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Detection of *Mycobacterium tuberculosis* and Drug Resistance: Opportunies and Challenges in Morocco

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1. Introduction

Tuberculosis (TB) was responsible for millions of human deaths in the past, when there were no adequate treatment methods for infected patients. Introduction of chemotherapy and prophylactic measures led to drastic death reduction, which was maintained for various decades. However, "the good times" waned, as this disease became worldwide recognized as the one responsible for most human deaths caused by a single infectious agent: *Mycobacterium tuberculosi*s (MTB). TB resumption is basically a consequence of anthropic factors, such as the recent HIV/AIDS pandemic and the development of drug resistant strains (stemmed from inappropriate treatments and/or patient non-compliance).

International attention has turned toward the evolving burden of multi-drug resistant tuberculosis (MDR TB) that has emerged in epidemic proportions in the wake of widespread HIV infection in the world's poorest populations, including sub-Saharan Africa. Extensively drug-resistant TB (XDR TB) was first reported in 2006 but has now been documented on six continents [WHO the global laboratory initiative]. These trends are critically important for global health, since drug-resistant TB mortality rates are high and second-line agents for the treatment of drug-resistant TB are less potent and less tolerable than first-line therapies.

Global control of tuberculosis is hampered by slow, insensitive diagnostic methods, particularly for the detection of drug-resistant forms and in patients with human immunodeficiency virus infection. Failure to quickly and effectively recognize and treat patients with drug-resistant tuberculosis (TB), particularly MDR and XDR tuberculosis, leads to increased mortality, nosocomial outbreaks and resistance to additional antituberculosis drugs. We believe that early detection is essential to reduce the death rate and interrupt transmission, but the complexity and infrastructure needs of sensitive methods limit their accessibility and effect.

Therefore, there's a critical need for methods that can rapidly detect *M. tuberculosis* and identify drug-resistant cases to optimize TB treatment with appropriate drugs.

The present review describes the main techniques used to detect TB and related resistant strains as well as the issues and challenges associated with implementing molecular techniques in Morocco to enhance the National Program against Tuberculosis.

2. History of tuberculosis

2.1 Looking at past: Before the discovery of BCG

First reference of a disease similar to TB in humans dates back to ancient Egypt. Examinations of mummies and tomb paintings reveal that tuberculosis was present at that time (around 5000 BC). The ancient Egyptian paintings portray spinal tuberculosis, indicating the presence of the disease. The reference of a disease called "phthisis" is found in ancient Greek literature by Hippocrates. In 1680, F. Sylvius carried out anatomic-pathologic studies in pulmonary nodules from TB patients, which he named as "tubercula", observing their evolution to lung ulcers (cavities). However, most of the great pathologists of his time believed that these knots were some type of tumor or abnormal gland, rejecting any probable infectious origin. The first credible speculation of the infectious nature of TB was performed by B. Marten, who proposed in 1722 that TB could be caused by micro-organisms. R. Morton used the term "consumption" to specifically denote TB, and finally, in 1819, the inventor of the stethoscope, R. Laennec identified for the first time the TB manifestation unit. As the disease became completely established among every European social level, afflicting many of the intellectual and artists of the continent by the half of the XIX century, TB was romanticized, as typical symptoms like thin and pale faces of the infected ones became signs of beauty.

In 1865, JA Villemin demonstrated formally that TB is a contagious disease; although his experimentation could be effectively reproduced in rabbits, the finding was ignored by his contemporaries for a long time.

2.2 BCG as prophylactic strategy

One of the greatest works on TB was performed in 1882 by Robert Koch, who isolated and cultured *M. tuberculosis* from crushed tubercles. His experimental work identified the bacterium as the TB etiological agent (Bloom & Murray 1992, Daniel 1997). In August of 1890, during The First Ordinary Session of the International Medical Congress, in Berlin, he announced the discovery of a TB therapeutic drug. Three months later, M. Wochenschkift published a new statement of Koch, revealing that although interested in the therapeutic properties of his findings, he observed that the referred liquid, named tuberculin, could be useful as a diagnostic tool to detect the disease due to the intensified reaction developed by sick animals inoculated with this drug, as no measurable effect was ever observed in healthy ones. This concept was perpetuated for several years, until it was observed that even healthy animals could react to the drug. The veterinarians clarified the fact by demonstrating that the healthy ones could be simply infected, although not ill. As a result, it was established that *M. tuberculosis*-infected animals will react to tuberculin infusion, whereas the non-infected ones will not. This drug, the first industrialised one, was called old tuberculin; subsequently, other tuberculins were produced, such as purified protein derivate (PPD), PPD-S, and PPD RT23, among others (Vaccarezza 1965, Ruffino- Netto 1970). The tuberculin skin test became the principal tool for infection

diagnosis. In the same period, Koch developed staining methods for the identification of the bacillus; these techniques were subsequently improved by the bacteriologist "Paul Ehrlich", whose method for detection of the bacillus provided the basis for the development of the Ziehl-Nielsen staining, which still is an important tool to diagnose TB. Koch's discovery allowed researchers to focus their efforts on the development of new and more efficient therapies to treat TB patients. One of the first attempts to fight the disease in 1884 was the introduction for TB patients of "sanatorium cure" concept, where the patients were isolated and taken care of, the treatment was based on rest, fresh air and a healthy diet.

In 1896, the bacteriologist T. Smith demonstrated that bovine TB was not caused by *M. tuberculosis*, but rather by another species, *M. bovis*. Twelve years later, the scientist-couple A. Calmette and C. Guérin isolated the bovine variant from its host and grew the bacilli in dispersed culture. By the 39th passage they observed a morphological variant that was avirulent in several animal models and which conferred immunological protection against subsequent challenges with virulent *M. tuberculosis*. Thirteen years of experimentation led to the obtaining of the 231st passage, the variant that was administered for the first time in humans (orally), as an attempt to immunize a child whose mother died in childbirth victim of TB. Currently known as BCG (Bacille Calmette-Guérin), the "intradermal" vaccine has become widely used to combat TB; it relies on a prophylactic administration of live attenuated bacilli to children.

2.3 Tuberculosis chemotherapy

Significant progress has been made in TB chemotherapy. In the pre-antibiotic era, before 1940, there were no drugs against the disease. The tuberculosis treatment at the time consisted mainly of cod liver oils (which by the way, include vitamin D), bed rest and fresh air.

The first TB drug, Streptomycin (SM), was discovered in 1944. It was followed by Para-aminosalicylic acid (PAS), which was discovered in 1946. Then, in 1952, two important first-line TB drugs were discovered, Isoniazid (INH) and Pyrazinamide (PZA). The last TB drug discovered was Rifampin, in 1963.

Hence, the introduction of TB chemotherapy in 1950's led to a significant decline in the incidence of the disease, particularly in developed countries. This reduction has prompted some public health professionals, mainly in the US, to claim that tuberculosis no longer poses a problem in developed countries. They have even eliminated many TB control programmes in the country. However, in the late 1980's, a major outbreak of MDR-TB occurred in New York City, which has cost USD 1 billion to control. This outbreak has led to a renaissance in TB research.

2.4 Drug combinations

It is noteworthy that the current TB therapy is based on the principle of drug combination. The first advantage of using drug combinations is that it reduces drug resistance. A second advantage of drug combinations is that they can enhance the efficacy of the therapy. This point is illustrated by the Mitchison hypothesis, also referred to as the Special Bacterial

Populations Theory. According to this theory, TB bacteria found in the lesions consist of four different sub-populations. Population A, which is actively growing, is killed by Isoniazid. In case of Isoniazid resistance, it is killed by Rifampicin, Streptomycin, or Ethambutol. Population B, which has a slower metabolism, is killed by Rifampicin. Population C, which resides in an acidic environment, is killed by PZA. Finally, population D is a dormant population, and there are currently no drugs that can effectively kill this population (Paramasivan, 2005).

2.5 Directly Observed Treatment Short course strategy

TB persists as a global public health problem and the main focus for the twentieth century is firstly to cure the individual patient and secondly to minimise the transmission of *M. tuberculosis* to other persons (WHO, 2003; Blumberg, 2003). The ongoing TB problem has been due to the neglect of TB control by governments, inadequate access and infrastructure, poor patient adherence to medication, poor management of TB control programs, poverty, population growth and migration, and a significant rise in the number of TB cases in HIV infected individuals. Treatment of patients with TB is done according to the following five key components of the Directly Observed Treatment Short course (DOTS) strategy recommended by World Health Organization (WHO) (Walley, 1997):

- Government commitment
- Case detection by sputum smear microscopy
- Standardised treatment regimen of six to eight months
- A regular, uninterrupted supply of all essential anti-TB drugs
- A standard recording and reporting system.

Since the introduction of the DOTS strategy in the early '90s by the WHO, considerable progress has been made in global TB control (Sterling, 2003). In 1997, the estimated average treatment success rate worldwide was almost 80%. However, less than 25% of people who are sick with TB are treated through the DOTS strategy (Bastian, 2000). A total of 180 countries (including both developed and developing countries) had adopted and implemented the DOTS strategy by the end of 2002 and 69% of the global population are living in areas covered by the DOTS strategy (Blumberg, 2003). However, even though DOTS programs are in place, treatment success rates are very low in low income countries due to poor management of TB control programs and patient non-compliance (Lienhardt and Ogden, 2004; Bastian, 2003). Furthermore, the effectiveness of DOTS is facing new challenges with respect to the spread and increase of MDR-TB and the co-epidemic of TB/HIV (WHO, 2003). Since 1999, WHO and partners have addressed these new challenges and have developed DOTS-Plus strategy which prevent further development and spread of MDR-TB and help to manage MDR-TB using second line drugs in low- and middle-income countries within DOTS strategy. Morocco joined the global project in 2004 and carried out its first simultaneous survey on primary and acquired drug resistance in tuberculosis patients exactly according WHO/IUATLD recommendations.

Subsequently, 41 million of TB patients have been successfully treated in DOTS programs and up to 6 million lives saved since 1995. Moreover, 5 million more lives could be saved up to 2015 by fully funding and implementing The Global Plan to Stop TB 2011-2015.

3. Epidemiology of tuberculosis

3.1 Tuberculosis throughout the world: A catastrophic situation

More than two billion people, equal to one third of the world's total population, are infected with TB bacilli. A total of 1.7 million people died from TB in 2009 (including 380 000 people with HIV), equal to about 4700 deaths a day. TB is a disease of poverty, affecting mostly young adults in their most productive years. The vast majority of TB deaths are in the developing world, with more than half occurring in Asia. There were 9.4 million new TB cases in 2009, of which 80% were in just 22 countries. Per capita, the global TB incidence rate is falling, but the rate of decline is very slow (less than 1%) (Wilson, 2011).

TB is a worldwide pandemic. Among the 15 countries with the highest estimated TB incidence rates, 13 are in Africa, while a third of all new cases are in India and China. There were an estimated 440 000 new MDR-TB cases in 2008 with three countries accounting for over 50% of all cases globally: China, India and the Russian Federation (WHO, 2010). Extensively drug-resistant TB (XDR-TB) occurs when resistance to second-line drugs develops. It is extremely difficult to treat and cases have been confirmed in more than 58 countries. The world is on track to achieve two TB targets set for 2015:

- The Millennium Development Goal, which aims to halt and reverse global incidence (in comparison with 1990);
- The Stop TB Partnership target of halving deaths from TB (also in comparison with 1990).

3.2 Tuberculosis status in Morocco

During last years, the incidence of TB has stagnated and is reported to be 81 per 100,000 overall. However, the incidence was significantly higher in several urban areas, or "hot spots": Casablanca, Tangier and Rabat (together 43% of all notified cases in 2010). Statistical data show that 59% of TB patients are male and 65% are 15-34 years old. Of the roughly 28,000 new TB cases reported annually, 12% are re-treatment cases. Moreover, the prevalence of tubeculosis in HIV individuals is 1.7% in 2008 (Dooley, 2010, WHO, 2010). The prevalence of MDR TB is 0.5% within new cases and reaches 12.2% among previously treated patients with failure treatment, relapse or chronical cases (Othmani, 2003; WHO, 2010).

National TB treatment guidelines in 2007 and 2008 recommended a Category I treatment regimen – 2 months of INH, RIF, PZA, and SM followed by 4 months of RIF and INH (2SHRZ/4RH) – for new smear-positive cases and a Category II regimen – 2HRZES/1RHEZ/5RHE (E = Ethambutol) – for re-treatment cases. By the beginning of 2009, ethambutol has replaced streptomycin in Category I regimen.

The follow up of tuberculosis which lasts from 6 to 18 months is done in specialised centres of TB diagnosis of the ministry for Health. The late consultations and the no observance of treatments are responsible for TB resistance in Morocco. To strengthen its efforts, the ministry for Health is planning to carry a national plan of acceleration of the fight against tuberculosis.

4. Drug resistance

4.1 Drug resistance and global surveillance: History

Shortly, after the first anti-tuberculosis (TB) drugs were introduced, streptomycin (STR), para-aminosalicylic acid (PAS) and isoniazid (INH), resistance to these drugs was observed in clinical isolates of *Mycobacterium tuberculosis* (Crofton and Mitchison, 1948). This led to the need to measure resistance accurately and easily. The Pasteur Institute introduced the critical proportion method in 1961 for drug susceptibility testing in TB and this method became the standard method of use (Espinal, 2000; 2003). Studies on drug resistance in various countries in the 1960s showed that developing countries had a much higher incidence of drug resistance than developed countries (Espinal, 2000; 2003). By the end of the 1960s rifampicin (RIF) was introduced and with the use of combination therapy, there was a decline in drug resistant and drug susceptible TB in developed countries. This led to a decline in funding and interest in TB control programs. As a result, no concrete monitoring of drug resistance was carried out for the following 20 years (Espinal, 2000; 2003). The arrival of HIV/AIDS in the 1980s resulted in an increase in transmission of TB associated with outbreaks of multidrug- resistant TB (MDR-TB) (Edlin, 1992; Fischl, 1992). In the early 1990s drug resistance surveillance was resumed in developed countries, but the true incidence remained unclear in the developing world (Cohn, 1997).

The emergence of MDR TB is the third epidemic, complicating the epidemics of acquired immune deficiency syndrome (AIDS) and tuberculosis, and is requiring urgent attention to achieve more rapid diagnosis, to develop new therapeutic regimens and to address the social and hospital environment to care for these patients (Neville, 1994).

4.2 Primary and acquired resistance: Definition and data

WHO estimated that 50 million people were infected with drug resistant MTB. Single-drug resistance is defined as resistance to only one antituberculous agent.

MDR-TB, or multidrug-resistant TB, is a specific form of drug-resistant TB. It occurs when the TB bacteria are resistant to at least isoniazid and rifampicin, the two most powerful anti-TB drugs. XDR-TB is an MDR TB strain that is resistant to any fluoroquinolone, and at least one of three injectable second-line drugs (capreomycin, kanamycin or amikacin).

Traditionally, patients with drug-resistant tuberculosis are classified as having primary or acquired drug resistance on the basis of a history of previous treatment. WHO criteria define acquired drug resistance as the isolation of drug-resistant *M. tuberculosis* from a patient with a record of previous treatment for 1 month, and primary drug resistance as the isolation of a drug-resistant strain from a patient without a history of previous treatment.

The classification of drug resistance as primary or acquired is used as an indicator of the efficiency of national tuberculosis programs and in the adjustment and development of these programs. The rate of primary drug resistance is interpreted as an epidemiological indicator for long-term surveillance of the quality of tuberculosis treatment in the community. The rate of acquired drug resistance reflects the efficacy of management of individual patients.

5. Molecular mechanisms of drug resistance

As a consequence of the increase in DR TB and the relatively restricted number of therapeutic agents, there has been a renewed effort during the 2 past decades to define the molecular basis of drug resistance in MTB.

MTB acquires drug resistance by antibiotic selection of mutations that occur randomly at chromosomal loci. No plasmids or transposable elements (horizontal gene transfer) are involved in this process. Individual nucleotide changes (point mutations) confer resistance to single drugs, and the stepwise accumulation of these mutations leads to MDR TB. drug resistance strains emerge when chemotherapy is intermittent or otherwise inadequate (Ducati, 2006; Zhang and Yew, 2009).

5.1 First line drugs

First-line drugs are mainly bactericidal and combine a high degree of efficacy with a relative toxicity to the patient during treatment.

Currently, a five-drug regimen is used consisting of INH, RIF, SM, PZA and EMB. Resistance to first line anti-TB drugs has been linked to mutations in at least 10 genes; *katG, inhA, ahpC, kasA* and *ndh* for INH resistance; *rpoB* for RIF resistance, *embB* for EMB resistance, *pncA* for PZA resistance and *rpsL* and *rrs* for STR resistance.

5.1.1 Isoniazid

***katG* gene alterations**. INH or isonicotinic acid hydrazide, was synthesized in the early 1900s but its anti-TB action was first detected in 1951 (Heym, 1999; Slayden and Barry, 2000; Rattan, 1998). INH enters the cell as a prodrug that is activated by a catalase peroxidase encoded by *katG*. The peroxidase activity of the enzyme is necessary to activate INH to a toxic substance in the bacterial cell (Zhang, 1992). This toxic substance subsequently affects intracellular targets such as mycolic acid biosynthesis which are an important component of the cell wall. A lack of mycolic acid synthesis eventually results in loss of cellular integrity and the bacteria die (Barry, 1998). Middlebrook *et al*. (1954) initially demonstrated that a loss of catalase activity can result in INH resistance. Subsequently, genetic studies demonstrated that transformation of INH-resistant *Mycobacterium smegmatis* and *M. tuberculosis* strains with a functional *katG* gene restored INH susceptibility and that *katG* deletions give rise to INH resistance (Zhang, 1992; Zhang, 1993). However, mutations in this gene are more frequent than deletions in clinical isolates and these can lower the activity of the enzyme.

Most mutations are found between codons 138 and 328 with the most commonly observed gene alteration being at codon 315 of the *katG* gene (Slayden and Barry, 2000). The Ser315Thr substitution is estimated to occur in 30–60% of INH resistant isolates (Ramaswamy and Musser, 1998; Musser, 1996; Slayden and Barry, 2000). The *katG* 463 (CGG-CTG / Arg-Leu) amino acid substitution is the most common polymorphism found in the *katG* gene and is not associated with INH resistance.

Resistance to INH could be also due to mutations in the promoter region of the *ahpC* gene. Indeed, it has been observed that a loss of *katG* activity due to the S315T amino acid substitution is often accompanied by an increase in expression of an alkyl hydroperoxide

reductase (*ahpC*) protein that is capable of detoxifying damaging organic peroxides (Sherman, 1996). Five different nucleotide alterations have been identified in the promoter region of the *ahpC* gene, which lead to over expression of *ahpC* and INH resistance (Ramaswamy and Musser, 1998). *AhpC* overexpression exerts a detoxifying effect on organic peroxides within the cell and protects the bacteria against oxidative damage but does not provide protection against INH. *KatG* expression can also be up regulated under conditions of oxidative stress. The correlation between polymorphic sites in the *ahpC* regulatory region with INH resistance in *M. tuberculosis* requires further examination.

***inhA* gene alterations**. One of the targets for activated INH is the protein encoded by the *inhA* locus. *InhA* is an enoyl–acyl carrier protein (ACP) reductase which is proposed to be the primary target for resistance to INH and ethionamide (ETH) (Banerjee, 1994). ETH, a second line drug, is a structural analogue of INH that is also thought to inhibit mycolic acid biosynthesis and several studies have suggested that low-level INH resistance is correlated with resistance to ETH. Activated INH binds to the InhA-NADH complex to form a ternary complex that results in inhibition of mycolic acid biosynthesis. Six point mutations associated with INH resistance within the structural *inhA* gene have been identified (Ile16Thr, Ile21Thr, Ile21Val, Ile47Thr, Val78Ala and Ile95Pro) (Ramaswamy and Musser, 1998; Basso and Blanchard, 1998). A Ser94Ala substitution results in a decreased binding affinity of *inhA* for NADH, resulting in mycolic acid synthesis inhibition. Although these mutations in the structural *InhA* gene are associated with INH resistance, it is not frequently reported in clinical isolates. *InhA* promoter mutations are more frequently seen and are present at positions -24(GT), -16(A-G), or -8(T-G/A) and -15(C-T). These promoter mutations result in over expression of *inhA* leading to low level INH resistance. To date approximately 70–80% of INH resistance in clinical isolates of *M. tuberculosis* can be attributed to mutations in the *katG* and *inhA* genes (Ramaswamy and Musser, 1998).

***kasA* gene alterations**. There seems to be considerable contreverse within the literature as to the role of *kasA* as a possible target for INH resistance (Sherman, 1996). This gene encodes a β-ketoacyl-ACP synthase involved in the synthesis of mycolic acids. Mutations have been described in this gene that confer low levels of INH resistance. Genotypic analysis of the *kas*A gene reveals 4 different amino acid substitutions involving codon 66 (GAT-AAT), codon 269 (GGT-AGT), codon 312 (GGC-AGC) and codon 413 (TTC-TTA) (Ramaswamy and Musser, 1998; Mdluli, 1998). However, similar mutations were also found in INH susceptible isolates (Lee, 1999; Piatek, 2000). Nevertheless, the possibility of *kasA* constituting an additional resistance mechanism should not be completely excluded.

***Ndh* gene alterations**. In 1998 another mechanism for INH resistance in *M. smegmatis* was described by Miesel *et al*. (1998). The *ndh* gene encodes NADH dehydrogenase that is bound to the active site of *inhA* to form the ternary complex with activated INH. Structural studies have shown that a reactive form of INH attacks the NAD(H) co-factor and generates a covalent INH-NAD adduct. Mutations in the *ndh* gene, encoding NADH dehydrogenase, cause defects in the enzymatic activity. Thus, defects in the oxidation of NADH to NAD result in NADH accumulation and NAD depletion (Lee, 2001). These high levels of NADH can then inhibit the binding of the INH-NAD adduct to the active site of the InhA enzyme (Rozwarski, 1998; Miesel, 1998). Prominent point mutations in the *ndh* gene at codons 110 and 268 (T110A and R268H) were detected in 9.5% of INH resistant samples. These similar mutations were not detected in the INH susceptible group (Lee, 2001).

5.1.2 Rifampicin

Rifampicin (RIF) is a major compound of anti-tuberculosis chemotherapy. A resistance to RIF is rarely found without associated resistance to other tuberculostatics. RIF resistance is a good marker for MDR-TB. Moreover, RIF resistance is a good predictor of poor treatment outcome. The mode of action of RIF is based on the inhibition of the elongation of transcripts by RNA polymerase in MTB, by covalent binding to the Beta sub-unit of RNA polymerase, thus leading to cell death. The RNA polymerase Beta sub-unit is encoded by the rpoB gene. 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. More than 95% of RIF^R *M. tuberculosis* has a mutation in this specific region (Telenti, 1993; Telenti, 1997).

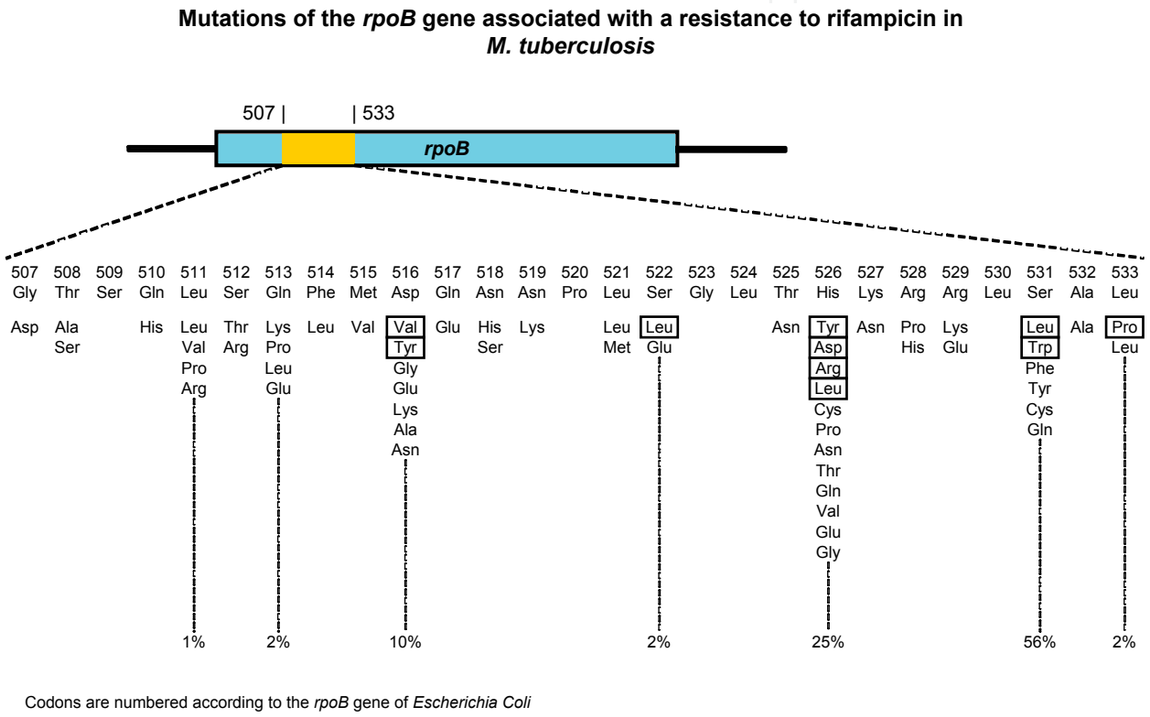

Fig. 1. Mutations of the *rpoB* gene associated with a resistance to rifampicin in *M. tuberculosis*

Resistance to RIF 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, 2001; Sekiguchi, 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, 2000; Zhang, 2005). Mutations at codons 511, 516, 518 and 522 result in a low-level resistance to RIF and rifapentine; and some susceptibility to rifabutin (Zhang, 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, 1996) or variable resistance (L533P) (Kim, 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, 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, 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, 2007).

Finally, less than 5% of resistant strains do not show a mutation in the *rpoB* resistance region (Riska, 2000; Mani, 2001). Rare loci found outside the hotspot region of *rpoB* are associated with resistance without associated mutation known for conferring resistance (Taniguchi, 1996; Fang, 1999; Schilke, 1999; Yuen, 1999; Heep, 2001; Zhang, 2005; Rigouts, 2007; Prammananan, 2008).

Mutated strains in Val146Phe (Heep, 2001; Rigouts, 2007; Prammananan, 2008) show a low-level resistance (MIC 4 μg/ml) (Rigouts, 2007). The Ala381Val mutation (Taniguchi, 1996) is described on a strain of MIC 200, with no other mutation on the *rpoB* gene.

Most susceptible strains show no mutation, except for a few: seven susceptible Japanese strains are mutated in TCG Ser 450 Leu TTG, ATG Met511 Val GTG, CTG Leu 521 Pro CCG, CTG Leu 533 Pro CCG, GCC Ala 679 Ser TCC (two strains) and CGC Arg 687 Pro CCC (Taniguchi, 1996; Yang, 1998). 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, 2000). Other studies describe some sensitive strains with mutations as Ser 450 Leu (Sekiguchi, 2007), Leu 511 Arg or Ser 512 Thr (Moghazeh, 1996) or Gln CAA 513 Gln CAG (Kim, 1997). Genotypic detections would therefore be more sensitive in certain circumstances.

5.1.3 Streptomycin

SM is an aminocyclitol antibiotic that is one of the first drug used to treat TB, SM binds to 16S rRNA, inhibits translational initiation and detrimentally affects translation fidelity. Mutations associated with SM resistance in MTB have been identified mainly in *rpsL* gene encoding ribosomal protein S12 and in the 16S rRNA gene (*rrs*) in 65–67% of STR resistant isolates (Ramaswamy and Musser, 1998).

In the *rrs* gene a C-T transition at positions 491, 512 and 516, and a A-C/T transversion at position 513 were observed in the highly conserved 530 loop. The 530 loop region is part of the aminoacyl–tRNA binding site and is involved in the decoding process (Carter, 2000). The C-T transition at codon 491 is not responsible for resistance to STR as it occurs in both STR resistant and susceptible isolates but is strongly associated with the global spread of *M. tuberculosis* with a Western Cape F11 genotype (van Rie, 2001; Victor, 2001). Other mutations in the 915 loop [903 (C-A/G) and 904 (A-G)] have also been reported to have an association with STR resistance (Carter, 2000). Mutations in the *rpsL* gene at codon 43 (AAG-AGG/ACG) (Lys-Arg/Thr) and codon 88 (AAGAGG/ CAG) (Lys-Arg/Gln) are associated with STR resistance. MIC analysis of STR resistant isolates indicate that amino acid replacements in the *rpsL* genes correlate with a high level of resistance, whereas mutations in the *rrs* gene correlate with an intermediate level of resistance (Cooksey, 1996; Meier, 1996). In addition, it has been suggested that low levels of STR resistance are also associated with altered cell permeability or rare mutations which lie outside of the *rrs* and *rpsL* genes.

5.1.4 Pyrazinamide

Pyrazinamide (PZA) is a structural analog of nicotinamide that is used as a first –line TB drug. PZA kills semi-dormant tubercle bacilli under acidic conditions. PZA targets an enzyme involved in fatty-acid synthesis and is responsible for killing persistent tubercle bacilli in the initial intensive phase of chemotherapy (Somoskovi, 2001). However, during the first two days of treatment, PZA has no bactericidal activity against rapidly growing bacilli (Zhang and Mitchison, 2003). PZA on the other hand has effective sterilizing activity and shortens the chemotherapeutic regiment from 12 to 6 months. PZA is a prodrug which is converted to its active form, pyrazinoic acid (POA) by the pyrazinamidase (PZase) encoded by *pncA*. The activity of PZA is highly specific for *M. tuberculosis,* as it has no effect on other mycobacteria. *Mycobacterium bovis* is naturally resistant to PZA due to a unique C-G point mutation in codon 169 of the *pncA* gene. PZA is only active against *M. tuberculosis* at acidic pH where POA accumulates in the cytoplasm due to an ineffective efflux pump. Accumulation of POA results in the lowering of intracellular pH to a level that inactivates a vital fatty acid synthase (Zimhony, 2004). Cloning and characterization of the *M. tuberculosis pncA* gene by Scorpio *et al.* (1997) showed that some *pncA* mutations conferred PZA resistance. Various *pncA* mutations have been identified in more than 70% of PZA resistant clinical isolates scattered throughout the *pncA* gene but thus far no mutational hot spot has been identified (Scorpio and Zhang, 1996; Sreevatsan, 1997b; Scorpio, 1997). PZA susceptibility testing is not done routinely in many countries due to technical difficulties. Thus the extent of PZA resistance globally is largely unknown. PZA resistant isolates had diverse nucleotide changes scattered throughout the *pncA* gene. However, PZA resistant isolates without *pncA* mutations were also observed suggesting that another mechanism may be involved in conferring PZA resistance in these isolates. In addition, not all mutations (e.g. Thr114Met) were associated with PZA resistance. In summary, the complexity of PZA resistance makes the development of molecular methods for rapid diagnosis difficult.

5.1.5 Ethambutol

Ethambutol (EMB) is a very specific and effective drug that is used in combination with INH to treat *M. tuberculosis* infection (Ramaswamy and Musser, 1998). EMB inhibits an arabinosyl transferase (*embB*) involved in cell wall biosynthesis (Takayama and Kilburn, 1989).Three genes designated *emb*C, A and B (Telenti, 1997) encode homologous arabinosyl transferase enzymes involved in EMB resistance. Various studies have identified five mutations in codon 306 of *embB* gene [(ATG-GTG), (ATG-CTG), (ATG-ATA), (ATG-ATC) and (ATG-ATT)] which result in three different amino acid substitutions (Val, Leu and Ile) in EMB-resistant isolates (Lee, 2002; Sreevatsan, 1997c; Mokrousov, 2002b; Ramaswamy, 2000). These five mutations are associated with 70–90% of all EMB resistant isolates (Ramaswamy and Musser, 1998). Missense mutations were identified in three additional codons: Phe285leu, Phe330Val and Thr630Ile in EMB resistant isolates. MIC's were generally higher for strains with Met306Leu, Met306Val, and Phe330Val and Thr630Ile substitutions than those organisms with Met306Ile substitutions. Mutations outside of codon 306 are present but quite rare. However a number of EMB phenotypic resistant isolates (about 30%) still lack an identified mutation in *embB*. There is therefore a need to fully understand the mechanism of EMB resistance in clinical isolates.

5.2 Second line drugs

5.2.1 Fluoroquinolones

Ciproflaxin (CIP) and ofloxacin (OFL) are the two fluoroquinolones (FQs) used as second-line drugs in MDR-TB treatment (WHO, 2001). These FQs are bactericidal against MTB. Their target is the DNA gyrase, an ATP-dependent type II DNA topoisomerase that catalyses negative supercoiling of DNA. DNA gyrase is a tetrameric protein composed of two A and two B subunits encoded by the gyrA and gyrB genes, respectively. FQs bing to gyrase and inhibit supercoiling of DNA, thereby disrupting cellular processes dependent on DNA topology (Ramaswamy and Musser, 1998).

The quinolone resistance-determining region (QRDR) is a conserved region in the *gyrA* (320bp) and *gyrB* (375bp) genes (Ginsburg, 2003) which is the point of interaction of FQ and gyrase (Ginsburg, 2003). Missense mutations in codon 90, 91, and 94 of *gyrA* are associated with resistance to FQs (Takiff, 1994; Xu, 1996). A 16-fold increase in resistance was observed for isolates with a Ala90Val substitution, a 30-fold increase for Asp94Asn or His94Tyr and a 60-fold increase for Asp94Gly (Xu, 1996). A polymorphism at *gyrA* codon 95 is not associated with FQ resistance, and is used, with the *katG*463 polymorphism, to classify *M. tuberculosis* into 3 phylogenetic groups (Sreevatsan, 1997a).

5.2.2 Ethionamide

Ethionamide (ETH) is a derivative of isonicotinic acid with potent activity against MTB and other mycobacteria. Like INH, ETH is also thought to be a prodrug that is activated by bacterial metabolism. The activated drug then disrupts cell wall biosynthesis by inhibiting mycolic acid synthesis. Mutations in the promoter of the *inhA* gene are associated with resistance to INH and ETH (Morlock, 2003). *EthA* catalyses a two step activation of ETH and gene alterations leading to reduced EthA activity lead to ETH resistance (Engohang- Ndong, 2004; Morlock, 2003; Vannelli, 2002). The expression of *ethA* is under the control of the neighbouring *ethR* gene encoding a repressor. *EthR* negatively regulates the expression of *ethA*, by binding upstream of *ethA* to suppress *ethA* expression (Engohang-Ndong, 2004).

5.2.3 Kanamycine and amykacine

KAN and AMY are aminoglycoside antibiotics that inhibits protein synthesis by inhibiting the normal function of ribosomes (Taniguchi, 1997; Ramaswamy and Musser, 1998). These drugs are used as second line anti-TB agents. Nucleotide substitutions in the region of *rrs* especially at position 1400 (between the *rrs* gene and 23S rRNA gene) are a major cause of resistance to KAN and AMY in *M.tuberculosis*. It seems that nucleotide substitutions at rrs position 1400 is implicated in high-level resistance to KAN and AMY (Taniguchi, 1997).

5.2.4 D-Cycloserine

D-cycloserine (DCS) is a cyclic analog of D-alanine which is one of the central molecules of the cross linking step of peptidoglycan assembly (Ramaswamy and Musser, 1998; Feng and Barletta, 2003). DCS inhibits cell wall synthesis by competing with D-Alanine for the enzymes D-alanyl-D-alanine synthetase (Ddl) and D-alanine racemase (Alr) and also inhibiting the synthesis of these proteins. Overexpression of *alr* causes DCS resistance. The

G→T transversion in the *alr* promoter may lead to the overexpression of *alr* (Feng and Barletta, 2003; Ramaswamy and Musser, 1998).

5.2.5 Peptides

Viomycin (VIO) and capreomycin (CAP) are basic peptide antibiotics that inhibit prokaryotic protein synthesis and have shown that resistance to VIO in *M. smegmatis* is caused by alterations in the 30S or 50S ribosomal subunits (Taniguchi, 1997). Mutations in the *rrs* gene that encodes the 16S rRNA is associated with resistance to VIO and CAP, specifically a G→A or G→T nucleotide change at codon 1473 (Taniguchi, 1997).

6. Availables tests for tuberculosis diagnosis

6.1 History

Since the 1880s with the development of the sputum smear microscopy, the most commonly used for TB diagnostic, several new and established methods were developed and implemented in many laboratory services worldwide to enhance MTB diagnosis and tuberculosis management.

The sputum smear microscopy has remained largely unchanged and is often described as a simple technology. However, it requires a high level of training and diligence.

Because microscopy is both cumbersome to implement and inherently insensitive, many patients remain undiagnosed and many non-TB patients are incorrectly treated with TB drugs on the basis of clinical suspicion alone. In endemic countries, simply obtaining an accurate diagnosis often takes weeks or months from the time a patient first visits a health centre. This delay prevents prompt treatment of TB and leads to continued disease transmission, at substantial cost to the individual and at huge cost to society.

Mounting drug resistance, including MDR-TB and extensively drug-resistant (XDR) TB, coupled with a growing number of people co-infected with TB and HIV, have highlighted the urgent need for more accurate and rapid diagnostic tests. Many patients are never diagnosed and contribute to the astonishing number of yearly deaths from TB worldwide.

The global control of tuberculosis remains a challenge from the standpoint of diagnosis, detection of drug resistance, and treatment. Thus, there recently has been a marked increase in the development and testing of novel assays designed to detect MTB complex and/or MDR MTB based either on conventional techniques or on molecular approaches. The Figure 2 summarise the main techniques used and under development for TB diagnosis.

Recently, the WHO has endorsed some of these novel methods, and they have been made available at discounted prices for procurement by the public health sector of high-burden countries. In addition, international and national laboratory partners and donors are currently evaluating other new diagnostics that will allow further and more rapid testing in point-of-care settings. While some techniques are simple, others have complex requirements, and therefore, it is important to carefully determine how to link these new tests and incorporate them within a country's national diagnostic algorithm. Finally, the successful implementation of these methods is dependent on key partnerships in the international laboratory community and ensuring that adequate quality assurance programs are inherent

in each country's laboratory network. Moreover, it's widely accepted that if left untreated, each person with active TB infects an average of 10 to 15 people each year. Interrupting disease transmission will require early and accurate detection paired with appropriate treatment.

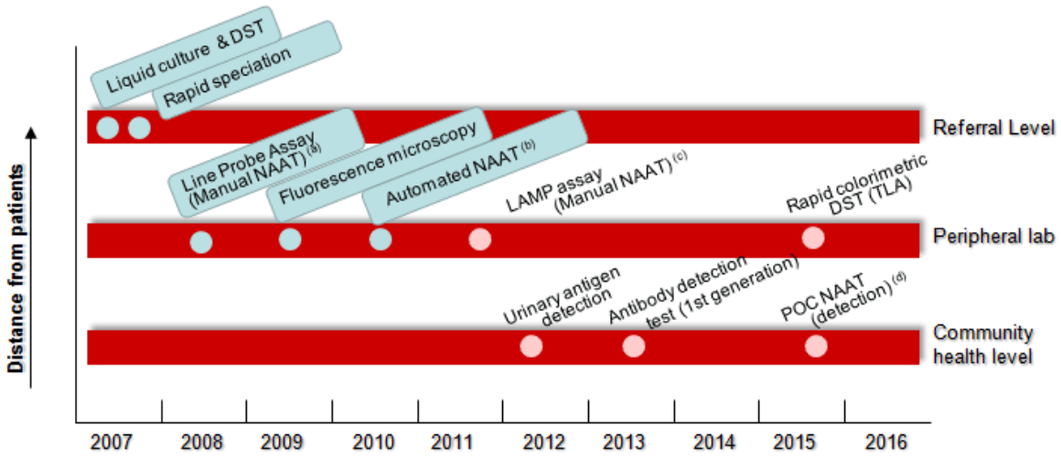

Fig. 2. Tuberculosis product deliverables 2007 2016

6.2 Conventional tests

Tuberculosis is generally diagnosed by traditional laboratory procedures, including microscopic examination of samples for the presence of acid-fast bacilli (AFB) and/ or isolation by culture followed by identification using biochemical tets.

6.2.1 Microscopic examination

The etiological diagnosis of tuberculosis is based on the appearance of bacilli in clinical samples (sputum). Direct sample examination according to Ziehl-Neelsen staining is 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 103 bacilli / ml or more in the sample. In addition, it is not species specific. As a result, false-positives occur, particularly in heavily colonized samples from children patients with other chronic pulmonary infections and paucibacillary cases common among HIV-infected individuals. 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.

Because of the limitations of conventional light microscopy using stains such as Ziehl-Neelsen, fluorochrome stains such as auramine were introduced that improve the sensitivity of the test and take less time to perform. However, fluorescence microscopy has the limitations of requiring a fluorescent microscope, a dark room, and an expensive light source.

Mercury vapor light sources used for this type of microscopy can also pose a hazard if bulbs are broken. To overcome these limitations, light emitting diode (LED) microscopy was

developed. This type of microscopy uses LED technology as a light source but still allows for the advantages of using a fluorescent stain while eliminating most of the disadvantages of fluorescent microscopy. LED microscopy is more sensitive and equally specific, compared with either conventional light or fluorescent microscopy.

The World Health Organization (WHO) recommends that conventional fluorescence microscopy could be replaced by LED microscopy, and that LED microscopy should be ''phased in as an alternative for conventional light microscopy''.

6.2.2 Culture

It's widely accepted that TB culture is substantially more sensitive, but is very slow, and results often do not get back to the health care workers until too late to be clinically useful.

Culture is needed to screen for bacilli either on solid (Lowenstein-Jensen [LJ]) or liquid (mycobacterial growth indicator tube [MGIT 960]) media.

In Morocco, as it's the case in many countries worldwide, culture on solid media using Lowenstein-Jensen is the main technique used to detect TB in samples. The culture-dependent laboratory procedures may take 4 to 6 weeks to have MTB cultures both for TB diagnosis and for further analyses e.g. Drug susceptibility testing (Eing, 1998).

The liquid culture (LC) gives an alternative opportunity to enhance TB diagnosis by conventional techniques. In fact, LC is significantly faster: the average time-to-growth detection with liquid culture is 10 to 14 days. Controlled trials have demonstrated that the performance of liquid media culture (LMC) is superior to that of solid media culture for diagnosis of MTB (Srisuwanvilai, 2008), but there is limited evidence about its performance in resource-limited settings.

TB culture on liquid media uses the Mycobacterium Growth Indicator Tube (MGIT). The MGIT is a commercial liquid culture system and the leading rapid culture method in the developed world. MGIT is manufactured in unbreakable plastic tubes containing enriched culture media. At the bottom of the tube is a silicone plug containing chemicals that become fluorescent when bacteria consume oxygen during the process of growth, making detection possible using either manual or automated systems. MGIT was approved by the World Health Organisation in 2007 and is already in use in high income countries and in the private sector. Its implementation in endemic countries is ongoing.

The roll-out of MGIT for case detection is especially important for patients with low numbers of TB bacteria in their sputum, such as children and individuals infected with HIV.

6.3 Molecular tests for diagnosis and identification of mycobacterial species

Because traditional techniques have several limitations, considerable progress has recently been made in developing novel approaches and tools, especially molecular methods (commercial and 'in-house'), for direct detection and identification of *M. tuberculosis* in clinical specimens within a single day after sputum collection. The potential advantages of molecular assays are the ability to (1) design assays that are highly sensitive and specific; (2) manufacture some assays in large quantities, allowing for decreased cost and ease of standardization in field use; (3) yield rapid results; and (4) be used more widely, because

they require less training and infrastructure than do conventional mycobacterial cultures and AST.

These potential advantages must be weighed against the disadvantages of these assays, some of which are common to all molecular techniques and others specific to particular assays. Among the disadvantages of molecular assays are (1) a need for laboratory infrastructure that can accommodate molecular testing; (2) cost; (3) a continued need of cultures for AST; and (4) most work better with smear-positive than with smear-negative specimens.

These methods, based on nucleic acid amplification (NAA) of different targets, aim to identify the M. tuberculosis complex, and eventually drug resistant strains. In general, commercial methods are recommended since they have a better level of standardization, reproducibility and automation. Although some aspects such as cost-efficiency and the appropriate setting for the implementation of these techniques are not yet well established, organizations such as the WHO are strongly supporting the implementation and universal use of these new molecular methods (Moure, 2010).

The available molecular methods for direct detection of MTB from clinical samples include in house polymerase chain (PCR) using essentially IS6110, hsp65 and 16SrRNA as target. Two Commercial nucleic acid amplification (NAA) tests for MTB detection in clinical specimens are available: the Enhanced MTB Direct Test (E-MTD), the Amplicor MTB test and its automated version the Cobas Amplicor MTB test, the BDProbe tec ET test, GeneXpert MTB/RIF Assay and the INNO-LiPA-Rif (Innogenetics, Ghent, Belgium).

6.3.1 Classical PCR using essentially IS6110, Hsp 65 and 16SrRNA as targets

The polymerase chain reaction (PCR) has been most widely used for the detection of *M. tuberculosis* in clinical specimens including sputum, blood, bone marrow, and biopsy samples.

The MTB Complex-specific insertion sequence 6110 is commonly used as a target for detecting MTB. The overall sensitivity and specificity of the assay range from 84.2-100% and 83-100%, respectively, for respiratory specimens (Shamputa, 2004).

The performance of the in house IS6110 PCR in direct detection of MTB on sputum has been evaluated in Morocco and showed promising results (under publication).

However, in non-respiratory samples, lower sensitivities were recorded in most studies with the exception of successful detection in pleural biopsy specimens in one study, and even in blood samples in another study.

The main advantage of the IS6110 targeted NAATs is the fact that most MTBC isolates carry more than five copies of this transposon, thereby increasing the sensitivity of the test. However, in some Asian regions MTBC isolates with no or few IS6110 copies are more prevalent.

The 16S rRNA and the Hsp65 genes have also been used to detect MTBC in respiratory and non-respiratory clinical specimens with high sensitivity and specificity values. In Morocco, the use of *hsp656* gene as a PCR target was evaluated as a direct method for the diagnosis of MTB in 70 clinical specimens (62 sputum, 6 cerebrospinal fluids, and 2 biopsies). Results

showed a sensitivity of 81.13 % with specificity of 88, 24 % as compared with conventional techniques. Moreover, the positive and negative predictive values were 95.56 %, 60% respectively (Zakham, 2011).

6.3.2 The amplicor MTB test and its automated version the Cobas Amplicor MTB test

The amplicor test is based on the PCR. In this assay, mycobacterial DNA is amplified with genus-specific primers formulated on the basis of the 16S rRNA gene. After denaturation, the amplicons are added to a microtiter plate containing a bound, *M tuberculosis* complex-specific oligonucleotide probe. An avidin-horseradish peroxidase conjugate then binds to the bound, biotin labelled amplicons. The conjugate then reacts with with peroxide and 3,39,5,59-tetramethylbenzidine in dimethylformamide to form a color complex. The results are measured with a photometer (D'Amato, 1995; Soini and Musser, 2001; Ozkutuk, 2006).

The Amplicor results are available in 6.5 h. An automated version of this test is available (Cobas Amplicor).The overall sensitivity of the Amplicor test (compared with culture) for respiratory specimens is 79.4 –91.9%, the specificity is 99.6 –99.8%. However, the sensitivity for smear negative specimens is somewhat lower, 40.0–73.1% (Bergmann, 1996; Stauffer, 1995; Tevere, 1996; Eing, 1998). Therefore, the Amplicor test has been approved by the Food and Drug Administration (FDA) only for direct detection of *M. tuberculosis* in AFB smear-positive respiratory specimens. Chin *et al.* (Chin, 1995) reported that the sensitivity of the Amplicor test was similar to that of culture (58% vs 56%) for detecting *M. tuberculosis* from respiratory specimens when the clinical case definition of TB was used as the reference standard. However, Al Zahrani *et al.* (2000) reported that although the Amplicor test had excellent specificity (100%), it was less sensitive than culture (42% vs 73%) for diagnosis of minimal active pulmonary TB (patients suspected of having TB but without spontaneous sputum or with AFB-negative smears).

6.3.3 Enhanced MTB direct test (E-MTD)

The E-MTD test is based on the transcription-mediated amplification system developed by Kwoh *et al*. (1989). In this assay, rRNA is released from the target cells by sonication, and a promoter-primer binds to the rRNA target. Reverse transcriptase is then used to copy rRNA to a cDNA-RNA hybrid. The initial RNA strand is degraded, and a second primer binds to the cDNA and is extended, leading to the formation of double-stranded cDNA, which is then transcribed by DNA-directed RNA polymerase to produce more rRNA molecules. The new transcripts serve as templates for reverse transcription and further amplification. The RNA amplicons are detected with an acridinium ester-labeled DNA probe in a solution hybridization assay. Importantly, the amplification procedure is isothermal and the reaction is performed in a single tube, which helps to reduce carryover contamination. After standard decontamination of the clinical specimen, the E-MTD test can be completed in 3.5 h.

The E-MTD test is FDA-approved for detection of *M. tuberculosis* in both AFB smear-positive and smear-negative specimens. The overall sensitivity (compared with culture) for respiratory specimens is 90.9 –95.2%, the specificity 98.8–100% (Bergmann, 1999; Gamboa, 1998; Smith, 1999).The performance of the E-MTD and the Cobas Amplicor is the same (Scarparo, 2000). However, it was noted that although the turnaround time is shorter for the

E-MTD test, the Amplicor test can be fully automated and has an internal control for monitoring amplification inhibitors.

6.3.4 BDProbeTec ET test

The BDProbeTec ET system allows amplification and detection of *M. tuberculosis* complex (MTBC) DNA in 1 h and simultaneously detects the presence of inhibitors as well. The target of the BDProbeTec ET system is a 95-bp region of IS*6110*, a highly specific insertion element in the MTBC DNA where it is present in multiple copies. Nucleic acid amplification is isothermal and is based on homogeneous strand displacement amplification (SDA) (Spargo, 1993), while detection is based on real-time fluorescent energy transfer (Little, 1999). An internal amplification control (IAC) is run with each sample to confirm the validity of the amplification reaction and to identify potential inhibitory factors from the processed specimen.

Of the published studies on the BDProbeTec system, sensitivities and specificities for respiratory samples were ranging from 82.7% to 100% and from 96.5% to 99.8% respectively (Mazzarelli, 2003). The extrapulmonary specimens represent a major diagnostic problem, mainly as they are often paucibacillary and at times contain inhibitors. With such samples, the resolved sensitivity of the BDProbeTec ET system is lower (77.8%) than with pulmonary specimens (91.5%), but nevertheless higher than with microscopy (63.1%) (Mazzarelli, 2003; Cho, 2007).

Other tests, Xpert MTB/RIF Assay (Cepheid) and INNO-LiPA-Rif (Innogenetics, Ghent, Belgium), used both for TB diagnosis and TB drug resistance screening will be discussed in later.

7. Diagnosis of drug resistant tuberculosis

7.1 Conventional tests

7.1.1 Microscopic Observation Drug Susceptibility (MODS) assay

The MODS assay is a broth microtiter method designed to detect *M. tuberculosis* complex and to detect resistance to isoniazid and rifampin (Moore, 2006; Mello, 2007; Ha, 2010). The method uses standard microtiter plates and other materials that are readily available in larger diagnostic laboratories. The method is straightforward: microtiter plates are prepared that contain Middlebrook 7H9 broth medium, growth supplements, and antimicrobial agents to prevent overgrowth of bacterial contaminants. Anti-TB drugs, at different concentrations, are added to some of the wells (Wilson, 2011).

The performance characteristics of the MODS assay were summarized in a recent meta-analysis. For detecting lowlevel resistance to isoniazid the pooled sensitivity of the assay is 97.7% and specificity is 95.8%. For detecting high-level isoniazid resistance, the sensitivity decreases to 90.0%, but the specificity increases to 98.6%. For detection of rifampin resistance, the pooled sensitivity is 98.0% and the specificity is 99.4%. This meta-analysis did not summarize the ability of the assay to identify the presence of *M. tuberculosis* in sputum specimens (Ha, 2010; Minion, 2010).

The published sensitivity of the assay varies from 87.4% to 97.8%, although the assay was compared with different gold standards in these studies. The contamination rate for the MODS assay, although lower than that of solid media, is higher than that of liquid media (Minion, 2010).

7.1.2 Drug susceptibility testing

The standard methods using the Lowenstein-Jensen (LJ) or agar proportion method (PM) (Canetti 1963, 1969, Kent & Kubica 1985) and the radiometric method in BACTEC TB-460 system (Becton-Dickinson) (Roberts 1983) are the current standard methods recommended to perform susceptibility testing of *M. tuberculosis*. The absolute concentration method is also commonly used on account of its technical simplicity for inoculums preparation and for reading results.

In order to shorten the turnaround time and make it more convenient for case management, numerous new techniques have appeared, aiming to detect growth inhibition as early as possible. The most commonly used systems are detection of CO_2 production, such as BACTEC 460 (Hawkins, 1991) or MB/Bact (Diaz-Infantes, 2000), and oxygen consumption, such as Mycobacteria Growth Indicator Tube (Bemer, 2002); there are others in developmental stage. However, many of those new techniques are difficult to implement in the developing countries where they are needed the most, because of high costs, technical complexity and absence of appropriately trained human resources.

7.1.3 DST on liquid medium

MGIT can also be used to perform drug susceptibility testing (DST), which is done by comparing the growth of mycobacteria with and without the addition of drugs used to treat TB. The combined use of MGIT for both TB detection and DST can shave months off the conventional process of identifying multidrug-resistant (MDR) TB (Hanna, 1999; Rusch-Gerdes, 1999).

Despite having been developed over a decade ago, the advantages of MGIT for TB detection were not reaching most endemic settings for several reasons. This was primarily due to the cost of the test, the lack of a simple means to confirm the growth of *M. tuberculosis* species in positive tubes, and the lack of data demonstrating that the use of liquid culture was feasible in resource-constrained settings. FIND has partnered with BD to overcome these obstacles and introduce MGIT as a solution for case detection and DST in developing countries.

7.1.4 Colorimetric assays

A colorimetric method for detecting microbial growth in drug-resistant strains was described in 1998 and subsequently evaluated in a limited number of clinical trials (Martin, 2005; Abate, 2004; Montoro, 2005). The assay is based on the observation that growing tubercle bacilli convert a yellow dye [3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyl tetrazolium bromide or MTT] to a purple color that can be detected visually or by use of a spectrophotometer. In field trials, the method has been shown to have a high degree of concordance with conventional AST (Martin, 2005; Abate, 2004; Montoro, 2005). The method has been compared with a nitrate reduction assay and a resazurin assay for detecting

resistance to isoniazid, rifampin, ethambutol, and streptomycin; similar results were obtained for isoniazid and rifampin, but only the nitrate reduction assay showed a high level of concordance with all of the first-line drugs. Although these methods are conceptually straightforward, they are likely to be useful primarily in larger laboratories with the capacity to perform more complex assays (Montoro, 2005; Wilson, 2011).

7.2 Molecular tests

Several molecular detection methods for drug resistance are of great value; all these methods are based on the observation that resistance to anti-TB drugs develops through the sequential accumulation of mutations in mycobacterial genes targeted by different drugs. Mutations in specific codons can therefore be used to rapidly detect drug resistance, since drug susceptible samples lack the corresponding gene mutation. These Molecular methods are fast and reliable and can potentially reduce the diagnosis time from weeks to days, These methods include PCR-based sequencing, PCR-Restriction Fragment Length Polymorphism, PCR-Single Strand Conformation Polymorphism (PCR-SSCP), Heteroduplex Analysis, DNA Microarrays and Probe methods. Moreover, a commercially available DNA strip assay (Genotype MTBDR; Hain Lifescience, Nehren, Germany) for detection of mutations conferring resistance to Rifampin (RMP) and Isoniazid (INH) in clinical *Mycobacterium tubercu*losis isolates is now widely used. GeneXpert MTB/RIF Assay and the INNO-LiPA-Rif (Innogenetics, Ghent, Belgium), are two rapid assays for simultaneous detection of MTBC and determination of the rifampicin (RIF) resistance profile (a marker for multidrug resistance).

7.2.1 PCR-based sequencing

PCR-based sequencing is the main technique used to elucidate the genetic mechanisms of drug resistance in *M. tuberculosis*. It is the most direct and reliable method for studying mutations and allows for detection of both previously recognized and unrecognized mutations. Unfortunately, the method is not as readily applicable for routine identification of drug resistance mutations as it is for identification of mycobacterial species because many different genes may be involved, as is the case in INH resistance, or the mutations may be scattered in a large segment of the gene. This means that several sequencing reactions need to be performed for each isolate. However, for targets such as *rpoB*, where mutations associated with RIF resistance are concentrated in a very short segment of the gene, PCR-based sequencing is a useful technique (Soini, 2001; Johnson, 2006; Kourout, 2009).

A previous study was done in Morocco to characterize mutations in rpoB gene associated with rifampicin (RMP) resistance in 47 RMP-resistant and 147 RMP-susceptible clinical strains of *Mycobacterium tuberculosis* by DNA sequencing. RMP-resistant mutations were identified in 85% of RMP-resistant isolates. Sequence analysis identified 10 alleles, including two deletions not previously reported: 514-515 Δ (Phe-Met → Leu) and 519-520 Δ (Asn-Pro). Nucleotide changes at codons 531, 526 and 516 were the most prominent, accounting for 74.4% of our RMP-resistant strains. These results demonstrate that DNA sequencing is an efficient tool for rapid detection of RMP resistance (Kourout, 2009).

7.2.2 Probe based hybridization methods

DOT BLOT strategy

It is a technique which can detect any known or newly described mutations and which can fulfil the criteria of accuracy, speed and simplicity. The hybridization with wild type probes can be used to efficiently screen for all mutations conferring drug resistance. The method includes:

- PCR amplification for target genes
- Confirmation of PCR amplification by gel electrophoresis
- Blotting PCR product on a filter using dot blot apparatus
- Labelling of probes for region of interest: the probes are 5′ end-labelled by phosphorylation with [∂^{32}P]-ATP or 3′ end-labelled with digoxigenin by terminal transferase.
- Dot Blot hybridization
- Autoradiography.

Although radioactive and non-radioactive detection procedures gave similar results, the radioactive procedure was favoured to empirically evaluate stringent hybridization washes during the development phase of this strategy.

The dot –blot procedure may be specifically useful in countries with a high incidence of TBand where procedures such as automated DNA sequencing are not readily available. As the predictive value of any test is dependent on prevalence, a mutational screening strategy should initially focus on the mutations most frequently diagnosed in the geographic area studied.

Codons 315 (KatG), 516,526 and 531 (rpoB), 43 (rpsL), 491,513 (rrs) and 306 (emb) are frequently altered in clinical isolates from many studies (Victor, 1999, Sabouni, 2008; Kourout, 2009; Chaoui, 2009). Such methods are needed to determine the most important mutations associated with drug resistance in different geographical regions, since it is known that drug resistant mutations may vary with the geographical origin of the sample.

The wild type probe strategy is unable to provide a precise understanding of the different mutations occurring at a specific codon, however, it is known that 99% of mutations within these loci confer resistance and therefore the absence of a hybridization signal has been interpreted to directly reflect drug resistance. The application of specific mutant probes allows the identification or the confirmation of the nature of this mutational event. The method is reproducible, not technically demanding and it takes about two normal working days to obtain results. This technique could be adapted to amplify and detect drug resistant mutations directly from sputum samples or microscopy stained slides.

7.2.3 Reverse line probe assay

This approach involves a combination of DNA amplification and reverse-line blot hybridisation. This home made and low cost test was first developed to detect RIF resistant isolates (RIFO): in this test , the DNA of *rpoB* gene of MTB is amplified by PCR using specific primers. Then, the PCR products are hybridized to oligonucleotides on a DNA membrane, encoding the consecutive parts of of the *rpoB* gene sequence and the consecutive parts of of

the *rpoB* gene sequence with the most frequently occurring mutations in rifampicin-resistant strains. The rpoB PCR products of in rifampicin-resistant strains will fail to hybridize to one or more of the wild type oligonucleotides, and will in most case show affinity to a mutant oligonucleotide. With this method, rifampicin resistance in MTB isolates can be detected within a few hours. In principle, the method can also be applied directly to clinical material and Ziehl-Neelsen (ZN) slides containing sufficient numbers of acid –fast bacilli, as has been demonstrated for spoligotyping, a PCR reverse-line blot assay to detect and type *M.tuberculosis*.

The accuracy, the high positive predictive value and the high sensitivity of the RIFO assay make it a useful tool for the early detection of MDR-TB cases (Morcillo, 2002; Senna, 2006). Even starting from early primary cultures, several important weeks can be saved with the application of the RIFO assay in comparaison with conventional laboratory methods.

The cost of the RIFO assay is 10 times lower than that of the commercially available kit to determine the RIF resistance of M.tuberculosis complex bacteria.

Later on, Mokrousov *et al* (1994) developed a home made reverse line blot (RLB) assay targeting a wide range of mutations in six genes (*rpoB*, *inhA*, *ahpC*, *rpsL*, *rrs*, *emb*B) associated with resistance to four first line anti-TB drugs (RIF, INH, SM and EMB).this macroarray based technique presnts in fact a rapid alternative to sequencing and may be recommended for use in TB reference laboratories. Its implementation can start with detection of RIF resistance as MDR marker and shoud focus on locallypredominant rpoB mutations. It is open to further development and it permits easy incorporation of new probes targeting mutations related either to newly uncovered mechanisms of resistance to the first-line anti-TB drugs, or to the second line drugs and newer anti-TB compounds. Analysis of the additional genes, such as, *gyrA* and *gyrB* (FQ resistance), other *rrs* mutations in the 530 and 912 regions (SM resistance), and *rpoB* mutations outside RRDR, eventually using a multiple co-amplification/co-hubridisation approach, seems promising.

7.2.4 PCR-Single Strand Conformation Polymorphism (PCR-SSCP)

SSCP is a gel based method that can detect short stretches of DNA approximately 175–250bp in size. Small changes in a nucleotide sequence result in differences in secondary structures as well as measurable DNA mobility shifts that are detected on a non-denaturing polyacrylamide gel.

PCR-SSCP analysis is increasingly useful. To date various studies have applied PCR-SSCP to identify mutational changes associated with drug resistance in *M. tuberculosis* for frontline drugs like, RIF and INH (Kim, 2004; Cardoso, 2004; Fang, 1999; Heym, 1995; Pretorius, 1995) In particular, the development of nonisotopic PCR-SSCP analysis has simplified the procedure, enhancing its utility in routine laboratories (Kalia, 1997; Lee, 2003). However, PCR-SSCP analysis has been found to be technically demanding and not sufficiently sensitive. Furthermore SSCP conditions must be carefully evaluated since not all mutations will be detected under the same conditions. Also, results obtained with SSCP analysis should be interpreted with caution as the technique only detects mutations and gives no information on the nature of associated mutation. For example, silent mutations in the *rpo*B gene have been identified that give altered mobility patterns on SSCP analysis but have no

association with RIF resistance, which underlines the need for caution in interpreting results and phenotypic or genotypic correlation (Kim, 1997).

7.2.5 PCR-Restriction Fragment Length Polymorphism

Mutations associated with resistance can be identified by digestion of amplified PCR products with a restriction enzyme that cuts at the specific polymorphic DNA sequence followed by gel electrophoresis. Since not all mutations result in the gain or loss of a restriction site, general use of RFLP to screen for mutations associated with drug resistance is limited (Victor *et al*., 2002).

7.2.6 Microarrays

Although technically a solid-phase-type hybridization assay, microarrays, also known as biochips, have been proposed as new molecular methods for detecting drug resistance in *M. tuberculosis*. They are based on the hybridization of DNA obtained from clinical samples to oligonucleotides immobilized in a solid support, such as miniaturized glass slides. They have been mainly used to detect resistance to rifampicin. In a recent evaluation using oligonucleotide microarrays for analysis of drug resistance, Gryadunov et al. (2005) has detected over 95% rifampicin resistant and almost 80% isoniazid resistant *M. tuberculosis* isolates within 12 h in a sample of drug-resistant isolates and clinical samples. For the time being and due to the high cost involved, the use of microarrays for detecting drug resistance in TB is still beyond the reach of most clinical mycobacteriology laboratories.

7.2.7 Line-probe assays

This technology involves a series of steps including extraction of DNA from mycobacterial isolates or directly from clinical specimens, polymerase chain reaction (PCR) amplification of nucleic acid sequences, hybridization of labeled PCR products with oligonucleotide probes immobilized on a strip, and colorimetric development that allows for lines to be seen where the probes are located (hence, the term ‘‘line-probe’’ assay) .

In 2008, the WHO issued a policy statement regarding the molecular line-probe assays for use in detection on *M. tuberculosis* and for detection of drug resistance.

The first line-probe assay was the INNO-LiPA Rif TB (Innogenetics NV) (Rossau, 1997). The results of clinical evaluations of the assay indicated that it accurately detects resistance to rifampin, but some of the evaluations showed that the assay was less sensitive for the detection of *M. tuberculosis* complex. A meta-analysis performed in 2005 showed that 12 of 14 published studies showed 95% of sensitivity with a specificity of 100% but that, in studies in which the assay was applied to clinical specimens, the sensitivity ranged from 80% to 100%. One study showed that the assay could be used successfully in a resource-poor setting, compared with reference laboratories (Wilson, 2011).

The second line-probe assay was the GenoType MTBDR® developed by Hain Lifescience. Initially, this assay was developed as the GenoType MTBDR assay, but early evaluations showed that the assay did not detect drug resistance to a satisfactory degree, detecting only 90%–95% of isolates with rifampin or low-level isoniazid resistance. The assay was eventually modified to include detection of more rpoB and inhA mutations under the name

GenoType MTBDRplus®. Although 2 evaluations of the new assay showed improvement of the detection of isoniazid resistance (Hillemann, 2007), 3 other evaluations showed that detection of isoniazid resistance remained suboptimal (particularly for strains with low-level resistance) (Helb, 2010).

GenoType MTBDRplus® is a DNA strip test that allows simultaneous molecular identification of tuberculosis and the most common genetic mutations causing resistance to rifampicin and isoniazid. This technology can diagnose MDR-TB directly from smear-positive sputum samples, providing results in just five hours - an enormous improvement on the 1 to 2 months needed for conventional DST (Figure 3).

A meta-analysis performed in 2008 confirmed these findings; the assay shows high sensitivity and specificity for detecting resistance to rifampin but variable results for detecting resistance to isoniazid (Barnard, 2008, Ling, 2008). A second meta-analysis performed the subsequent year showed similar results, although in this analysis, the pooled sensitivity of the GenoType MTBDRplus assay showed better sensitivity for detecting isoniazid resistance (Wilson, 2011). Overall, this genotyping kit is a rapid, manual nucleic acid amplification test (NAAT) and capable of both detecting *M. tuberculosis* and carrying out drug susceptibility testing (DST), however, results of studies' evaluations indicate that the assay is of limited use with smear-negative specimens and that detection of isoniazid resistance is more variable but generally lower than detection of rifampin resistance (Barnard, 2008).

More recently, another version, named GenoType MTBDRsl®, was developed to detect resistance to fluoroquinolones, ethambutol, kanamycin, amikacin and capreomycin. Two evaluations of this assay have shown promising but variable results for detection of resistance to the second-line drugs (Brossier, 2010).

As with any new diagnostic test, the impact of GenoType MTBDRplus® Assay will depend on the reproducibility of the results under actual field conditions, the manner and extent of their introduction, the strength of the laboratories and the degree to which access to appropriate therapy follows access to diagnosis.

LPA is now being rolled out by FIND and partners in 27 high MDR-TB burden countries under the EXPANDx-TB programme, supported by UNITAID.

Currently, the implementation of the GenoType MTBDRplus® as a tool for detection of MDR / XDR strains is under evaluation in two regions of Morocco and is supported by WHO under EMRO – COMSTECH projects. The first assay concerned Rabat and neighbours cities which is the highest rate of pulmonary tuberculosis in Morocco and containing the major and the reference hospital of pulmonary diseases. In fact, this hospital receives patients from hall the country and concentrate patients infected with MDR and XDR TB strains

The second assay concerned Tangier and neighbours cities, with a high prevalence of tuberculosis. Moreover, Tangier area has been the theater of a new phenomenon that can affect the epidemiology of tuberculosis in this region; immigration from the sub-Saharan region to attend Europe.

We believe that the implementation of the GenoType MTBDRplus® will be of a great interest to enhance drug resistant TB diagnosis and therefore to improve TB management in Morocco.

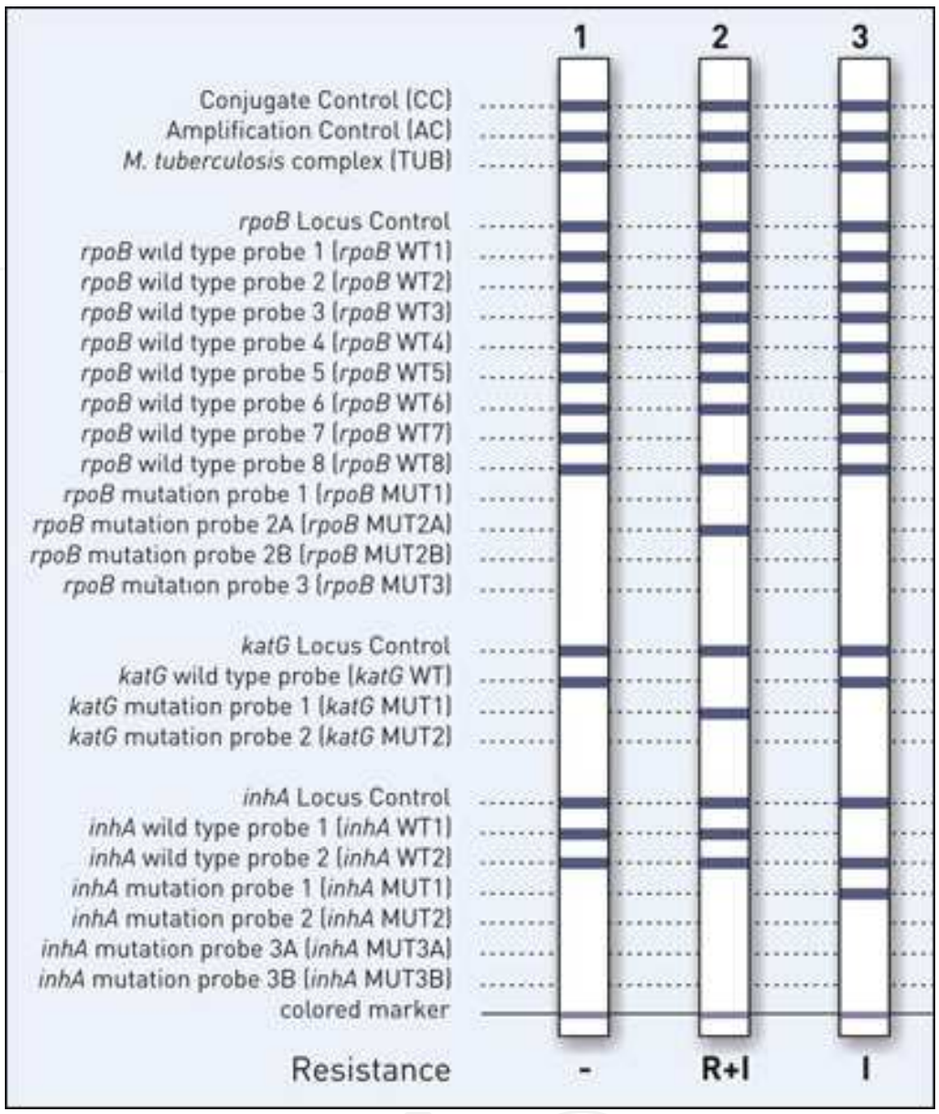

Fig. 3. Schematic representation of results obtained with the GenoType MTBDRplus® test
- : Sensitive strain
R+I: Strain resistant for both rifampicin and isoniazid,
I: Strain resistant only for isoniazid

7.2.8 Cartridge-based automated NAAT

This assay, usually called Xpert MTB/RIF, is a self-contained and fully automated technological platform that integrates sputum processing, DNA extraction and amplification, TB and MDR-TB diagnosis. This assay, which was CE (Conformité Européenne) marked in 2009, avoids many of the pitfalls of conventional nucleic acid amplification tests (Blakemore, 2010; Helb, 2010; Boehme, 2010; Hillemann, 2011). This is largely because the device is self-enclosed and, therefore, requires less sophisticated infrastructure in terms of laboratory facilities, user training, and supply chain management.

The assay has recently undergone performance evaluation to detect TB and rifampicin resistant strains on respiratory specimens (Wilson, 2011; Helb, 2010; Boehm, 2010; Rachow, 2011) as well as on non-respiratory samples (Rachow, 2011). The sensitivity of the test to detect smear-positive isolates reached 100%. However, the sensitivity for the identification of smear-negative culture positive isolates ranged from 71 to 72.5%. Xpert MTB/RIF test was shown to be specific in 99.2% of patients without TB. Moreover, its usefulness in detecting sputum smear and culture negative patients needs further studies (Helb, 2010; Marlowe, 2011).

On the other hand, and as compared with phenotypic drug-susceptibility testing, Xpert® MTB/RIF test was shown to be highly sensitive for detecting rifampin resistance, correctly identifying 97.6% of rifampicin-resistant isolates and 98.1% of rifampicin susceptible isolates (Boehme, 2010).

The main disadvantage of this system is the inability to test for and detect isoniazid resistance. Other potential disadvantages are related to the cost and a continued need for adequate laboratory infrastructure and training of personnel.

As for GenoType MTBDRplus® test, the Xpert MTB/RIF Assay will depend on the reproducibility of the results, the manner and extent of their introduction, the strength of the laboratories and the degree to which access to appropriate therapy follows access to diagnosis.

Thus, due to its high sensitivity and specificity, time consuming and the facility to use, the Wold Health Organisation endorsed in December 2010 Xpert® MTB/RIF technology and released a roadmap to guide its rapid adoption in endemic countries. In this context, the Xpert® MTB/RIF technology will be implemented in different cities of Morocco and should be used as the initial diagnostic test in individuals suspected of having MDR-TB or HIV-associated TB and may be considered as a follow-on test to microscopy in settings where MDR-TB or HIV is of lesser concern, especially in further testing of smear-negative specimens.

8. The place of molecular approaches in the TB management policy in Morocco

Guidelines for the use of nucleic acid amplification (NAA) tests for the diagnosis of tuberculosis (TB) were published in 1996 (CDC, 1996) and updated in 2000 (CDC, 2000). Since then, NAA testing has become a routine procedure in many institutions for the diagnosis of TB, because NAA tests can rapidly and reliably detect *Mycobacterium tuberculosis* bacteria directly in a specimen one or more weeks earlier than culture. Earlier laboratory confirmation of TB can lead to earlier treatment initiation, better patient care and outcomes, greater opportunities to interrupt transmission and improved public health interventions.

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laboratory confirmation of TB can lead to earlier treatment initiation, better patient care and outcomes, greater opportunities to interrupt transmission and improved public health interventions.

In Morocco, TB biological diagnosis is mainly limited to conventional techniques. These techniques (AFB smear, bacterial culture on solid and liquid media) are well established across the network of tuberculosis control laboratories. However, 2 laboratories, considered as reference laboratories, are authorized to perform drug susceptibility tests to antibiotics. According to WHO guidelines, the tested antibiotics are: INH, RIF, SM and EMB.

In some cases, molecular techniques based on PCR amplification are used to detect TB especially in extra-pulmonary tuberculosis.

Moreover, molecular techniques are well used in research and epidemiology (RFLP, spoligotyping, MIRUs,…). Currently, manual and automated techniques based on molecular approaches are achievable in some well-equipped laboratories in major centrs in Casablanca, Rabat and Tangier.

As in many developing countries, where the incidence of tuberculosis is declining slowly, molecular based tests should be introduced in the national program of Morocco; or at least tested for a period to verify the impact of such tests on the annual incidence of tuberculosis. In response to a request from many researchers in the field of tuberculosis, an advisory council should be set up to evaluate the place of NAA tests in the national program; against tuberculosis in Morocco. This committee must include TB clinicians, TB control officials; laboratory directors or supervisors from small, medium and large public health laboratories, hospital laboratories, and commercial laboratories and representatives from the Regional Training and Medical Consultation Centres. This suggestion is based on general observations for the use of NAA test to diagnose tuberculosis infections and drug resistance of *Mycobacterium tuberculosis* (CDC, 2009).

NAA testing has significant potential added value for clinicians and TB control officials.

a. Earlier diagnosis leads to earlier initiation of treatment, a reduced period of infectiousness and improved patient outcomes.
b. Earlier notification of TB cases to public health authorities should permit public health interventions sooner and may engage a TB expert sooner in the care of the TB patient.
c. Earlier detection of *M. tuberculosis* bacteria in sputum specimens can facilitate earlier infection control (respiratory isolation) decisions.
d. Earlier differentiation of AFB-smear positive specimens containing *M. tuberculosis* from those containing other Mycobacteria can eliminate unnecessary contact investigations.

NAA tests can provide substantial savings

i. for the patient (earlier diagnosis, improved outcomes, reduced health-care costs);
ii. for the health care provider (definitive diagnosis earlier, focused diagnostic testing, optimum patient care);
iii. for the hospital (less potential for nosocomial transmission, briefer period of respiratory isolation if TB is excluded);
iv. for the public health program (interrupt transmission earlier, abbreviated period for transmission, focused contact investigations).

For achieving this Implementation, that we believe will have benefic on the national program for TB in Morocco, research projects are needed to:

1. Conduct operational, translational, and implementation research for developing, evaluating, and selecting the most effective and efficient NAA testing algorithms for routine use and for specific scenarios.
2. Develop and evaluate suitable tests for use with non-respiratory specimens (e.g., cerebrospinal fluid, gastric aspirates, or biopsies).
3. Develop and evaluate tests that will enhance the diagnosis of TB in children.
4. Develop and evaluate optimal specimen collection, transport, and processing methods.
5. Determine the influences of specimen quality and quantity on NAA test performance.
6. Characterize the ability of NAA tests to detect *M. tuberculosis* bacteria in mixed infections, specimens and cultures.
7. Develop, evaluate and deploy NAA tests with improved performance and ease-of-use.

9. Conclusion

Truly rapid results for drug susceptibility tests are particularly important in the management of drug-resistant tuberculosis. Thus, the improvements made by molecular biology suggest that effective diagnostic strategies could be used to identify patients with or without MDR and even XDR TB strains. However, these techniques suffer from the problem that the genetic basis of resistance is not fully understood for any TB drug for all *M. tuberculosis* isolates.

Conventional techniques will yet be considered as a gold and reference methods. The currently available molecular methods may aid in rapid detection of mutations associated with drug resistance, but the test results must always be confirmed by phenotypic methods.

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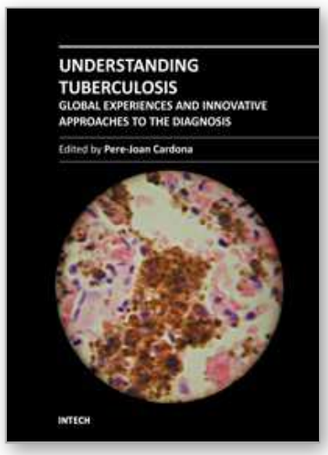

Understanding Tuberculosis - Global Experiences and Innovative Approaches to the Diagnosis

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

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