The contribution of molecular biology in diagnosing tuberculosis and detecting antibiotic resistance.

Catherine Pierre-Audigier[1,2] and Brigitte Gicquel[1]
(1) Institut Pasteur and (2) Hospital Bichat-Claude Bernard

1 Tuberculosis throughout the world: a catastrophic situation

With more than 2 million deaths and 9.4 million new cases per year (Corbett et al. 2003; Dye 2006; Harries, Dye 2006; Anonymous 2008b; WHO 2011), tuberculosis (TB) remains a major public health concern throughout the world. Even though the World Health Organization (WHO) declared tuberculosis an international emergency in 1993, the situation has worsened due to the deterioration of socioeconomic conditions in many countries and the acquired immune deficiency syndrome (AIDS) pandemic, as well as the recent increase in drug-resistant tuberculosis strains. As an example, the prevalence of tuberculosis in Sub-Saharan Africa is estimated to have increased from 290/100,000 inhabitants in 2000 to 487/100,000 in 2004, with a mortality rate of 81/100,000 in 2004.

Antibiotic-resistant strains of *Mycobacterium tuberculosis* are emerging due to a variety of reasons, such as the failure to identify resistance to a tuberculosis drug, interrupting treatment, omitting one or more antibiotics, suboptimal dosage, defective absorption, and not using enough active compounds on the strain. Thus, *M. tuberculosis* can become resistant to multiple antibiotics in the space of a few months. Susceptible bacteria are eliminated, giving way to resistant bacilli (Iseman 1993; Espinal, Dye 2005).

In the 1990s, the United States reported strains of multidrug resistant (MDR) *M. tuberculosis*, defined as being resistant to isoniazid (INH) and rifampicin (RIF), which were responsible for serious epidemics with a high mortality rate, particularly in countries with a high rate of HIV (human immunodeficiency virus) and *M. tuberculosis* co-infection. Programs for fighting tuberculosis targeted the strains of tuberculosis resulting from these cases of co-infection (Moore M 2000; Corbett et al. 2003). In 1995, WHO established a Direct Observed Therapy Short course treatment program (DOTS) as a strategy for fighting tuberculosis. The program's goal was to ensure a cure rate of at least 85% and prevent further resistance to TB drugs. Unfortunately, the management of multi-drug resistant TB (MDR-TB) was left to the individual initiatives of already strained national programs, which lacked funding and specific WHO guidelines (Meya, McAdam 2007). In addition, although the tuberculosis rate fell in many countries, the total number of tuberculosis cases worldwide increased 1.8% between 1997 and 2000, and grew more than 6% in the former countries of the Soviet Union and Africa. In 1999, WHO put together a work group called "DOTS-plus" to oversee the use of antibiotics in treating drug-resistant tuberculosis (Bastian et al. 2000; WHO 2000b; Aziz et al. 2006). In 2000, WHO reported that there were drug-resistant

M. tuberculosis strains in 72 countries at a frequency of 3% to 41% (WHO 2000a; Espinal, Dye 2005). It estimated the number of new MDR-TB cases per year to be between 300,000 and 600,000, with an overall prevalence of at least one million cases (Aziz et al. 2006; Anonymous 2008a).

Resistance to tuberculosis drugs creates major problems. Patients infected with multi-resistant strains are difficult to treat. Instead of being able to treat pulmonary tuberculosis with quadruple therapy followed by double therapy for a total of six months, treatments may last up to two years. Patients are treated for 18 to 24 months with four to six drugs to which the strain is susceptible, selected from second-line antibiotics (SLD for second line drug) if necessary, which significantly increases cost and the risk of side effects. These patients are far more likely to remain contagious for a longer period of time than patients with a strain which is susceptible. With second-line antibiotics, therapeutic success in treating MDR-TB varies from 48% to more than 80% of patients. The mortality rate is estimated at 0 to 37% in studies with seronegative HIV patients, and up to 89% in seropositive patient populations.

SLDs have been increasingly used to treat the growing number of MDR-TB strains. Since then, there has been an escalation of tuberculosis strains that are extremely resistant to antibiotics (XDR-TB) (XDR for Extremely Drug Resistant). These strains are defined as being resistant to INH, RIF, fluoroquinolone (FQ) and at least one second-line injectable TB drug (amikacin, kanamycin or capreomycin) (CDC 2006).

Between 2000 and 2004, an international network of laboratories identified 17,690 isolates of *M. tuberculosis* worldwide, of which 20% were MDR and 2% XDR (MMWR 2006; Shah et al. 2007). These XDR strains were observed in several regions of the world (in particular South Korea, Eastern Europe and Western Asia (countries of the former Soviet Union)). They were also the cause of epidemics reported in Russia and South Africa. The epidemic of Kwazulu Natal, in South Africa, caused the death of 52 out of 53 patients who developed XDR tuberculosis, with an average diagnosis time of 25 days (Gandhi et al. 2006). The prevalence of XDR-TB infections in other African countries (Mendelson 2007) is unknown, due to the failure of infrastructures and laboratories to identify them. Only 50 to 60% of patients suffering from XDR tuberculosis recover (Meya, McAdam 2007).

The convergence of the AIDS and tuberculosis pandemics in developing countries is an unprecedented disaster in medical history. In many countries with limited resources, the rate of tuberculosis has quadrupled since the AIDS epidemic and more than 80% of new tuberculosis cases are related to patients infected with HIV (Corbett et al. 2006; MMWR 2006; Friedland 2007). 12 to 14 million people will be co-infected by HIV and *M. tuberculosis*, of whom the majority live in Sub-Saharan Africa (Friedland 2007). Tuberculosis is the leading cause of death in patients infected by HIV (Harries et al. 2001), and what is alarming is that less than half of these patients are diagnosed with tuberculosis before their death (Mendelson 2007).

There are many reasons for failed early diagnosis and treatment. For example, patients co-infected with HIV and *M. tuberculosis* develop active tuberculosis more easily, diagnosis time should be shorter, and the countries

concerned are often among the poorest and most lacking in care. However, the main reason for late diagnosis in seropostive HIV patients is the shortage of a rapid and sensitive TB-specific test. The World Health Organization's DOTS program diagnoses tuberculosis using direct microscopic examination (DE – direct examination) of sputum specimens. However, this test is not specific and its sensitivity is diminished by impaired host responses, which significantly reduces the formation of cavities and bacilli excretion. Thus, negative tuberculosis results upon direct examination (DE) are correlated with poor prognosis, including death (Hargreaves et al. 2001; Mendelson 2007). In addition, tuberculosis symptoms in patients infected with HIV are not specific for this infection. Moreover, it tends to take on the clinical form of extrapulmonary TB. Diagnosis errors may lead to the wrong treatment, which can lead to side effects, drug interactions, and drug-resistant bacillus.

Thus, it is vital that tuberculosis be diagnosed and antibiotic resistance identified as quickly as possible. Early diagnosis can improve the patient's chance of survival and reduce the spread of the strain. The persistence of undiagnosed tuberculosis patients, as well as drug-resistant TB carriers who have not been treated properly, particularly in an environment where there are many patients infected with HIV, can have disastrous consequences.

Mycobacteriology laboratories play an important role in monitoring tuberculosis by detecting mycobacteria, identifying them and measuring their susceptibility to antibiotics. Because the tuberculosis bacillus grows slowly, several weeks may be needed for these results. Culture in liquid medium has significantly reduced growth time, but measuring antibiotic susceptibility still takes time and is sometimes impossible, particularly for second-line antibiotics. However, many molecular tests for detecting, identifying and testing antibiotic susceptibility have been reported over the last 20 years. These methods do not require a culture and can significantly reduce diagnosis time from a few weeks to a few days. Implement molecular tests that can sensitively detect the presence of *M. tuberculosis* in clinical samples, as well as its possible resistance to antibiotics are very needful (2012).

The regions of the world most threatened by antibiotic-resistant tuberculosis are among the poorest and cannot afford costly tests. How can the most suitable methods be provided to the most affected countries? Have needs changed? The purpose of this review is to examine the main molecular diagnostic methods being proposed to detect tuberculosis and its drug resistance, as well as how these methods are being applied to manage tuberculosis patients more quickly and appropriately.

2 Diagnosing Tuberculosis

There are clinical symptoms for diagnosing tuberculosis; however, etiological diagnosis is based on the appearance of bacilli in clinical samples of the infected site. Traditional diagnostic methods remain inadequate because they are long and painstaking. Direct sample examination can be used to screen for positive bacilli results. For pulmonary tuberculosis, direct

examination of sputum is fast and reveals the quantity of acid-fast bacillus, and thus the risk of contagiousness. However, direct examination has a low susceptibility of 22% to 78% and can only detect a concentration of 10^3 bacilli/ml or more in the sample. In addition, it is impossible to identify the mycobacterium species in question during direct examination. As a result, false-positives occur, particularly in heavily colonized samples from patients with other chronic pulmonary infections. Nevertheless, many countries use direct examination of samples as a quick test for diagnosing tuberculosis. Early diagnosis can improve patient survival and reduces the spread of the *M. tuberculosis* strain.

When microscopic exams are negative, culture is needed to screen for bacilli. Although there are molecular tests that should theoretically detect and identify very small quantities of bacilli, it is always necessary to culture *M. tuberculosis* to identify and test for antibiotic susceptibility. Automatic methods for detecting growth in a liquid medium have improved speed and sensitivity; however, these methods still take several weeks. Likewise, 30% of all tuberculosis strains combined, and 22% of pulmonary tuberculosis, remain bacteriologically unproven. Thus, selecting a treatment using traditional methods is empirical, and takes more than a month to confirm with the results of the antibiotic susceptibility test.

Traditional antibiotic susceptibility tests are conducted using the proportions method in a Löwenstein-Jensen medium (Grosset J 1990), or more rarely in 7H10, 7H11 or MGIT (Inderlied CB 1995). When unsuitable treatments are selected before results from antibiotic susceptibility tests are known, and a strain turns out to be resistant, it can lead to the emergence of multidrug resistant bacilli. Quickly evaluating the susceptibility of *M. tuberculosis* to TB drugs is vital in countries with a high rate of resistance.

The first gene amplification applications for diagnosing tuberculosis appeared 20 years ago (Hance et al. 1989). Since then, numerous developments have occurred.

3 Direct Molecular Detection in Clinical Samples

Since the use of gene amplification in 1986, several specific *M. tuberculosis* base sequences were able to be identified directly from clinical samples (Forbes 1997). However, until recently, this method worked primarily on positive samples under microscopic exam. The improved detection sensitivity of recent tests now allows use on smear-negative samples. The targets can be DNA or RNA, and those that are the most frequently amplified in *M. tuberculosis* are the *IS6110* insertion sequence, 16S ribosomal DNA, and 16S ribosomal RNA (16S r-RNA).

3.1 The first generation of Gene Amplification tests (NAA for Nucleic Acid Amplification)

There have been many studies published about detecting *Mycobacterium tuberculosis* Complex (MTB) in clinical samples. It is difficult to draw accurate conclusions about their sensitivity, specificity, and predictive values, given the wide disparity of DNA extraction techniques used, the selection of amplification targets, the amount of inoculum, amplification parameters, detection of amplification products, and populations studied. In the United States, only two marketed kits were approved by the FDA (Food and Drug Administration): the "Amplified *M. tuberculosis* Direct Test" (AMTD) (Gen-Probe, Inc, SanDiego, Calif.) and "Amplicor" (Roche Molecular Systems, Branchburg, N.J.). AMTD uses a constant temperature strategy based on ribosomal RNA, and Amplicor uses a polymerase chain reaction (PCR) to amplify a part of the 16S ribosomal RNA gene. These are the most widely used tests, but there are also various "in-house" tests.

Most studies have confirmed that amplification detects 5 to 100 mycobacterium colony-forming units (CFU) when it is applied to decreasing dilutions. However, this threshold is much higher when clinical samples are involved. The presence of inhibitors interferes with the amplification reaction and bacteria from the sample could be more difficult to lyse or concentrate by centrifuging, compared to bacilli in culture. This is particularly the case for fixed tissue samples. Moreover, patients undergoing tuberculosis treatment were found to be positive using NAA due to the stability of mycobacterial DNA.

- Respiratory Samples

Many studies have compared NAA tests with culture tests as the "gold standard"; clinical information can then clear up any uncertainties. NAA tests detect *M. tuberculosis* in nearly all pulmonary samples for which direct smear is positive (susceptibility from 95% to 96%; specificity from 99 to100%) and only half of those that are direct smear negative (susceptibility from 48% to 53%) (ATS 1997; Forbes 1997). The test result is closely correlated with the bacilli load and its breakdown in the sample. Therefore, the FDA only used the result of these tests when pulmonary samples were positive upon direct examination. Genprobe modified the AMTD test by increasing the volume of the sample, making it possible to widen the search for bacilli. This second generation AMTD was approved for detecting *M. tuberculosis* in case of negative results upon direct examination and a strong suspicion based on other criteria (basically clinical).

Another flaw in NAA tests is the occurrence of "false-positives". The use of Uracyl N Glycosylase (UNG) is aimed at eliminating contaminations by destroying amplicons generated by previous amplification tests.

Furthermore treated patients could be NAA positive because of stability of *M. tuberculosis* DNA.

- Extrapulmonary Tuberculosis

There is significant interest in using NAA tests when extrapulmonary tuberculosis is suspected because clinical diagnosis is often questionable and culture diagnosis is not sensitive enough. There have been many studies conducted, with susceptibility ranging from 54% to 92%. Clearly, the problems related to inhibitors found in the sample (particularly pleural fluid), sample volume, DNA extraction methods and so on, have a significant impact on results. Thus, the use of NAA tests may be inadequate and may do little to effectively diagnose TB, especially where diagnosis is difficult to implement (meningitis, pleuritis, aso).

- Clinical Use and Result Interpretation

Test effectiveness cannot be fully evaluated without taking the disease's prevalence into consideration. Sensitivity, specificity, positive predictive value (PPV), and the negative predictive value (NPV) of amplification tests were determined by comparing culture results with the clinical diagnosis; this was discussed in two literature reviews (Woods 2001; Piersimoni, Scarparo 2003). The clinical value of NAA tests depends largely on their PPV and NPV, which vary considerably according to the known probability of tuberculosis in the studied population. Very few studies have included this clinical probability in evaluating amplification tests. The performances of the AMTD2 test on samples from different patients classified according to clinical suspicion were studied (Catanzaro et al. 2000). The test's sensitivity for the low, average and high suspicion group varied 83%, 75% and 87% respectively, and specificity varied 97%, 100% and 100% respectively. These values are higher than those of direct smear. For the low-risk group (5% previous risk), direct smear and AMTD2 exclude tuberculosis as an etiology with an NPV of 96% for direct smear and 99% for the AMTD2. Neither of these tests conducted alone can provide reliable tuberculosis diagnosis, but the PPV of AMTD2 is better (59%) than that of DE (36%). For the high-risk group (87% previous risk) the PPV is even better: 94% for DE and 100% for AMTD2. However, only the AMTD2 effectively rules out tuberculosis in this group: NPV of 91% versus 37% for DE. The AMTD2 demonstrates the most effectiveness in the average suspicion group (29% of previous risk) with a PPV of 100% (30% for DE) and an NPV of 91% (71% for DE). This is a diverse group because it includes, more than in other groups, patients infected with atypical mycobacteria, seropositive HIV patients, and patients exposed to TB who have a positive intracutaneous reaction. The Piersimoni study (Piersimoni et al. 2005) on the Abbott LCx test (Abbott Park, IL) also concluded that the test provides a faster and more effective diagnosis in a group of patients with a high to moderate probability of tuberculosis.

Overall, studies that have been published have minimized the need for a more tailored use of gene amplification based on the clinical type of tuberculosis and the interpretation of the results based on the patient: previous history, clinical symptoms, and risk factors. To help laboratories and clinicians in this regard, the CDC has developed an algorithm. Three sputum specimens are collected three days in a row and then analyzed. If there are

three positive direct examinations (DE) and a positive NAA test, the patient is presumed to have tuberculosis without additional NAA testing. However, if only one DE is positive and the NAA test is negative, a test for inhibitors in the sample should be done. If there is no inhibitor, an NAA test should be performed on additional specimens (no more than three). If everything is negative, the patient is presumed to be infected with atypical mycobacteria. If the DEs are negative, an NAA test is conducted on the first specimen. Regardless of whether the patient is TB positive or negative, he/she must be monitored and other specimens should be tested. A patient is presumed not to be infectious if all of the direct exams and NAA tests are negative. This does not exclude the possibility of active TB and the clinician must rely on clinical symptoms and cultural tests to prescribe tuberculosis treatment and stop the etiological investigation. Ultimately, the patient's response to treatment and culture results will confirm or refute a diagnosis of TB.

It was initially though that gene amplification tests would be used when they had the greatest chance of influencing diagnostic and therapeutic decisions, as in the case where the probability of tuberculosis is neither high nor low (which does not correspond with FDA recommendations following an evaluation of these tests). However, these tests are only recommended when direct examination is positive, and therefore probability is high.

- Specific Laboratory Conditions

Using 3 separate rooms is compulsory. In order to prevent contamination, the technician must always use the 3 rooms in the following order: 1, 2 then 3, on a one-day period. The rooms must contain the necessary equipment. It is to stay in this same dedicated room and shall never be transferred to from room nr 3 to nr 2, from nr 2 to nr 1, or from nr 3 to nr 1. The thermal cycler cannot be placed in either room 1 or 2.

1. The DNA-free clean "white" room.

It is used to prepare the PCR mix.

Rules:

- The technician wears disposable lab coat, nurses cap, shoe covers and gloves.
- The pipettes, racks, and all equipments are new ones and are to stay in this room and be solely dedicated to PCR mix preparation.
- The mix is prepared and distributed in the tubes by a technician not having been in contact with amplified DNA on that same day.

Equipment:

- Disposable lab coat, nurses cap, shoe covers and gloves
- PCR cabinet (protection of the product thanks to a vertical laminar flow) or, if not available, a

clean work area dedicated to PCR mix.

- New 1000, 200 and 20 µL pipettes
- Disposable sterile pipette tips with filter
- Absorbent paper (if no PCR safety cabinet available)
- 200 µL PCR micro tubes (+ 96 wells microtiter plates or strips of 8 PCR tubes)
- Eppendorf tubes for mix preparation
- Eppendorf racks and disposable micro tube racks
- Refrigerator et -20°C freezer for PCR reagents conservation

2. The "grey" room dedicated to non-amplified DNA handling.

It is used for the introduction of DNA in the PCR mix.

Rules:

- The technician wears a clean lab coat
- The pipettes, racks, and all equipments are new ones and are to stay in this room and be solely dedicated to non-amplified DNA handling.
- The reaction tubes prepared in the white room enter the gray room on a disposable rack that is to never re-enter the white room.
- The technician not having been in contact with amplified DNA on that same day will be the one to handle the non-amplified DNA in this room.

Equipment:

- Clean working surface or laminar flow safety cabinet protecting both the product and the technician
- 20 µL Pipette
- Disposable sterile pipette tips with filter
- Absorbent paper (if no safety cabinet available)
- Eppendorf racks and disposable micro tube rack
- Container for infectious clinical waste

3. The "black" room for amplified DNA handling.

It is used for the detection of amplified DNA

Rules:

- The technicians wears black room dedicated lab coat and gloves
- Pipettes, racks and all equipments are to stay in this room and be solely dedicated to amplified DNA handling.
- After amplification, the tube enters the black room on a disposable rack that is to never re-enter the white and grey rooms.
- The technician handling amplified DNA can not return to the white and grey rooms on the same day in order to prevent contamination by introducing already amplified DNA in a new amplification process.

Equipment:

- Clean working surface
- Gloves
- 1000, 200 and 20 µL Pipettes
- Disposable sterile pipette tips with filter
- Absorbent paper
- Micro tubes racks
- Plastic tweezers to handle the test strips
- Container for infectious clinical waste
- Refrigerator for GenoType kits and amplified DNA conservation
- Twincubator

It is extremely important that molecular biology laboratories undergo rigorous inspections. These inspections begin with the evaluation, in each laboratory, of susceptibility values and the specificity of the tests performed. Systems are accurate and undergo regular inspections. Inhibitors are detected by setting up an internal control and a positive control in each test. Outside quality control validates the results. This critical need is underscored by a European study that found widely varied results in 30 different laboratories (Noordhoek et al. 1994; Noordhoek, van Embden, Kolk 1996). Only five of these 30 laboratories screened correctly for *M. tuberculosis* in all of the clinical specimens.

The cost of NAA test is much higher than the cost of traditional methods. Conducting all of these tests in just a few "expert" laboratories within a network appears to be a good way to lower the cost.

3.2 Recent NAA tests

- **The Cepheid GeneXpert System (Sunnyale, CA)**

The Cepheid Gene Xpert System is a single-use sample-processing cartridge system with integrated multi-color real-time PCR capacity that has the potential to greatly simplify nucleic acid amplification tests.

Based upon analysis of the core region of the rpoB gene, it simultaneous detects M. tuberculosis and RIF resistance directly from smear-negative and smear-positive sputum samples, within 2 hours 20 mins (including sample decontamination) (Helb et al 2010).

- **The GenoType MTBDRplus version 2.0 test (Hain Lifescience, Germany)**

GenoType MTBDRplus version 2.0 test detects MTC DNA and mutations commonly associated with RIF-R and INH-R with a greater sensitivity than previous version 1.0 (see paragraph Rifampicin resistance and isoniazide resistance).

4 Molecular Identification of Mycobacterial Species

Traditional methods for identifying mycobacteria rely on growth characteristics and certain biochemical tests. These methods are tedious, slow, and inconclusive. In addition, results from the same test can vary among isolates of the same species and the phenotype profiles will overlap and make identification impossible.

New molecular biology techniques have made significant progress in this field. The hybridization of ribosomal RNA gene probes has made it much easier to identify the most frequent mycobacterial species. AccuProbes (Gen-Probe, Biomérieux, France), combined with a non-radioactive marker, acridinium ester, can identify tuberculosis complex bacilli obtained in culture in solid or liquid medium in less than two hours (Lebrun et al. 1992; De Beenhouwer et al. 1995).

The nucleic amplification tests on the market effectively recognize the main mycobacteria species, particularly for early species identification in liquid medium cultures. This approach reduces the identification time of *M. tuberculosis* in culture and significantly improves detection sensitivity by bypassing the problem of inhibitors. There was a test that was first marketed (INNO-LiPA Mycobacteria; Innogenetics) in which the 16S-23S intergenic region was amplified and then hybridized to a membrane on which are attached probes that recognize mycobacterial species revealed by colorimetry (Cooksey et al. 1997). This same reverse hybridization principle was used to develop GenoType MTBC tests (Hain Lifescience GmbH, Nehrin, Germany), which use multiplex amplification of DNA fragments (*23S r-RNA*

gene, RD1 region, and *gyrB* gene) to differentiate *M. tuberculosis* complex species (*M. tuberculosis*, *M. bovis ssp bovis, M. bovis BCG, bovis ssp caprae, M. africanum, M. canettii* and *M. microti*) (Richter et al. 2003). Moreover, GenoType Mycobacterium (Hain Lifescience GmbH, Nehrin, Germany) CM/AS is used to identify atypical species from *23S r-RNA*. The GenoType MTBDRplus test is explained in the chapter on TB drug resistance. This test can identify *M. tuberculosis* complex and determine its resistance to rifampicin and isoniazid by detecting the primary mutations in question.

Direct sequencing of hypervariable regions of the *rrs* gene, which codes for 16S r-RNA, has become a universal method (Kirschner et al. 1993; Han et al. 2002). The first 500 bases of the gene include two hypervariable regions among the species, particularly at slow growth. The MicroSeq 500 16S rDNA Bacterial ID system uses a gene database that codes for 16S r-RNA, developed by Perkin Elmer/Applied Biosystems (Foster City, Calif.), for the PCR-sequence identification of more than 80 mycobacterial species. When the conserved character of the *rrs* gene restricts the differentiation of certain species, sequencing other genes will provide specificity: especially sequencing a heat shock protein gene (65 kD *hsp65*) (Telenti et al. 1993b; Ringuet et al. 1999; McNabb et al. 2006), RNA polymerase (*rpoB*) (Kim et al. 1999; Adekambi, Colson, Drancourt 2003) (GeneXpert MTB/RIF test that uses this target is explained at Rifampicin resistance chapter), the 16S-23S intergenic sequence (Roth et al. 1998), superoxide dismutase (*sodA*) (Zolg, Philippi-Schulz 1994), or the *gyrB* gene, particularly for recognizing *M. tuberculosis* complex species (Kasai, Ezaki, Harayama 2000; Richter et al. 2003).

The PCR sequencing technique has become the most reliable method for identifying mycobacteria due to data banks on the World Wide Web, to which increasingly more bacterial sequences are being added. These are several examples: Ribosomal Differentiation of Medical Microorganisms (RIDOM, http://www.ridom-rdna.de/) Ribosomal Database Project (http://rdp.cme.msu.edu:html/) (Harmsen et al. 2002), European Molecular Biology Laboratory (http://www.ebi.ac.uk/embl/), BIBI (http/pbil.univ-lyon1.fr/bibi/query.php) (Cloud et al. 2002; Devulder et al. 2003; Devulder, Perouse de Montclos, Flandrois 2005) and GenBank (http:/www.ncbi.nlm.nih.gov/). Sequence results are compared to bank data. Creating a phylogenetic tree makes it possible to measure the degree of homology with known sequences in order to identify the analyzed species or recognize the closest one (Devulder et al. 2003; Adekambi, Drancourt 2004). However, these analyses have certain limitations due to incorrect results in the banks, inconsistent sequence quality or unverified sequence submissions, which could lead to identification errors. Even when working with a "perfect" data bank, it is not necessarily easy to interpret homology with a sequence indexed in the data bank. It may be impossible to differentiate species from the same complex or same mycobacterial group. If the bank only contains one sequence for a specie, it cannot be used to find information about the intra-species genotypic variation. In addition, no set threshold has been established for the number of base pair differences required to differentiate

one species from another. For mycobacteria, values from <0.8 to 2.0% have been reported for the *rrs* gene (Turenne et al. 2001; Cloud et al. 2002). Studies are needed to determine whether or not thresholds would be specifically assigned to the phylogenetic diversity of species (Hall et al. 2003; Hall, Wohlfiel, Roberts 2003).

For lack of sequencing, some teams analyze the restriction profile of amplification targets such as the *rrs* or *hsp65* gene. This tedious method cannot easily standardize electrophoresis conditions, which makes it difficult to exchange data from one lab to another. In contrast, nucleotide sequences can be reproduced and easily shared between labs. They can recognize known and unknown mutations.

Overall, the cost of molecular biology identification methods must be compared with traditional methods (need for cultures in different mediums, incubated at different temperatures, biochemical tests, and other methods). These procedures take several weeks during which the clinician has no result, which could delay specific treatment and extend hospitalization. Sesuencing cost is become very competitive.

Thus, molecular methods have significantly improved the identification of mycobacteria. They completely replace biochemical tests at an early stage in culture growth.

Comment: analyzing DNA typing can help identify the species name. Such is the case, for example, of the special profile of *M. bovis* in spoligotyping. However, epidemiology methods are not presented in this review.

5 Molecular Detection of Drug-Resistant *M. tuberculosis*

M. tuberculosis becomes resistant to antibiotics by chromosomal mutation, which confers resistance to a TB drug. The accumulation of these mutations leads to multi-drug resistance. Unlike horizontal transfers, which involve the acquisition of DNA fragments with different origins, mutations that confer resistance to TB drugs affect isolated portions of bacterial chromosome. Thus by detecting them, the strain's resistance can be predicted. This is particularly easy in the case of antibiotics to which resistance is caused by the alteration of an essential gene matching the antibiotic target. Very few mutations could cause a loss of interaction between the target and the antibiotic without affecting the gene's function. Thus, only a few permissive mutations of the antibiotic's target will be observed. In the case of resistances to rifampicin (RIF) or fluoroquinolones (FQ), permissive mutations primarily affect a small fragment of *rpoB* (coding for RNA polymerase) and *gyrA* (coding for DNA gyrase) genes respectively. This is also the case for aminoglycoside resistance, which is caused by mutations in the translation machinery genes (*rrs and rpsL*) and can only be caused by permissive mutations. These mutations, which affect vital genes, lead to a loss of affinity for the antibiotic without destroying the gene's function. However, when antibiotic resistance is caused by the loss of function of a non-essential gene, a broad spectrum of mutations will be observed, which makes detecting them difficult. This will involve any mutation that inactivates the gene. In order to detect these mutations, the complete sequence of the mutated genes will need to be known. In addition, mutation will not necessarily mean the loss of gene function, and therefore antibiotic resistance. This is the case for example, of the *pncA* gene, which is inactivated by mutation in the event of resistance to pyrazinamide (PZA), and the genes involved in isoniazid (INH) resistance. The *pncA* gene is a small gene that can be easily sequenced to find mutations. However, a large number of mutations have been observed in strains susceptible to PZA. These mutations do not affect gene function. High-level resistance to INH is often due (approximately 75% of cases) to the same point mutation in *katG*. Thus isolating it for a large number of strains is easy and can be done to identify the resistance. Here we have described mutations that determine resistance to most first-line antibiotics and a few second-line antibiotics. Resistance can only be linked to a mutation on one gene or be the result of single or multiple complex mutations on different genes as in the case for INH resistance for example.

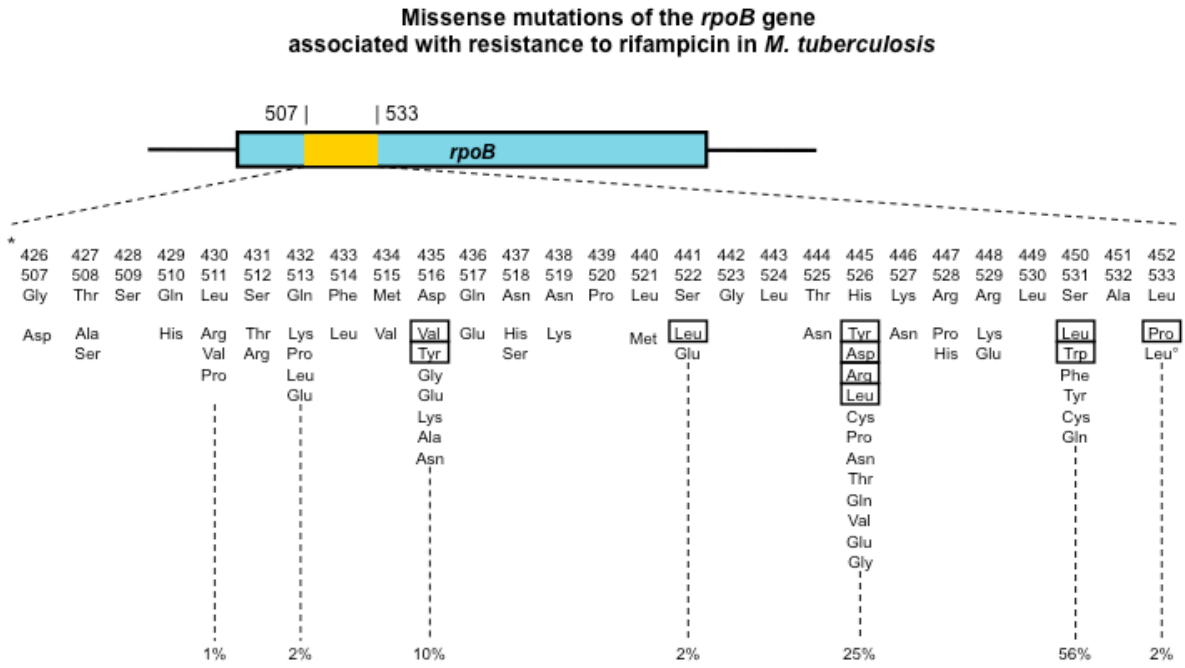
There are many molecular techniques available for studying antibiotic susceptibility. Most of them are based on amplifying a specific target zone of an antibiotic, followed by the analysis of an amplified product to identify the gene mutation associated with the resistance to this antibiotic. The presence or absence of mutation can be detected by several methods. A large portion of this review has been devoted to sequencing, which is the most accurate method. Although other techniques for identifying gene mutations

have not been ruled out, not all of them have been mentioned here. A review describes these methods (Eisenach 1999).

An update on drug resistance mechanisms in *M. tuberculosis* and areas that need further studies has recently been published (Zhang, Yew 2009).

5.1 Rifampicin Resistance

In use since 1966, Rifampicin (RIF) is an important component in treating tuberculosis. It is a broad spectrum rifamycin derivative that interferes with the synthesis of mRNA synthesis by binding to the ß subunit of RNA polymerase (RpoB) in bacterial cells. The RIF binding site is a pocket in the upper wall of the main channel for double-stranded DNA entry just upstream of the polymerase catalytic center. The various RIF-resistant mutations are clustered around this pocket (Zhang 2000). Thus, RIF resistance is associated with a hotspot (codon 507 to 533) core region called RRDR, for "rifampicine resistance determining region" (81 bp) of the *rpoB* gene (Telenti et al. 1993a; Telenti et al. 1997a). More than 95% of RIF^R *M. tuberculosis* has a mutation in this specific zone (Zhang Ying 2005).



* *M. tuberculosis rpoB* gene
Codons are numbered according to the *rpoB* gene of *Escherichia Coli*

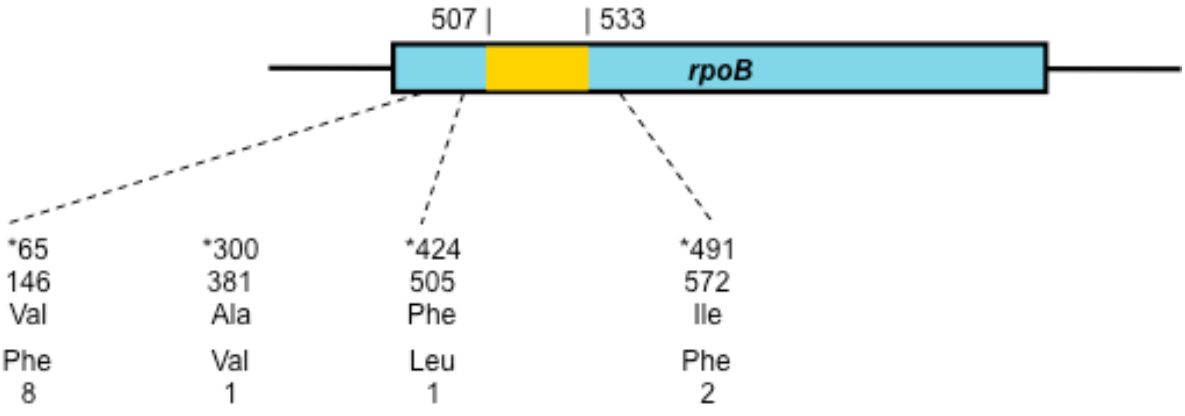
Resistance occurs at a frequency of 1 out of 10^7 to 10^8 bacterial cells. Most RIF-resistant strains show one mutation in the gene. Two to four mutations are rarely reported (Mani et al. 2001; Sekiguchi et al. 2007). The most prevalent mutations (81%) affect codons 531 and 526 and usually lead to a **high level** of phenotypical resistance (MIC > 64 µg/ml) as well as cross resistance to other rifamycins (Riska, Jacobs, Alland 2000; Zhang Ying 2005). Mutations 511, 516, 518 and 522 result in a **low-level** resistance to RIF and rifapentine; and some susceptibility to rifabutin (Zhang Ying 2005).

At the same time, mutations in this hotspot region seem to confer **low phenotypical resistance** (deletion of codon 508-509, mutation at 515) (Taniguchi et al. 1996) or **variable resistance** (L533P) (Kim et al. 1997), which could lead to an overly hasty interpretation of resistance. The latest observations of Asian strains suggest a geographic variability that can influence the accuracy of genotypic tests (Riska, Jacobs, Alland 2000).

Silent mutations (Leu511 and Leu521) have been reported in resistant strains. Interestingly, the L511L mutation is always associated with other mutations that confer resistance (Siddiqi et al. 2002). In rare cases, double mutations appear to have an **additive effect** on the degree of resistance. The role of mutations, combined with those known to confer resistance, is uncertain, as in the case of S509R described with H526R (Sekiguchi et al. 2007).

Finally, less than 5% of resistant strains do not show a mutation in the *rpoB* resistance region (Riska, Jacobs, Alland 2000; Mani et al. 2001). Rare **loci found outside the hotspot region** of *rpoB* are associated with resistance without associated mutation known for conferring resistance.

Missense mutations of the *rpoB* gene *(outside of the core region)* associated with resistance to rifampicin in *M. tuberculosis*



**M. tuberculosis rpoB* gene
Codons are numbered according to the *rpoB* gene of *Escherichia Coli*

Mutated strains in Val146Phe (Zhang 2000; Heep et al. 2001) and Leu251Phe show a low-level resistance (MIC 4 µg/ml) (Rigouts et al. 2007). The Ala381Val mutation (Taniguchi et al. 1996) is described on a strain of MIC 200, with no other mutation on the *rpoB* gene. Phe505Leu confers cross-resistance to both RIF and rifabutin (Fang et al. 1999).

Most **susceptible strains** show no mutation, except for a few: six susceptible Japanese strains are mutated in TCG Ser 450 Leu TTG, CTG Leu 521 Pro CCG, CTG Leu 533 Pro CCG, GCC Ala 679 Ser TCC (two strains) and CGC Arg 687 Pro CCC (Taniguchi et al. 1996).

Missense mutations of the *rpoB* gene associated with sensitivity to rifampicin in *M. tuberculosis*

* 369	430	431	432	434	440	452	598	606
450	511	512	513	515	521	533	679	687
Ser	Leu	Ser	Gln	Met	Leu	Leu	Ala	Arg
Leu	Arg	Thr	Gln	Val	Pro	Pro	Ser	Pro
1	1	1	1	1	3	1	2	1

* *M. tuberculosis rpoB* gene
Codons are numbered according to the *rpoB* gene of *Escherichia Coli*

The CTG Leu 533 Pro CCG mutation has been shown on two strains of low-level resistance (MIC 12.5 µg/ml) and on a strain that is susceptible according to phenotypic tests, yet clinically resistant (Riska, Jacobs, Alland 2000). Other studies describe some sensitive strains with mutations as Ser 450 Leu (Sekiguchi et al. 2007), Leu 511 Arg or Ser 512 Thr (Moghazeh et al. 1996) or Gln CAA 513 Gln CAG (Kim et al. 1997). Genotypic detections would therefore be more sensitive in certain circumstances.

Resistance to rifampicin, which usually develops with resistance to INH, thus defines the strain as multi-resistant (MDR). However, there are rare isolated mono resistances to rifampicin (Ridzon et al. 1998).

The first mutation detection studies were based on PCR sequencing or PCR-SSCP (single strand conformational polymorphism analysis (SSCP)). Now, resistance is frequently screened by one of the methods on the market (INNO-LIPA Rif.TB or GenoType MTBDRplus, HAIN) (Hillemann, Rusch-Gerdes, Richter 2007; Crudu et al. 2012). In these tests, a sample is amplified with biotin-labeled primers. This amplification product is hybridized on a test strip containing oligonucleotides. The reaction identifies the signals as parallel marks on the test strip. Thus the presence or absence of a mutation is easily detected in a few hours. To identify rifampicin resistance, the Hain MTBDRplus test strip has 8 probes that recognize wild sequence and four that correspond to the most frequently observed mutations (S531L, H526Y, H526D, and D516V of *rpoB*, mutations observed in 75% of RIF^R). The test is rapid and easy, and can be applied to clinical samples or to advanced cultures. There is strong correlation with results obtained through phenotypic susceptibility measurements (De Beenhouwer et al. 1995), automatic sequencing (Cavusoglu et al. 2002; Brossier et al. 2006; Hillemann, Rusch-Gerdes, Richter 2007), or RNA-RNA mismatch assay (Mokrousov et al. 2002a).

Based upon *rpoB* core region analysis, the new Cepheid gene Xpert System's hands-free sputum processing and real-time PCR system (with rapid on-demand, near-patient technology), simultaneously detects *M. tuberculosis* and RIF resistance within 2 hours from smear-negative and smear-positive clinical sputum samples. The self-contained cartridge fluidics of the Xpert MTB/RIF assay make it possible to design a hemi-nested PCR assay with a sensitivity that approaches culture-based diagnostics in a closed system that avoid PCR contamination. An internal control target detects false-negative results. The specificity of the MTB/RIF test in the diagnosis of TB has been shown

to be very high (97-100%) in demonstration studies coordinated by the Foundation for Innovative New Diagnostics (FIND). (Boehme et al. 2010; Boehme et al. 2011) The sensitivity differed between pulmonary TB patients whose sputum was positive on smear microscopy and culture and those who were positive on culture only. Taking culture as the gold standard, the sensitivity is >95% for direct sputum smear-positive samples, and varies between 65% and 77% if direct sputum sputum smear is negative (Boehme et al. 2010; Boehme et al. 2011; Marlowe et al. 2011; Moure et al. 2011; Vadwai et al. 2011) with an incremental gain in sensitivity when the number of tests is increased from one to three. Results of RIF resistance need to be interpreted according to the prevalence of MDR-TB. Given the 95% sensitivity and 98% specificity of the MTB/RIF test in the detection of RIF resistance (Boehme et al. 2010), the positive predictive value of the test varies from >90% to 32% if the prevalence of RIF resistance is >15% or 1% (Trebucq et al. 2011).

In summary, the RIF binding site to polymerase is a pocket around which the mutations observed on resistant strains are clustered. These mutations are found in nearly all of the strains (>95%). Thus, analyzing this core sequence makes it easy to detect RIF^R strains and predict their resistance level. It also provides strong concordance among results from different geographic regions. Furthermore, the frequency of mutations among resistant strains is very high: 100% (n=28) in Japan (Sekiguchi et al. 2007), 100% (n=93) or 98% (n=44) in two Indian studies (Mani et al. 2001; Siddiqi et al. 2002). Some silent mutations are observed. Diagnostic difficulty could be caused by rare inconsistencies where mutated strains are phenotypically susceptible. One of them may even be clinically resistant, which could suggest greater genotypic test sensibility.

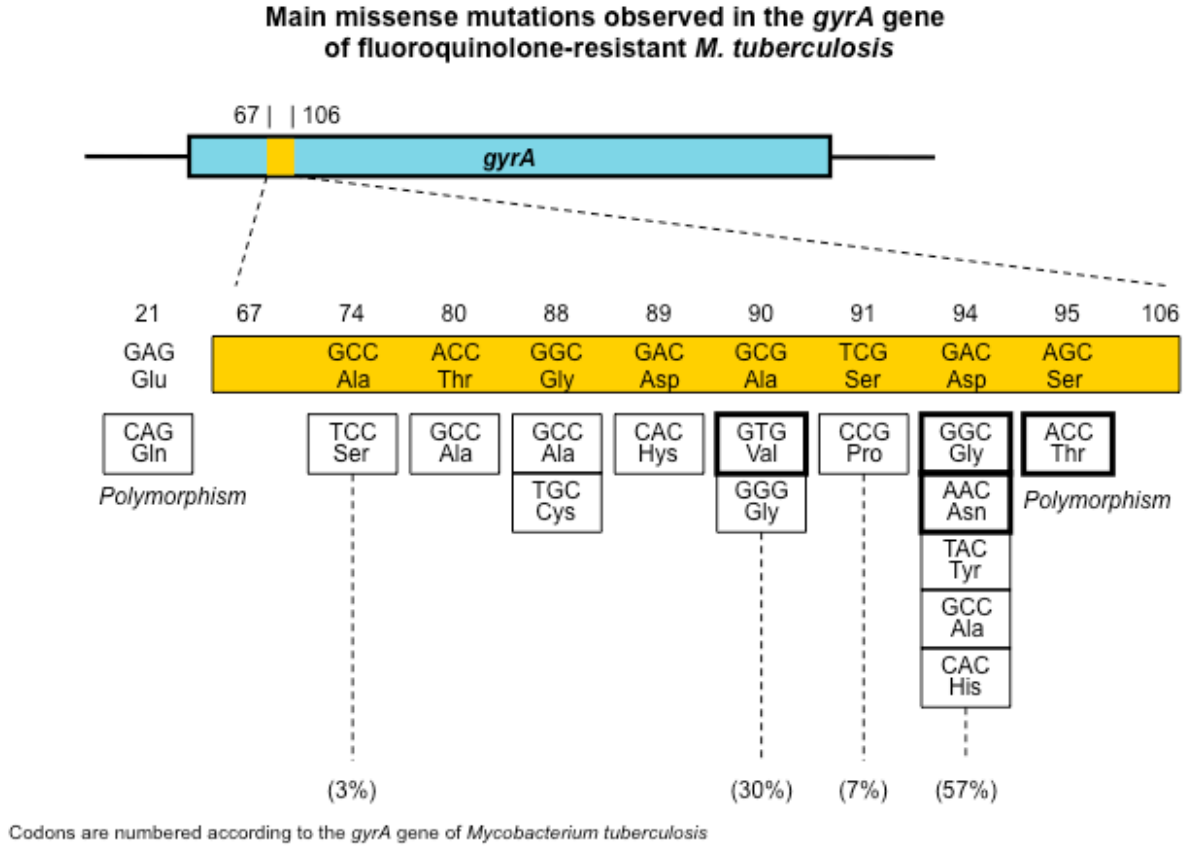
Commercial amplification/reverse hybridization kits or real-time PCR system provide rapid, effective detection of the mutations most commonly responsible for rifampicin resistance.

5.2 Fluoroquinolone Resistance

The antimycobacterial activity of Fluoroquinolones (FQ) has been demonstrated since the 1980s. These are second-line antibiotics whose indications are restricted to multi-resistant tuberculosis because of a very high rate of mutations (frequency 1 out of 10^7 to 10^8 bacteria), which confers resistance to quinolones. Moxifloxacine is the most active on *M. tuberculosis* in vitro.

All of the quinolones act by inhibiting DNA supercoiling, thus preventing replication and cell division. They block topoisomerase II (called DNA gyrase) of *M. tuberculosis*, a heterotetramer with two A and B subunits coded by the *gyrA* and *gyrB* genes.

The majority of FQ-resistant strains shows mutations in a conserved core region (codon 67 to 106), known as the "QRDR" for Quinolone Resistance-Determining Region, of the ***gyrA*** gene (2517 bp) (Takiff et al. 1994; Musser 1995; Xu et al. 1996; Ramaswamy, Musser 1998; Riska, Jacobs, Alland 2000; Zhang 2000; Ginsburg, Grosset, Bishai 2003; Maruri et al. 2012)



Codons are numbered according to the *gyrA* gene of *Mycobacterium tuberculosis*

Strains with high levels of resistance (MIC > 2 µg/ml to ofloxacine) show mutations at codons 88, 89, 90, 91, 94 and 95. The most **frequent** mutations are in position 94 (D94G or D94A) and position 90 (A90V) (Siddiqi et al. 2002; Cheng et al. 2004; Bozeman et al. 2005; Aubry et al. 2006; Shi et al. 2006; Cheng, Xu, Liu 2007). Mutations are often multiple and are thus associated with a high level of resistance (Shi et al. 2006; Sekiguchi et al. 2007). Oddly

enough, **a polymorphism at codon 95** of *gyrA*, found in 15% of strains, is a genetic evolution marker, unrelated to an increase in resistance level (Sreevatsan et al. 1997a). In addition, the S95T variation was the most frequently found polymorphism in several studies, yet had no direct role in resistance development (Siddiqi et al. 2002; Huang et al. 2005; Shi et al. 2006). In 2007 Sekiguchi reported a new mutation, E21Q, upstream from the area of known mutations, which was missing from the H37Rv strain, but found among all of the strains in his study (n=138), both susceptible (n=135) and resistant (n=3) (Sekiguchi et al. 2007). A line probe assay is now able to detect quinolones resistant strains (Hillemann, Rusch-Gerdes, Richter 2009).

Quinolone-resistance sites have rarely been found in the ***gyrB* gene** of *M. tuberculosis (Yoshida et al. 1991; Takiff et al. 1994; Lee et al. 2002; Veziris et al. 2007; Wang et al. 2007; Mokrousov et al. 2008; Maruri et al. 2012)*. D426N, N469D, D505A, N510D and Q549H mutations (*E coli* numbering) were described on clinical isolates of quinolone-resistant strains, but because of the numbering used, *gyrB* mutations positions remain ambiguous. Maruri (Maruri et al. 2012) reviewed mutations in DNA gyrase. Four different GyrB numbering were reported, resulting in mutation location dicreapancies. The authors proposed the 2002 numbering system (Camus et al. 2002)as consensus.

Overall, the frequency of mutations on the *gyrA* and *gyrB* genes among FQ-resistant strains varies between 10.3% (n=69) in India (Siddiqi et al. 2002), 50% in Taiwan (Huang et al. 2005), 55.2-58.8% in Hong Kong (Yew, Chau 2002; Cheng et al. 2004), 60% in Thailand (Pitaksajjakul et al. 2005), 89.5% (n=29) in Italy and Abkhazia (region of Georgia) (Giannoni et al. 2005), and up to 100% (n=3) in Japan (Sekiguchi et al. 2007).

Moreover, some low-level resistance strains **have no mutation** in the *gyrA* or *gyrB* loci, which suggests the use of other mechanisms (Riska, Jacobs, Alland 2000).

It is interesting to note that the *M. tuberculosis* genome does not contain the counterpart to topoisomerase IV, whose mutation leads to other quinolone-resistant bacteria.

Surprisingly, *gyrA* T80A and A90G mutations confer quinolone **hypersensitivity** (Aubry et al. 2006).

FQs are active *in vitro* on *M. tuberculosis*. They must remain indicated for use as a second-line treatment for multi-resistant tuberculosis because of their high mutation rate, as well as contraindications and drug interactions (rifampicin and retroviral treatments for example). The primary mechanism of resistance is a modification in the DNA gyrase through the acquisition of permissive mutations of the *gyrA* gene, on a small gene fragment, which confers a high level of resistance without significantly affecting gene function. The position of *gyrA* mutated amino acids can determine the level of resistance to FQ (Kocagoz et al. 1996; Guillemin, Jarlier, Cambau 1998). The mutations observed are connected with the bonding site of FQs with the DNA

gyrase complex (Yoshida et al. 1991; Takiff et al. 1994). Rare mutations on *gyrB* have been described as conferring a low level of resistance.

The fact that MICs are only higher for MDR strains (Huang et al. 2005) suggests that taking FQs for bacterial infections is not responsible for increasing FQ resistance. Rather, factors related to multi-resistance risks (intermittent compliance with treatments and AIDS) could be the cause. Recently, a correlation was demonstrated between resistance to a first-line TB drug and past tuberculosis treatment history (Wang et al. 2007). Multi-resistance was the strongest correlation observed (Xu et al. 2009). Neither previous use, nor exposure time to fluoroquinolones, influenced *M. tuberculosis* resistance to these drugs.

Currently, the possibility of the clonal dissemination of FQ-resistant MDR strains has been ruled out by the observation of different resistance profiles.

Other resistance mechanisms are likely. The **efflux pump**, LfrA, confers a low level of resistance to *M. smegmatis*; however, a resistance mechanism for quinolones has not been demonstrated with *M.* tuberculosis (Zhang Ying 2005). However, when this gene is induced, a low level of resistance to quinolones develops and *gyrA* mutations occur more easily, which leads to a high level of resistance (Riska, Jacobs, Alland 2000).

It is troublesome that the FQ resistance rate is increasing. The discretionary use of FQs for MDR strains or in cases of intolerance to all other TB treatment (based on preliminary sensibility tests) could stop this increase (Riska, Jacobs, Alland 2000).

The presence of a limited diversity of single, specific mutations has enabled the use of molecular tests to detect resistance in a large number of strains. The genotype MTBDRsl (Hain) test is based on amplification/reverse hybridization and provides effective detection of the *gyrA* mutations most commonly responsible for fluoroquinolone (FQ) resistance (Ofloxacin and moxifloxacin). Wild type probes have been designed that hybridize to the most important resistance region of the *gyrA* gene. When all wild type probes of a gene stain positive, there is no detectable mutation within the examined region. Hence, the strain tested is sensitive to FQ. When a mutation exists, the respective amplicon cannot bind to the corresponding wild type probe. The absence of a signal with at least one of the wild type probes indicates resistance to FQ. The mutation probes detect some of the most common resistance mediating mutations. The banding pattern obtained with the *gyrA* probes allows a conclusion to be drawn about resistance to FQ

Mutations in the gene *gyrA* and the corresponding wild type and mutation probes according to .(Kocagoz et al. 1996; Cheng et al. 2004; Aubry et al. 2006; Matrat et al. 2006)

Failing wild type probe(s)	Codon analyzed	Mutation probe	Mutation	Phenotypic resistance*
gyrA WT1	85-90		G88S	FQ^R
gyrA WT1	85-90		G88T	FQ^R
gyrA WT2	89-93	*gyrA* MUT1	A90V	FQ^R
gyrA WT2	89-93	*gyrA* MUT2	S91P	FQ^R
gyrA WT3	92-97	*gyrA* MUT3A	D94A	FQ^R
gyrA WT3	92-97	*gyrA* MUT3B	D94N	FQ^R
gyrA WT3	92-97	*gyrA* MUT3B	D94Y	FQ^R
gyrA WT3	92-97	*gyrA* MUT3C	D94G	FQ^R
gyrA WT3	92-97	*gyrA* MUT3D	D94H	FQ^R

* FQ^R : fluoroquinolones-resistance

5.3 Isoniazid Resistance

Since 1952, Isoniazid (INH) has been one of the most effective and specific agents for treating *M. tuberculosis* infections. This is an essential TB drug that, along with RIF and PZA, forms the base for treating susceptible tuberculosis and is also widely prescribed for treating latent infections. INH is very active (MIC 0.02 to 0.2 µg/ml) on *M. tuberculosis* thanks to a simple structure made up of a pyridine ring and a hydrazide group, two essential components to its powerful activity against increasing *M. tuberculosis* (Zhang Min et al. 2005).

It has one of the most complex modes of action among all antibiotics. INH is a prodrug that requires activation of the catalase-peroxidase enzyme (KatG) coded by the *katG* gene (Zhang et al. 1992). Once activated, INH seems to destroy the synthesis of mycolic acids of the cell wall by inhibiting NADH-dependent enoyl-ACP reductase coded by the *inhA* gene. It encourages the bacterial cell to produce toxic free radicals.

Resistant strains appeared shortly after the use of INH in clinical treatment, many of whom had lost their catalase activity. The increasing observation of INH-resistant strains, up to 20-30% of strains in some regions (Cohn, Bustreo, Raviglione 1997), which is the first step towards multi-resistance, have cast doubts on its use as a first-line treatment of tuberculosis. Resistance develops readily with a high mutation frequency (1 for 10^6), higher than that of rifampicin (1 for 10^7 to 10^8). There are many molecular mechanisms causing INH resistance, but only some of them have been characterized. A recent review explains them by their molecular analysis (Vilcheze, Jacobs 2007).

Mutations in the ***katG*** gene, which hinder activation of the INH prodrug, are among the most frequent in INH-resistant strains: 20 to 95% INH-resistant strains have at least one mutation in the *katG* gene; (Zhang et al. 1992; Riska, Jacobs, Alland 2000; Ramaswamy et al. 2003; Zhang Min et al. 2005; Zhang Ying 2005; Hazbon et al. 2006; Jiao et al. 2007). The **S315T** mutation is the most frequent (75 to 90%) even up to 93.6% (Mokrousov et al. 2002b). It lowers catalase-peroxidase activity by 50% and leads to a relatively high level of resistance (MIC from 5 to 10 µg/ml), without cross-resistance to ethionamide. It alters INH activity by preventing bond formation between INH and KatG. Strains with a complete deletion of the *katG* gene may have an MIC equal to at least 50 µg/ml.

R463L substitution, found in *M. bovis*, *M. africanum* and *M. canettii*, is not considered to be a source of resistance in *M. tuberculosis*, where it is found in 15 to 30% of cases, regardless of whether the strain is susceptible or resistant (Sreevatsan et al. 1997a; Kiepiela et al. 2000). The Siddiqi study in 2002 (Siddiqi et al. 2002) analyzed 24 INH-resistant strains and reported other insertions, deletions, and substitutions on the *katG* gene: an AC deletion at position 30 on six strains, resulting in the synthesis of a small polypeptide; a G deletion at position 109 on two strains, resulting in a truncated polypeptide; an A inserted at 98 and a C at 185 on four and three strains respectively, causing premature polypeptide chain terminations. Only a few strains had new substitutions:

Ala61Thr, Thr12Pro, Thr11Ala, Asp73Asn and Asn35Asp. One strain had a partial gene deletion. All of the isolates in this study had the Arg463Leu mutation, which was not related to resistance.

Loss of catalase activity is not the only cause of resistance. Resistance can also develop through alterations or an overexpression of the **InhA** (Banerjee et al. 1994) target, which codes for NADH-dependent enoyl-ACP reductase. 0 to 5% of INH-resistant strains have a mutation in the structural gene while 8 to 20% have a mutation in the *InhA*, ***MabA*** (Zhang et al. 1992; Musser 1995; Banerjee et al. 1998; Zhang 2000; Ramaswamy et al. 2003) promoter. 20 to 34% of INH-resistant strains have at least one mutation in *MabA* promoter, which is either alone or combined with a mutation in the *InhA* gene. Hazbon (Hazbon et al. 2006) identifies 51 mutations in *inhA* in 3/608 (0.5%) of INH^S strains and 48/403 (12%) of INH^R strains. All of the promoter mutations (except G17T) are observed in INH^R strains, which are usually associated with the C-15-T mutation. All of the structural gene mutations (except I47T and I194T) were only detected on INH^R strains. The *inhA* S94A mutations, which seem to alter the INH-NADH bond with InhA, are restricted to INH^R strains (Leung et al. 2006; Vilcheze, Jacobs 2007). Most of the strains that have one mutation in *inhA*, with no mutation in *katG*, have a relatively low level of resistance > 0.2 µg/ml and < or equal to 1 µg/ml, without cross-resistance to ethionamide. In 1998, mutations of the ***kasA*** gene, which codes for ß-ketoacyl ACP synthetase, were reported in INH^R strains, with genetic complementation showing no proof that mutations were involved in resistance (Mdluli et al. 1998). These mutations were found in INH-resistant strains as well as INH-susceptible strains. In Hazbon's recent study (Hazbon et al. 2006), ***kasA*** mutations were identified at 66, 269 and 312 in 100/608 (16%) of INH^R strains and 44/403 (11%) of susceptible strains. The *kasA* G312S mutation is unrelated to resistance in the study of 98 strains (Sun et al. 2007). These results prove that none of the mutant *KasA* alleles are on INH-resistant strains alone. In three *M. tuberculosis* strains, overexpression of *KasA* did not increase resistance to INH or EMB, in contrast to InhA (Larsen et al. 2002). The role of *KasA* in resistance is still undetermined.

Mutations were found in the ***ahpC*** gene, which codes for an alkyl hydroperoxide reductase, for 10% to 13% of resistant strains (Riska, Jacobs, Alland 2000; Zhang 2000; Hazbon et al. 2006) and also in susceptible strains (Hazbon et al. 2006). Those that are located at position -46 (the most frequent), -9 (G at A) or D73H are not related to resistance. The only mutation found exclusively on INH-resistant strains is T51. These mutations are always related to mutations in *katG*, but rarely to those of codon 315 (Riska, Jacobs, Alland 2000).

Mutations in the ***ndh*** gene, which codes for NADH dehydrogenase, were found in a small number of clinical strains (3% of INH^R and INH^S strains for one study and 9% of INH-resistant strains in another study) (Lee, Teo, Wong 2001; Hazbon et al. 2006). These mutations confer resistance to INH and to ethionamide (see paragraph below). The most frequent is V18A, which was observed independently from susceptibility. Only one mutation in *ndh*, R268H, is found exclusively on clinical strains of INH-resistant *M. tuberculosis* (Lee, Teo,

Wong 2001; Hazbon et al. 2006; Vilcheze, Jacobs 2007). Other genes could be involved in resistance.

The ***NAT*** gene, coded for an arylamine N-acetyltransferase. The NAT protein of *M. tuberculosis* acetylates the INH nitrogen group, thus preventing the *katG* gene from activating it. *NAT* mutations observed in clinical strains of INH-resistant were always associated with mutations in *katG*, but also found in INH-susceptible strains (Ramaswamy et al. 2003; Vilcheze, Jacobs 2007).

Studies on clinical INH-resistant strains identified mutations on other genes such as *furA*, *Rv 0340-0343*, *Rv1772*, *fadE24*, *efpA* (Vilcheze, Jacobs 2007), and so on. These mutations were related for the most part to mutations in *katG* and/or in the *inhA* promoter, which makes it difficult to determine their role in INH resistance (Ramaswamy et al. 2003; Zhang Min et al. 2005).

Several studies have focused on the interactions between genes. Many of the strains (n=1,011) analyzed in Hazbon's study (Hazbon et al. 2006) were able to demonstrate a strong individual association between mutations of the *katG*, *inhA*, and *ahpC* genes and resistance to INH (P = 0.0001), while *kasA* mutations were associated with susceptibility. Remarkably, there were more mutations (particularly katG315) among MDR strains than among strains that were only resistant to INH. In this last case, mutations of the *inhA* gene were more frequent. Moreover, significant associations were described between mutations of the *katG*, *inhA*, and *ahpC* genes and RIF resistance, while reverse associations exist between *katG*315 and *inhA* or *ahpC* and between *kasA* and *ahpC* (Hazbon et al. 2006)

The phenotype for INH tolerance in *M. tuberculosis* strains is described as being genetically susceptible, but remaining active after INH treatment. The *iniA* gene of *M. tuberculosis* is associated with this phenomenon. Its overexpression confers a cross-resistance to INH and ethambutol (Colangeli et al. 2005).

In summary, there is high frequency of INH-resistant mutations (10^{-6}). INH-resistant strains most often show at least one mutation in the ***katG*** gene. This gene is located in a hypervariable region of the genome, containing repeated elements, and which as a result could contribute to the high frequency of mutations of this gene in resistant strains. There could be a boost in *katG* mutations at position 315 (the most frequent) because the mutations in this location seem to decrease INH activation without stopping catalase-peroxidase activity, which is a virulence factor in strains. *M. tuberculosis* can also offset mutations in the *katG* gene by overexpressing the *ahpC* gene (Hazbon et al. 2006).

The description of ***inhA*** S94A mutations, which seem to alter the bond between the INH-NADH compound and InhA, corresponds to known interactions between INH and InhA. This suggests that *inhA* mutations play a role in resistance (Hazbon et al. 2006). The role of the *KasA* gene in INH resistance remains undetermined. The outcomes of the mutations observed in the *ahpC* promoter or ndh gene suggest that these genes have very little or no effect on resistance.

Isoniazid resistance is thus complex. The genes involved are either non-essential, or extragenic mutations can compensate for their absence.

Multiresistance development could be influenced by the interactions between these genes. The complexity of detecting isoniazid resistance was highlighted in 2003 by the description of *katG* mutations, associated with resistance and identified on INH-susceptible strains. In addition, approximately 9 to 25% of resistant strains contain no mutations in targets known to confer resistance (Telenti et al. 1997a; Piatek et al. 2000; Lee, Teo, Wong 2001; Kim et al. 2003; Ramaswamy et al. 2003; Guo et al. 2006). The decline in the effect of INH may be caused by the persistence of INH-tolerant bacilli (Siddiqi et al. 2002; Wallis, Palaci, Eisenach 2007). This could explain the high rate of clinically-observed resistance.

The recently marketed GenoType MTBDRplus test (Hain) is able to detect resistance to isoniazid by mutation in the *katG* and *inhA* genes. Mutations are identified by the amplification method and reverse hybridization on test strips. One probe covers the wild-type S315 region of *katG*, while two others (probes katG MUT T1 and MUT T2) are designed to assess the AGC-to-ACC (S315T) and the AGC-to-ACA (S315T) mutations. For the promoter region of the *inhA* gene, an *inhA* WT1 probe covers the wild-type region from -9 to -22 and a second *inhA* WT2 probe covers the positions from -1 to-12. Four mutations (-15C/T, -16A/G, -8T/C, and -8T/A) can be targeted with the *inhA* MUT1, MUT2, MUT3A, and MUT3B probes. The absence of hybridization with a wild-type probe indicates resistance. This test can identify mutations in a few hours. Mutations in *katG* resulted in a high level of isoniazid resistance, and mutations in *inhA* resulted in low levels of resistance. The Hillemann evaluation (Hillemann, Rusch-Gerdes, Richter 2007) detected an association with *katG*315 or *InhA* mutations is 92% of INH resistant strains.

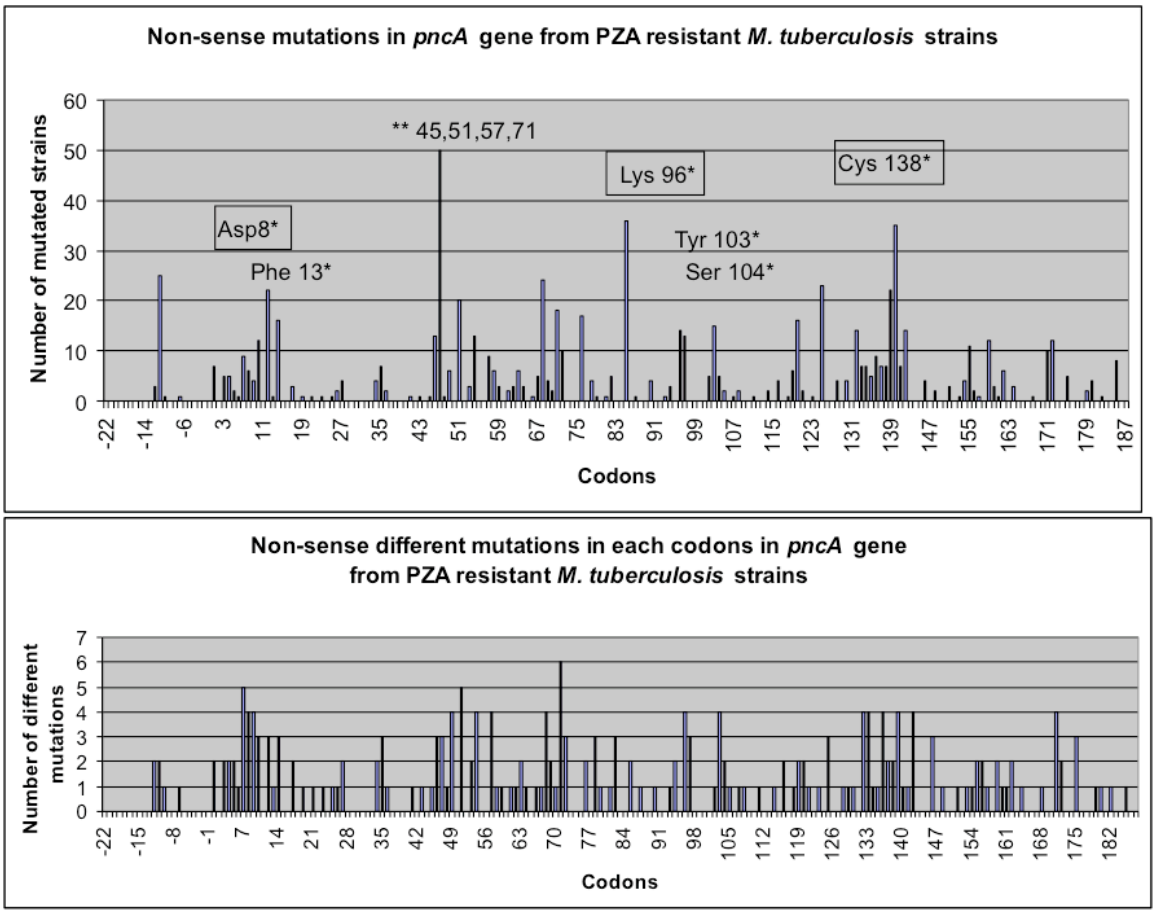
5.4 Ethionamide (ETH)

Ethionamide is a structural analog of INH. It is among one of the most effective and frequently used second-line tuberculosis drugs. INH and ETH target InhA, an enzyme involved in synthesizing mycolic acids. While mutations of the *katG* gene, particularly at 315, confer resistance to INH and not ETH, mutations in *inhA* or its promoter confer resistance to both INH and ETH. An EthA enzyme capable of activating ethionamide was identified. Mutations in *ethA* structural genes were associated with ethionamide resistance. Mutations of the *ethA* gene are spread out along the entire gene. In the Morlock study (Morlock et al. 2003), approximately 76% of high-level ethionamide-resistant strains showed such mutations.

5.5 Pyrazinamide

Pyrazinamide (PZA), like INH, is a synthetic derivative of nicotinamide, which has been used to fight tuberculosis since 1952. It is a bactericidal compound for a short-course, first-line tuberculosis treatment, used for its sterilizing effect against multiplying bacilli. In vitro, the effect of PZA only appears in an acid medium (possibly reproducing bactericidal activity within macrophages or granuloma), which is not conducive to *M. tuberculosis* growth and complicates evaluation of phenotypic sensitivity. Like isoniazid, it is a prodrug. It enters the cell through passive diffusion and is converted into pyrazinoic acid by the bacillum's cytoplasmic pyrazinamidase. Its mechanism of action is not fully known.

The only known resistance mechanism is the mutations present in the *pncA* gene, which codes for pyrazinamidase, associated with resistance. The loss of pyrazinamidase activity is strongly linked to PZA resistance (Cheng et al. 2000). 72% to 98% of PZA-resistant strains have at least one mutation in the *pncA* gene (Musser 1995; Scorpio et al. 1997; Riska, Jacobs, Alland 2000; Zhang 2000). The mutations are diverse (substitutions, insertions and deletions) and are spread out over the 561-bp *pncA* region and region of the promoter (-11). There are more favorable zones: Ala3, Cys14, Thr61, Leu85, Gly132, Thr142 (Scorpio et al. 1997; Lemaitre et al. 1999; Lemaitre et al. 2001; Zhang Min et al. 2005), which probably contain catalytic sites, metal ion binding and catalysis (Zhang Min et al. 2005).



Essential for activity zones : * catalysis ** metal ion binding

Several mutations in *pncA* are a property of PZA resistance in contrast with other mutations in the resistance associated genes. Although mechanisms are not so clear, the reason could be that pncA is not an essential gene so there is no selective pression on it and mutations could be well tolerated anywhere in the gene (Zhang Min et al. 2005).

Since 72% to 98% of PZA-resistant strains have a mutation in various loci *pncA*, which inactivates the gene, identifying resistance requires systematic sequencing of this gene. This remains possible given its small size. Detection of a wild-type gene indicates that the strain is probably susceptible to PZA. In contrast, detection of a known mutation indicates a potential resistance. The meaning of a new mutation is uncertain and has to be reliated to the AA change in the structure of the gene. NAA tests will be used however, especially since culture mediums are not always available with PZA.

All published mutations are indicated in the table at the end of this review. Line Probe Assays and microarrays were designated with all these mutations in order to detect PZA resistant strains.

BCG, known to be resistant to PZA, shows a substitution of C for G at position 169, leading to the H57D substitution of PncA, which makes the enzyme non-functional (Scorpio et al. 1997). *M. canettii* shows a A46A mutation suggesting that an alternative mechanism is responsible for its natural PZA resistance (Somoskovi et al. 2007).

Although most of the PZA-resistant strains have mutations in *pncA*, some resistant strains do not show any, particularly strains that have lost peroxidase activity (Lemaitre et al. 1999; Marttila et al. 1999; Cheng et al. 2000). This suggests that mutations in *pncA* regulation genes could affect resistance. Some strains demonstrate both a low level of resistance and pyrazinamidase activity, probably due to another resistance mechanism (Zhang Min et al. 2005). Finally, the fact that strains that are highly resistant to PZA do not lose their pyrazinamidase activity (Butler, Kilburn 1983) and can still have a wild *pncA* gene (Davies et al. 2000) suggests that other resistance mechanisms remain to be found (Musser 1995). Tran-translation (depending on ribosomal protein S1 (RpsA)) could be inhibited and explain the ability of PZA to eradicate persisting organisms (Shi et al. 2011).

Considering the prevalence of pyrazinamide resistance in different clinical settings, PCR-DNA sequencing, and other molecular assays targeting *pncA*, can detect PZA resistance in MDR *M. tuberculosis* isolates, with predictive values largely exceeding 90% and rule out PZA resistance in non-MDR isolates, with predictive values exceeding 99% (Chang, Yew, Zhang 2011).

5.6 Ethambutol

Ethambutol (EMB) is very specific. It has been used since 1966 in combination with INH in treating tuberculosis. It is used as an accessory drug in short-course therapy, particularly when resistance to at least one TB drug reaches 4% (Riska, Jacobs, Alland 2000). EMB inhibits the formation of mycobacterial membrane (Musser 1995). Three homologues of arabinosyltransferases, EmbC, EmbA, and EmbB (Telenti et al. 1997b; Zhang 2000), have been proposed as the targets of EMB. These enzymes are involved in the synthesis of arabinogalactan, one of the basic components of a mycobacterial cell wall.

Resistance to EMB is frequent among MDR strains (Pablos-Mendez et al. 1998). Phenotypic resistance is difficult to standardize in part due to the instability of EMB in both solid and liquid culture mediums. The overexpression of genes, leading to low intracellular rates of EMB, or mutation of the codon at position 306 of the *embB* gene can cause higher resistance to EMB (Telenti et al. 1997b). Mutation of codon 306 was found among 47% to 62% of strains with high EMB resistance (MIC > 20 µg/ml) (Sreevatsan et al. 1997b; Telenti et al. 1997b; Zhang 2000). Some reports have suggested that the EmbB306 mutation is not involved in EMB resistance but is instead associated with the development of other drug resistance including MDR-TB. A Russian study found mutations of *emb*B306 in 48.3% of resistant strains, as well as in 31.2% of susceptible strains (Mokrousov et al. 2002c).

Hazbon tested 1,020 strains from different regions of the world and concluded that *embB*306 mutations are associated with multi-resistance but do not directly cause resistance (Hazbon et al. 2006). Shortly afterwards, Plinke et al. reported that *embB*306 mutations were only detected on EMB-resistant strains, suggesting that they predict resistance (Plinke, Rusch-Gerdes, Niemann 2006). Finally, Shen in Shanghai studied 10,659 strains and described embB306 mutations in EMB^R and EMB^S strains (Shen et al. 2007). Using site-directed mutagenesis and allelic exchange in *M. tuberculosis*, Safi (Safi et al. 2008) found that mutations leading to certain aminoacid changes are indeed causing EMB resistance while others have little effect on EMB resistance.

Other mutations were observed: D354A and N296Y at codon 330 (n=1) (Sekiguchi et al. 2007) and 360 (n=2) of *embB*, which produced a high level of resistance (Sreevatsan et al. 1997b), or at codons 328, 406 and 497 (Zhang Min et al. 2005), and in *embA* and *embC* of some resistant strains (Sreevatsan et al. 1997b). However, there are substitutions of codon 981 in phenotypically susceptible strains (Sreevatsan et al. 1997b). Moreover, other mutations were observed in other genes such as *embR*, *Rv0340*, *rmlD*, and *rmlA2*, associated with resistance. Finally, for approximately 25% of low resistance strains (MIC < 10 µg/ml), no mutation in the known resistance region was observed (Telenti et al. 1997b). This low level resistance could result from an overexpression of Emb or mutations outside of the sequenced region (approximately 80% of the 3297 bp gene).

Multi-resistant strains have more *embB*306 mutants than non-MDR strains (Mokrousov et al. 2002c; Johnson et al. 2006; Plinke, Rusch-Gerdes, Niemann

2006; Ahmad, Jaber, Mokaddas 2007). The *embB*306 locus appears then as a marker candidate for detecting MDR and XDR *M. tuberculosis* strains.

A line probe assay is now proposed to detect ethambutol resistant strains (Hillemann, Rusch-Gerdes, Richter 2009). The genotype MTBDR*sl* (Hain) test is based on amplification/reverse hybridization and provides detection of the *embB* mutations most commonly responsible for EMB resistance. Wild type probes have been designed that hybridize to the most important resistance region of the *embB* gene. When all wild type probes of a gene stain positive, there is no detectable mutation within the examined region. Hence, the strain tested is sensitive to EMB. When a mutation exists, the respective amplicon cannot bind to the corresponding wild type probe. The absence of a signal with at least one of the wild type probes indicates resistance to EMB. The probes detect four 306 *embB* mutations. The banding pattern obtained with the *embB* probes could allow a conclusion to be suggested about resistance to EMB.

Mutations in the gene *embB* and the corresponding wild type and mutation probes according to (Johnson et al. 2006; Plinke, Rusch-Gerdes, Niemann 2006)

Failing wild type probe(s)	codon analyzed	Mutation probe	mutation	Phenotypic resistance*
embB WT	306	*embB* MUT1A	M306I*[1]	EMB^R
embB WT	306	*embB* MUT1B	M306V	EMB^R
embB WT	306		M306I*[2]	EMB^R
embB WT	306		M306I*[3]	EMB^R

* EMB^R : ethambutol-resistance
*[1] M306I : base exchange at codon 306 : ATG to ATA
*[2] M306I : base exchange at codon 306 : ATG to ATC
*[3] M306I : base exchange at codon 306 : ATG to ATT

5.7 Resistance to aminoglycosides and macrocyclic peptides

5.7.1 Streptomycin-resistance

Streptomycin (STR), an aminoglycoside antibiotic, was the first antibiotic used against tuberculosis in 1944 but its required parenteral administration, toxicity, and low effectiveness compared to other drugs has made it less prescribed as a first-line treatment. Its initial use in monotherapy led to the emergence of an STR-resistant *M. tuberculosis* strain (also associated with INH-resistance).

Aminoglycoside antibiotics act by preventing protein synthesis of bacterial ribosome. They inhibit the initiation of mRNA translation. The site of action is the small 30S ribosome subunit, a highly conserved part of the translation apparatus including S23 ribosomal protein, coded by the *rpsL* gene, and 16SrRNA coded by the *rrs* gene. Changes in the amino acid sequence of *rpsL* alter RpsL and 16SrRNA interaction and lead to STR resistance. S12 ribosomal protein interacts with the 530 loop of ARNr16S, and maintains its super-structure. Thus, the 16SrRNA loops that interact with the S12 protein are a mutation selective site for conferring resistance to STR. In bacteria, the presence of several rRNA operons and one copy of the *rpsL* gene prevent the isolation of STR^R mutants whose resistance is conferred by a mutation on the *rrs* gene. *M. tuberculosis* only has one rRNA operon (rrn), which explains the isolation of STR^R whose resistance is conferred by a mutation of the *rrs* gene.

50% [42% to 59%] of STR^R strains have at least one mutation in the ***rpsL*** gene, which codes for the S12 ribosomal protein associated with a high level of resistance (MIC > 1000 µg/ml) (Nair et al. 1993; Musser 1995; Zhang 2000). Two *rpsL* mutations, Lys(AAG)→Arg(AGG), at codons 43 and 88, contribute to these high levels of resistance in most cases (Finken et al. 1993; Meier et al. 1996). The *rpsL* mutants can host missense mutations at position 9 and 93 that have no obvious association to a role in STR resistance (Sreevatsan et al. 1996) since they are described in association with mutations that are already known at codons 43 and 88.

In certain strains of STR-R *M. tuberculosis*, the *rpsL* gene is a wild type and resistance is attributed to mutations of the *rrs* gene coding for 16S ribosomal RNA : 8 to 21% of STR^R strains have at least one mutation in the *rrs* gene associated with an intermediary resistance level (MIC 64 to 512 µ g/ml) (Spies et al. 2008) (Sekiguchi et al. 2007). The first mutation described is A904G (Douglass, Steyn 1993). Resistances are associated with single mutations, mainly in one of two conserved regions of 16SrRNA (around nucleotides 530 and 912), which form interactions with the S12 protein (Finken et al. 1993; Nair et al. 1993; Cooksey et al. 1996; Meier et al. 1996). One of them, the G524C mutation, severely disrupts 524G and 507C, a Watson-Crick type action that is essential to ribosomal function.

The nucleotide mutation previously described at position 491 of the *rrs* gene, in two strains resistant to streptomycin (Sreevatsan et al. 1996), is a polymorphism that is not related to resistance (Zhang Ying 2005).

30% of STR^R strains do not have a mutation in the *rpsL* or *rrs* genes. Resistance could be caused by membrane impermeability (Riska, Jacobs, Alland 2000).

Recently, it has been suggested that mutations in *gidB*, encoding a conserved 16S RNA specific 7-methylguanosine methyltransferase, or the presence of efflux pump inhibitors, appear to be involved in low-level SM resistance (Okamoto et al. 2007; Spies et al. 2008)

5.7.2 Amikacin-kanamycin-capreomycin-viomycin-resistance

Multi-resistant tuberculosis treatment requires the use of second-line antibiotics such as other aminoglycosides or peptide antibiotics. These antibiotics are considered together because of their same target gene.

Amikacin (AMK) and kanamycin (KAN) are aminoglycoside antibiotics (deoxystreptamines) with no cross-resistance to STR (streptidine). Capreomycin (CAP) is a macrocyclic peptide that is bactericidal for nonreplicating *M. tuberculosis* (Heifets 05). Its structure is similar to the structure of viomycin (VIO), a basic peptide, 4 times less active than STR (Fenagle 07).

Resistance is crossed between AMK and KAN and the genotypes associated with resistance to KAN and AMK and cyclic peptides (CAP and VIO) overlap.

The low number of mutants ($2x10^{-8}$) of *M. tuberculosis* H37Rv that are resistant to STR, a streptidine and to AMK or KAN ($2x10^{-6}$) a deoxystreptamine suggests that the resistance to two sub-classes of aminoglycoside antibiotics results in a single mutation.

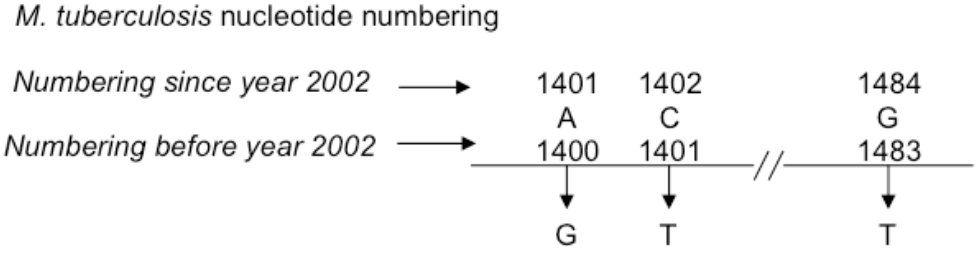
This A1401G mutation (formerly A1400G) of the *rrs* gene confers high-level resistance of *M. tuberculosis* to AMK and KAN (Alangaden et al. 1998). The substitution of A for G is found in 13 KAN/AMK^R strains studied, with only half of these strains being resistant to CAP also. Another study demonstrated the existence of rrs mutations in 67% of KAN^R strains, one of which was at position 1401, which when compared to what is known about *E. coli*, can cause cross-resistance with CAP (Suzuki et al. 1998). However, this A1401G mutation is found in clinical strains and mutants that remain susceptible to VIO.

Strains mutated in C1402T can remain susceptible to AMK (MIC ≤ 4 µg/ml) and have a low level of resistance to KAN (MIC 10-20 µg/ml).

The G1484T confers to it alone a high level of resistance to AMK, KAN, CAP and VIO. (Maus, Plikaytis, Shinnick 2005)

Main missense mutations responsible for amikacin and kanamicin resistance in *M. tuberculosis*

- *rrs* gene -



The mutation of the *tlyA* gene coding for a putative methyltransferase confers resistance to CAP and VIO in *M. tuberculosis*. However, some CAP-resistant clinical strains have a non-mutated *tlyA* gene and an A1401G mutation on the *rrs* gene.

CAP^R and VIO^R strains can show a mutation of the *tlyA* gene or *rrs* gene such as C1402T or G1484T.

In fact, none of the enzymes responsible for aminoglycoside resistance (through drug alteration in other bacteria) was found in *M. tuberculosis*. Finally, the MICs of resistant mutants suggest other additive or synergic factors for resistance.

The presence of a limited number of single, specific mutations had made possible to use molecular tests to detect resistance in a large number of resistant strains.

The genotype MTBDRsl (Hain) test is based on amplification/reverse hybridization and provides effective detective detection of the *rrs* mutations most commonly responsible for aminoglycoside resistance.

The wild type probes comprise the most important resistance region of the *rrs* gene. When all wild type probes of a gene stain positive, there is no detectable mutation within the examined region. Hence, the strain tested is sensible for the aminoglycosides. In case of a mutation, the respective amplicon cannot bind to the corresponding wild type probe. The absence of a signal for at least one of the wild type probes hence indicates a resistance of the tested strain for the aminoglycosides. The mutations probes detect some of the most common resistance mediating mutations. The banding pattern obtained with the *rrs* probes allows to draw a conclusion about a resistance to aminoglycosides (kanamycin and amikacin) and cyclic peptides (capreomycin and viomycin).

Failing wild type probe(s)	codon analyzed	Mutation probe	mutation	Phenotypic resistance*
rrs WT1	1401	*rrs* MUT1	A1401G	CAP^R, Vio^S, AMK^R, KAN^R
rrs WT2	1402		C1402T	CAP^R, Vio^R, AMK^S, KAN^R
rrs WT3	1484	rrs MUT2	G1484T	CAP^R, Vio^R, AMK^R, KAN^R

* CAP : capreomycin, Vio : viomycin, KAN : kanamycin, AMK : amikacin
^{R} = resistance, ^{S} = sensitivity

5.8 Multiresistance

The accumulation of mutations in each of the individual target genes mentioned above leads to multiresistance (Morris et al. 1995). It is, however, likely that low levels of resistance are due to other mechanisms. The possibility of identifying resistance by detecting mutations of target genes for high levels of resistance, *rpoB*, *gyrA* and *gyrB* in particular in the case of multiresistances, will improve diagnosis of MDR and XDR tuberculosis.

6 Conclusion

Molecular biology methods significantly improved the identification of mycobacteria, notably shortening response time. Amplification did not improve the sensitivity of bacilli screening as much as it was hoped; however, culture in liquid medium, combined with molecular biology identification, permits species diagnosis and resistance detection. These methods are applied to cultures and even to clinical samples. Thus, the marketed GenoType kit lets you identify the mycobacterial species and determine its susceptibility to RIF, INH, FQ, AMK-KAN and EMB. GeneXpert test allows detection of *M. tuberculosis* complex and RIF-resistance. Sequencing other antibiotic targets is a major improvement in evaluating other resistances. However, the correlation between phenotypic and genotypic measurement is usually necessary.

The improvements made by molecular biology suggest that effective diagnostic strategies could be used to identify patients with MDR and even XDR strains. In an epidemic situation of drug-resistant tuberculosis, identifying the mutations responsible for resistance to rifampicin and quinolones would be a rapid indicator, making it possible to isolate the patient and provide the most suitable treatment possible.

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