA-size ladder is formed by a set of double-stranded DNA fragments having known and different sizes. The size of PCR products is estimated by interpolation or simply

comparing the position of the DNA marker, having a size equivalent to the size expected for the PCR product of interest. The identification on the gel of the amplified product with the expected molecular weight indicates the presence of an MTBC member in the sample studied [55, 56].

6.3. Variations of PCR-final point of DNA

The main variations of the basic point final PCR are nested PCR, multiplex PCR, spoligotyping [57, 58] and RFLP-PCR. These variants were designed to improve the sensitivity and specificity of basic RFLP and PCR.

6.3.1. Nested PCR

Nested PCR is widely used, because this method significantly improves the sensitivity and specificity of basic PCR. The nested PCR consists of using two sets of primers, both directed against the same target DNA sequence, which may be a gene or some other region characteristic of a mycobacterial species [59]. The first set of primers is designed to hybridize to the most distal regions of the target sequence, the first amplification run is made and the resulting amplicons are used, in turn, to amplify a slightly inner region of the used original target sequence. Once performed the first series of PCR cycles, the PCR is used to carry out the second amplification procedure. Prasad et al. designed a nested PCR-based assay using the *hupB* gene of MTB (Rv2986c) and *M. bovis* (Mb3010c) as a method to differentiate these two closely related species [60]. Our research group developed a method for the diagnosis of TB based on nested PCR that targets the gene coding for the β -subunit of MTB RNA polymerase. This method is noticeably more specific and sensitive than all that have been published to date or have been approved by the FDA. Most current methods require cultivating mycobacteria for a few days and do not work with certain types of samples. In contrast, our method can work well with the genome of a single bacterium and samples of sputum [61] or cerebrospinal fluid [62], without the need to culture the biological samples. Currently, the patent is in a process.

6.3.2. Multiplex PCR

Multiplex PCR refers to the use of PCR to amplify, simultaneously, several different DNA sequences. That is to say, multiplex PCR allows performing many separate PCR reactions, all together, carrying out only one procedure. Otherwise, amplification by PCR of several DNA sequences would require several runs, as well as more reagents and longer time to perform them. On the other hand, if additional information is required from a particular DNA sequence, this may be gained by accomplishing a single, conventional PCR test.

PCR multiplex has enormous value in infectious disease that can be caused by a variety of microorganisms, for instance, meningitis. Meningitis can be caused by MTB [63], several other bacterial species (*Streptococcus pneumoniae*, *Haemophilus influenzae* type b, *N. meningitidis*, group b *Streptococcus* and *Listeria monocytogenes*) [64] and fungi [65]. Meningitis is a medical emergency, and immediate steps must be taken to establish the specific cause and initiate effective

therapy [66]. PCR multiplex is a rapid, sensitive and specific diagnostic test for acute bacterial meningitis. PCR is particularly useful for analyzing cerebrospinal fluid of patients who have been treated with antibiotics before lumbar puncture [64]. Furthermore, in 2002, Shah et al. [67] reported a multiplex PCR-based assay performing a rapid and specific differentiation of *M. bovis* and MTB. This procedure can be beneficial for medical and veterinary microbiological laboratories. The designed multiplex PCR to identify MTB or *M. bovis* is based on the differential amplification of *pncA* gene. This test is highly specific and sensitive. Only 20 pg of pure mycobacteria DNA are needed, and none PCR amplification product has been obtained from any atypical mycobacterial isolate.

There is a series of requirements to be met to perform a good multiplex PCR analysis. These are as follows: (1) Accomplish the standard requirements for primer design. (2) Design one pair of primers for each expected amplicon. (3) Apply suitable temperatures, allowing DNA polymerase works accurately in every multiplex PCR cycle. (4) The primer design must be optimized; so that, all primer pairs work properly, at the same time and in a single reaction. (5) Apply similar alignment temperatures for all primers. (6) Choose the size of those DNA regions to be amplified so that be possible obtaining amplicons having clearly different sizes among them. Allowing, in this way, to distinguish the bands between them in the electrophoresis gel, (7) in case of needing to differentiate amplicons having the same size, the primers must be labeled with fluorescent dyes having distinctive colors. For certain multiplex analyses, commercial multiplexing kits are available. It has been informed that multiplex PCR has a sensitivity and specificity of 95% [68].

6.3.3. Random fragment length polymorphism (RFLP)-PCR

RFLP-PCR is a fast and reliable method for identifying non-TB mycobacteria [69]. This method is based on amplifying fragments of diverse genes; for instance, *hsp65*, the gene for histone-like protein *hupB* and *pncA*. Gene *hsp65* are present in all species of mycobacteria. The selected target of *hsp65* gene is a 439 bp fragment. Using *hsp65* can be distinguished between MTB, *M. avium* and *M. intracellulare*, but not between MTB and *M. bovis* [70]. Gene *hupB* encodes for an histone-like protein. RFLP-PCR of *hupB* allows differentiation of MTB and *M. bovis*. The amplicons generated have 645 bp and 618 bp, respectively. *pncA* PCR assays were found specific in detecting MTB and *M. bovis*, as well as the *M. avium* complex in human sputum [71].

6.3.4. Real-time PCR

Real-time PCR, also known as quantitative PCR (qPCR), offers several advantages over end-point PCR, which are as follows: (1) as its name indicates, it is quantitative; (2) reduces the risk of cross-contamination because it minimizes the need to manipulate samples after performing PCR; (3) the manual processing time is reduced; (4) the run preparation times are also reduced and (5) offers high sensitivity and specificity [72]. Since the last decade, qPCR for the detection of MTB target genes in clinical specimens has contributed to improved diagnosis and epidemiological studies of TB. This is because qPCR offers the advantage of combining amplification and detection of molecular targets in a single step; and there is no need to use nested PCR [73].

Therefore, qPCR is rendering obsolete those techniques based on end-point PCR, which are much less sensitive. The qPCR technique involves the use of fluorescent chromophores (fluorophores) covalently attached to the primers. The fluorophore is placed at the 5' end of the primer (probe) and at the 3' end or internally, a fluorescence quencher is inserted covalently. During the PCR extension phase, the exonuclease activity of the Taq polymerase cuts the initiator in the 5'-3' direction, and the fluorophore and quencher are separated, which results in a detectable fluorescence that is proportional with the number of accumulated amplicons. A spectrofluorometer integrated with the thermocycler reads the emerging fluorescence. The PCR occurs in a dynamic way, and the results are read continuously. A computer integrated with the equipment, with the help of specialized software that accumulates and analyzes the data, generates a DNA amplification curve under study. The computer program allows monitoring the amplification curve on a screen. Usually, an analysis of qPCR is carried out in 1.5 h. The analysis described above can be performed with different platforms. SYBR green (cyanine colorant) is widely used for qPCR because it is very sensitive and high cost/benefit in uniplex formats. Besides of SYBR green, a number of options are available, such as TaqMan probes, molecular beacons, scorpion primers, fluorescence resonance energy transfer probes (FRETs) and primer-probe energy transfer. There are also different fluorescent chromophores, e.g. 6-carboxyfluorescein-aminohexyl amidite (6-FAM), carboxy-X-rhodamine (ROX) and cyanine (Cy5) as reporters at the 5' end and a quencher at the 3' end. Considering the advantages and disadvantages of IS6110 and of hsp65 discussed above, some authors have proposed using them in a multiplex qPCR format, which would presumably offer a more specific and inclusive diagnosis [2].

6.3.5. Real-time (RT) PCR

RT-PCR allows to amplify specific regions of the MTB genome or to obtain DNA starting from RNA by using a reverse transcriptase. This form of PCR is called RT-PCR (reverse transcriptase-PCR). The nested RT-PCR is a variation of the nested-PCR. Nested RT-PCR offers the same advantages than nested PCR, discussed above; with the difference that nested RT-PCR allows obtaining a cDNA strand, using the original mRNA sequence as template. A reverse transcriptase is used instead of a DNA polymerase to synthesize the cDNA. Then, like in nested PCR, the first set of primers is designed to hybridize to the most distal regions of the target sequence, the first amplification run is made and the resulting amplicons are used, in turn, to amplify a slightly inner region of the used original target sequence [59].

6.3.6. Spoligotyping

Besides of PCR multiplex, spoligotyping offers the possibility of distinguishing between *M. bovis* and MTB. Forty-three types of mycobacterial spacers are known. Of these, 37 are typical of MTB and other 6 of *M. bovis* BCG. An edition of the international spoligotyping database namely SpolDB4/SITVIT was introduced containing 1939 different spoligotypes (ST) identified. Furthermore, spoligotyping allows grouping MTB isolates according to the presence or absence of intermediate regions of the DR locus (direct repeats) in each MTB isolate, forming

specific patterns. The DR locus is a member of the CRISPR (clustered regularly interspaced short palindromic repeats) family of the MTBC.

Spoligotyping is used to analyze genetic diversity of DR locus. It is useful for clinical, molecular epidemiology, evolution and population genetics laboratories [74]. This method is relatively easy to use, robust and allows numerical analysis. Spoligotyping was designed to detect biotinylated, amplified products from MTB and *M. bovis* from the DR locus. This method is based on reverse hybridization to the spacer sequences (43 synthetic oligonucleotides), which are covalently attached as lines to a nylon membrane. The hybridization is performed by turning 90° the amplicon template concerning the immobilized synthetic oligonucleotides. To immobilize the perpendicular samples, an acrylic device is used. So that, each homology will give rise to a tiny square of hybridization. Non-hybridizing products are eliminated with successive washes and will leave a blank space where the correspondent synthetic oligonucleotide is placed. On the other hand, those hybridized products will be detected using the streptavidin-peroxidase system, which binds to the biotin present in the amplified products. Results are visualized by incubating the hybridization template with luminol, in such a way that peroxidase catalyzes the oxidation of the reagent, resulting in light emission, which is developed by exposing the membrane to an X-ray film. The patterns obtained to reveal absence or presence of the spacers is read as a binary code, which can be easily interpreted and computerized. A commercial kit for MTB spoligotyping is available. In addition, a spoligotyping databases from MTB and *M. bovis* isolates from worldwide are available online.

6.4. Methods to determine MTB-drug resistance

6.4.1. End-point PCR to determine MTB-MDR

Most PCR-based tests described above were designed exclusively to detect the presence of MTB in biological samples. Fortunately, there are other PCR-based tests designed to identify MTB in biological samples and to know whether these microorganisms are resistant to rifampicin or rifampicin and isoniazid. The most important tests are described below.

The methods for detecting resistance to anti-TB drugs are based on the detection of mutations in the genes that are associated with that resistance condition and this is achieved by the application of sequencing or hybridization techniques. Recent advances in the rapid and direct detection of mycobacteria, with an emphasis on MTB, are based on the analysis of 16S rRNA gene sequence or oligonucleotide hybridization (oligo hybridization), typing of strains and detection of patterns of drug susceptibility. Semi-automated systems for culture have greatly increased sensitivity and reduced the time required to perform the detection and identification of mycobacteria in clinical specimens. However, further research is still needed to assess the impact and cost/benefit of new diagnosis methods. In addition, well-designed clinical trials are still required to evaluate new diagnosis methods and thus enable medical staff to have methods to help them respond quickly [75]. PCR-based tests to identify mutations in the *katG* and *rpoB* genes that are associated with resistance to rifampicin and isoniazid, respectively, may assist in the early identification of resistance to these drugs in mycobacteria [40]. The WHO Stop TB Partnership's New Diagnosis Working Group and the Foundation for

Innovative New Diagnosis (FIND) ranked tools for diagnosing active and drug-resistant TB in three categories: (1) tools approved by WHO; (2) tools that are in the last phase of development or evaluation and (3) tools that are in the early stages of development [76]. Tools approved by WHO include LPAs for the diagnosis of MDR-TB, by GenoType[®] MTBDR plus, Hian Lifescience, Nehren, Germany; INNO-LiPA Rif. TB, produced by Innogenetics, Ghent, Belgium, an assay for rapid detection and speciation, whose manufacturer is Capilia TB-Neo, TAUNUS, Numazu, Japan and the GeneXpert MTB/RIF system, produced by Cepheid, CA, USA, a nucleic acid amplification test (NAAT) to screen for MTB-MDR. In the third group, tools that are in the early stages of development include an assay based on the detection of lipoarabinomannan, a breath analyzer, a loop-mediated isothermal amplification technology, called TB-LAMP, produced by Eiken Chemical Co Ltd, Tokyo, Japan, and a phage-based bioassay for the rapid diagnosis of MDR-TB [77]. Another very promising development is the one belonging to the company Hain Life-science, Nehren, Germany. This technique is used to screen resistance of MTB strains to second-line injectable drugs: amikacin, kanamycin and capreomycin.

6.4.2. RT-PCR and line probe assay (LPA) for detecting MDR

The LPA is based on the principle of reverse hybridization, in which the 16S-23S spacer region of the rRNA (ribosomal RNA) is amplified by PCR. The amplicons are hybridized with oligonucleotide probes which are placed on nitrocellulose strips and are detected by a colorimetric system (usually by the biotin/avidin system). The probe is biotinylated and the avidin is covalently attached to a chromophore. There is currently a system for an LPA called Inno-LiPA Mycobacteria, produced by Innogenetics, Belgium. Inno-LiPA Mycobacteria allows identification of CMTB species, *M. kansasii*, *M. xenopi*, *M. gordonae*, *M. avium* complex, *M. intracellulare*, *M. scrofulaceum* and *M. chelonae-M abscessus* complex species [78]. This system has demonstrated an accuracy of 99.2%. INNO-LiPA Rif has also proven its usefulness in examining the resistance/susceptibility of rifabutin in mycobacteria [79]. The LPAs allow obtaining results in 24 h, with a very high sensitivity (99%), as already mentioned above. This system is the only one under development that will allow detection of MTB resistant to second-line drugs. The limitation of LPAs is that in most resource-poor countries, the facilities required to avoid contamination of amplicons, laboratory supplies and the equipment necessary to perform amplification of genetic material by PCR are not available [80].

6.4.3. GeneXpert MTB/RIF

The method called GeneXpert MTB/RIF is based on real-time PCR and RT-PCR. The target is the *rpoB* gene (encoding the beta subunit of RNA polymerase). This gene has in its sequence a hot zone, which is very susceptible to mutations associated with resistance to rifampicin. GeneXpert MTB/RIF is the fastest and safest system known until now, being able to produce reliable results in less than 2 h. Therefore, GeneXpert MTB/RIF allows physicians making accurate diagnoses and prescribing appropriate treatments, practically during the patient's visit to his office. GeneXpert MTB/RIF is fully automated. The system consists of an instrument, a personal computer, a bar code reader and specialized preloaded software to interpret the results. The system requires disposable GeneXpert cartridges that contain everything

needed to perform nucleic acid amplification. As the cartridges are individual and watertight, the possibility of cross-contamination and false results is eliminated. Concerning to usefulness and availability of GeneXpert MTB/RIF, van Rie et al. [77] stated that is the first system that has a true point of care [POC] tool in regions with limited resources. The automation and simplicity of the GeneXpert MTB/RIF system allow non-mycobacteriology personnel to obtain reliable results. According to WHO, GeneXpert MTB/RIF should be used as an initial test for the diagnosis of TB and in symptomatic patients having a high risk of be sick of MDR-TB. On the other hand, one of the main limitations that GeneXpertMTB/RIF has is the fragility of the equipment, especially in places where the power supply fails continuously. On the other hand, it is doubtful whether the detection of resistance only to rifampicin is sufficient to guide a suitable treatment in areas where there is a high incidence of polyresistance [81].

6.4.4. Proteomic molecular tools

Proteomics is one of the most recent technological advances, which will surely bring enormous benefits in the diagnosis of fast and simple PTB. Significant progress has already been made in this regard. The sources of MTB proteins are easier to obtain and analyze the serum or plasma of patients with TB and to control the serum of healthy controls. Several focused papers on the above-mentioned sense have already been published: (1) Deng et al. in 2011 [82]; (2) Liu et al. in 2010 [83]; (3) Zhang et al. in 2012 [84] and (4) Liu et al. in 2011 [85]. However, this approach is neither accessible nor suitable for microbiological laboratories. The reasons are as follows: currently, the instruments required to perform a TB diagnosis are costly, very sophisticated systems and software and a high degree of specialization are required, not only for the correct handling of the equipment [mass spectrometers, generally associated with high-performance liquid chromatography (HPLC) equipment]. In addition, once the main proteins are known, other specialized databases must be identified and consulted to determine their function.

7. Immune molecular tools

Among molecular diagnosis methods, there are tools based on the detection of antibodies, for instance, the Enzyme Linkage Immuno Assay (ELISA). That is, these techniques are based on the use of serological tests [86]. The vast majority of patients with TB (90%) live in low- or middle-income countries. In these countries, the diagnosis of TB is based on the identification of AFB in unprocessed sputum samples and using conventional microscopes. Mycobacterial culture methods partially alleviate the low sensitivity of the ZN microscopy method. Nevertheless, we have already discussed the limitations of this method and those of techniques based on nucleic acids amplification.

7.1. Methods based on immunochromatography

Methods based on immunochromatography are suitable for use in low-income areas, because these tests can be done without specialized equipment and with minimal training. The most commonly used MTB detection tests are based on the detection of anti-MTB antibodies. One of

the aspects to be considered in the evaluation of commercial methods or the design of new methods based on the detection of anti-TB antibodies is that the profile of antigenic MTB proteins that are recognized by the antibodies generated in the immune response of the host. A possible limitation of these methods consists of that the set of antibodies used as biomarkers varies according to the progression of TB [87]. Therefore, a reliable diagnostic tool based on an adequate combination of antigens is still required. Currently, there are about 40 commercial serological tests for rapid TB diagnosis. These systems use various antigen compositions to detect patients with anti-MTB antibodies. However, there are insufficient data on its reliability in patients with HIV/AIDS and uninfected persons.

7.2. Immunoprecipitation in cellulose acetate strips

A usual format for rapid TB diagnosis is to use cellulose acetate strips with one line for the test to which MTB antigens have been prefixed and another line as a positive control having predefined MTB antigens and human antibodies anti-MTB antigens. At the end of the strip, a section is left where the patient's blood or serum sample is placed, plus a suitable volume of some saline buffer, and then, a conjugate of anti-human antibodies labeled with colloidal gold or some chromophore is placed. A positive reaction is observed when the antibodies from the patient and the conjugate migrate on the cellulose acetate strip, and immunoprecipitation occurs, and consequently, binding of the conjugate on the line with the preset MTB antigens. On the other hand, the human chromophore/anti-antibody conjugate also binds to the positive control line with the MTB antibody and the preselected human anti-MTB anti-antibody. These tests take only a few minutes. Due to its usefulness and simplicity, it is currently considered a high priority to rigorously evaluate these tests to take advantage of them with safety and advantage as soon as possible. These tests are conducted by the National TB Programs of the United States of America [86]. Even though immunoprecipitation in cellulose acetate strips is considered as a promising tool, the WHO disapproves the use of such immunological methods for the diagnosis of TB, especially when it is intended to replace the search for AFB with microscopy [88].

7.3. Enzyme Linkage Immuno Assay (ELISA)

ELISA serves as a presumptive test in cases of suspected TB. The ELISA plates are sensitized with a complete soluble extract of *M. bovis*, strain BCG. The test serum is diluted, and after addition of the immunoenzymatic conjugate and its substrate, the spectrophotometric reading is made and it is determined whether it is positive or negative, according to the cutoff value determined in 100 sera from apparent healthy subjects coming from an endemic area of TB. The result is reported as positive or negative. Given the individual differences in response and cross-reactions with antibodies induced by other mycobacteria, the negative result should be interpreted with caution as it does not necessarily rule out TB [55]. Therefore, commercial ELISA tests are considered to have limited sensitivity and inconsistent specificity for the diagnosis of TB. Nevertheless, this method is the basis for interferon-gamma release assays (IGRAs), which are useful to detect LTBI (see below), using interferon- γ detection, as we discuss below.

7.4. Interferon (INF)- γ

The basic utility of IFN- γ is the diagnosis of LTBI, although IFN- γ detection is also used intensively for the diagnosis of active TB. Tests based on the measurement of IFN- γ release for the diagnosis of TB are generically called IGRAs. The infection usually ends by infiltration into the lung tissue of CD4⁺ T lymphocytes, which release IFN- γ . In turn, IFN- γ activates macrophages [89]. Identification of LTBI is a difficult issue. Therefore, the current diagnostic methods are based on markers of infection. Because of this, the intradermal reaction described above has long been used. Currently, kits are available to measure the release of IFN- γ as an immunodiagnosis alternative.

7.5. Interferon-gamma release assays (IGRAs)

IGRAs are designed to diagnose LTBI. These detect a cellular immune response to MTB. A remarkable characteristic of IGRAs consists in that results produced by these methods are not affected by the status of vaccination with BCG. Thus, in contrast with Mantoux method, IGRAs are useful for evaluation of LTBI in BCG-vaccinated individuals, particularly in settings where BCG vaccination was administered after infancy or multiple times. On the other hand, the IGRA's limitations are as follows: (1) These are unable to distinguish between an LTBI and an active-TB disease. (2) A positive IGRA result may not necessarily indicate an MTB infection, because it can also be caused by a non-tuberculous mycobacteria infection. (3) A negative IGRA does not rule out an active TB disease; many researchers have shown that up to a quarter of patients with active TB have negative IGRA results.

The IGRAs mainly used in the world are base ELISPOT and Quanti-FERON Gold In-tube.

7.6. Enzyme-linked immunospot (ELISPOT) assay

One of the best-known IGRA is ELISPOT, produced by Abcam. This system is used to determine the number of IFN- γ -producing T cells. ELISPOT employs monoclonal or polyclonal antibodies—preferably monoclonal antibodies because of their greater specificity than polyclonal antibodies. The antibodies are immobilized under aseptic conditions in the bottom of polyvinylidene fluoride (PVDF) microplates. The microplates are blocked, usually with serum albumin, which does not react with any antibody in the assay. Patient's cells are deposited on the microtiter plates at different densities in the presence of an antigen (in this case, the ESAT-6 MTB protein or the CPF10 protein filtrate). Both targets are strongly specific for TH1 cells in an MTB infection and are absent in BCG [90], whereas *M. kansasii*, *M. szulgai*, *M. marinum*, *M. flavescens* and *M. gastric* react with MTB-ESAT-6 and MTB-CPF-10. Microplates are put in a wet chamber at 37°C in an incubator with 5% CO₂ atmosphere for the time indicated by each manufacturer [91]. The specificity in HIV-infected individuals is 90%, while the specificity of the intradermal reaction is 57% [92].

7.7. Quanti-FERON Gold In-tube™ (QFT-GIT)

QuantiFERON Gold In tube (QFT) was specifically designed to LTBI. QFT-GIT is produced by QIAGEN. The QFT-GIT assay is an ELISA-based method. The test uses whole blood samples

from LTBI-suspicious patients and peptides from three TB antigens (ESAT-6, CFP-10 and TB7.7) in an in-tube format. The result is reported as quantification of IFN- γ in international units (IU)/mL of blood. An individual is considered positive for LTBI if the concentration of IFN-gamma concentration secreted by the responsive cells to TB antigens is higher than the test cut-off. These estimations are made after subtracting the IFN- γ concentration found **in the negative control** [93]. Several authors have reported that QFT-GIT has a sensitivity of 64–93% with an average of 70% [94]. Diel et al. found an average of 88% sensitivity of QFT-GIT with a range of 85–90% [95].

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