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Advances in the molecular diagnosis of tuberculosis: From probes to genomes

Diana Machado, Isabel Couto, Miguel Viveiros*

Global Health and Tropical Medicine, GHTM, Instituto de Higiene e Medicina Tropical, IHMT, Universidade Nova de Lisboa, UNL, Lisboa, Portugal

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ABSTRACT

Tuberculosis, disease caused by *Mycobacterium tuberculosis*, is currently the leading cause of death by a single infectious agent worldwide. Early, rapid and accurate identification of *M. tuberculosis* and the determination of drug susceptibility is essential for the treatment and management of this disease. Tuberculosis diagnosis is mainly based on chest radiography, smear microscopy and bacteriological culture. Smear microscopy has variable sensitivity, mainly in patients co-infected with the human immunodeficiency virus (HIV). Conventional culture for *M. tuberculosis* isolation, identification and drug susceptibility testing requires several weeks owning to the slow growth of *M. tuberculosis*. The delay in the time to results drives the prolongation of potentially inappropriate antituberculosis therapy contributing to the emergence of drug resistance, reducing treatment options and increasing treatment duration and associated costs, resulting in increased mortality and morbidity. For these reasons, novel diagnostic methods are need for timely identification of *M. tuberculosis* and determination of the antibiotic susceptibility profile of the infecting strain. Molecular methods offer enhanced sensitivity and specificity, early detection and the capacity to detect mixed infections. These technologies have improved turnaround time, cost effectiveness and are amenable for point-of-care testing. However, although these methods produce results within hours from sample collection, the phenotypic susceptibility testing is still needed for the determination of drug susceptibility and quantify the susceptibility levels of a given strain towards individual antibiotics. This review presents the history, advances and forthcoming promises in the molecular diagnosis of tuberculosis. An overview on the general principles, diagnostic value and the main advantages and disadvantages of the molecular methods used for the detection and identification of *M. tuberculosis* and its associated disease, is provided. It will be also discussed how the current phenotypic methods should be used in combination with the genotypic methods for rapid antituberculosis susceptibility testing.

1. Introduction

Tuberculosis, caused by *Mycobacterium tuberculosis*, is the leading cause of death by a single infectious agent and the ninth leading cause of death worldwide (World Health Organization, 2017a). Despite the global incidence of tuberculosis steady decline, tuberculosis burden is still globally high. In 2016, the WHO estimated 10.4 million incident cases with 1.3 million deaths among human immunodeficiency-virus (HIV)-negative patients and an additional 374 000 deaths among HIV-positive patients (World Health Organization, 2017a). Drug-resistant tuberculosis is a permanent threat to the global control of the disease. The emergence of multi- and extensively drug resistant tuberculosis (MDRTB and XDRTB, respectively) is increasing in some regions of the world. MDRTB is defined as tuberculosis resistant to at least isoniazid and rifampicin and XDRTB is defined as MDRTB with additional resistance to any fluoroquinolone plus a second line injectable. In 2016, there were 600 000 new tuberculosis cases presenting resistance to rifampicin, of which 490 000 were MDRTB cases, resulting in 240 000 deaths. The average proportion of MDRTB cases with XDRTB was 6.2%, globally (World Health Organization, 2017a).

The increase of MDRTB and XDRTB cases demands accurate and reproducible early detection and drug susceptibility testing concerted with global efforts to contain this potentially incurable disease, very much depending on its rapid detection and implementation of effective treatment together with public health interventions. The spread of drug resistant tuberculosis reflects the partial failure of the tuberculosis control programs and the inefficacy of the health care system to contain the dissemination of the resistant forms of the disease. Failure in the

* Corresponding author at: Unidade de Microbiologia Médica, Instituto de Higiene e Medicina Tropical, Universidade Nova de Lisboa, Rua da Junqueira 100, 1349-008 Lisboa, Portugal

E-mail address: mviveiros@ihmt.unl.pt (M. Viveiros).

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antibiotic provision and supply in a direct observed treatment course, inadequate prescription, non-adherence to the treatment, inadequate training of health care providers and the association HIV-tuberculosis are some of the contributing factors to the development of drug resistance (World Health Organization, 2017a).

The early detection of active tuberculosis, particularly the detection of MDR and XDR *M. tuberculosis* strains, is essential since it allows the implementation of appropriate treatment and isolation measures, promoting cure and preventing the dissemination of the disease. Nevertheless, despite all the advances in tuberculosis diagnosis, only half of the pulmonary tuberculosis cases are laboratory-confirmed, globally. For example, in 2015 and 2016, only 57% of the pulmonary cases reported to the WHO were bacteriologically confirmed (World Health Organization, 2016a, 2017a). This figure rests practically unchanged since 2013 where only 58% of the pulmonary cases reported were bacteriologically confirmed (World Health Organization, 2014, 2015). The testing for rifampicin resistance was performed in 33% of new tuberculosis cases and 60% of previously treated tuberculosis patients with an overall coverage of 39% (World Health Organization, 2017a). Among the MDRTB and rifampicin-resistant cases notified in 2016, only 39% were tested for resistance to both fluoroquinolones and second-line injectable agents. Altogether, this data stresses the need for improvement in both tuberculosis detection and implementation of drug susceptibility testing on a routine basis all over the world. In this context, the mycobacteriology laboratory has a major role in the diagnosis of tuberculosis.

To develop guidelines for efficient clinical and laboratory diagnosis of all forms of tuberculosis and to optimize the appropriate treatment for drug resistant tuberculosis it is essential to perform accurate drug susceptibility testing and to understand the genetic basis of *M. tuberculosis* drug resistance. With the advances in the molecular biology field, rapid detection of *M. tuberculosis* and drug resistance has been shown to be an important tool for the development and implementation of more effective and sensitive diagnostic methods able to enhance tuberculosis control. Although not ideal, the tuberculosis pipeline for the development of new nucleic acid-based diagnostic methods is progressing (Fig. 1). This review will present the history, advances and forthcoming promises in the molecular diagnosis of tuberculosis. We will provide an overview about the general principles, diagnostic value and the main advantages and disadvantages of the molecular methods currently used or in late stage of development and evaluation for the early detection and identification of *M. tuberculosis* and the disease caused. It will be also discussed how the current phenotypic methods can be used in combination with the genotypic methods for rapid antituberculosis susceptibility testing in order to effectively guide tuberculosis treatment.

2. Current status of tuberculosis diagnosis and determination of drug resistance – an overview

The methods currently employed for the routine laboratory diagnosis of tuberculosis rely on acid-fast smear microscopy, culture-based methods and nucleic acid amplification tests (NAATs) (Fig. 2). *M. tuberculosis* can be isolated from a wide variety of biological samples such as bronchial secretions, sputum, bronchoalveolar lavage fluid, gastric lavage, blood, bone marrow, urine, pleural fluid, cerebrospinal fluid, biopsies, among others. The microscopic detection of acid-fast bacilli is the oldest diagnostic method used in the diagnosis of tuberculosis. Microscopic examination of smears for the detection of acid-fast bacilli by the Ziehl-Neelsen staining is simple, rapid, and inexpensive. In spite of the high specificity of the procedure, its sensitivity has been reported to vary between 20% to 80% (Steingart et al., 2006; Steingart et al., 2007) with a limit of detection ranging between 5,000 to 10,000 bacilli/ml of sputum in unprocessed direct smears (David, 1976). Smear microscopy sensitivity is particularly low in patients in paucibacillary tuberculosis commonly observed in children, patients with extrapulmonary tuberculosis and among HIV-infected patients (Parsons et al., 2011). Other staining methods have been developed to improve the sensitivity and specificity of the detection including the fluorescent auramine-rhodamine staining (Steingart et al., 2006). This technique is faster than the Ziehl-Neelsen staining and increases the sensitivity by almost 10% (Steingart et al., 2006). This increased sensitivity allows the reduction in the number of samples analysed, thereby decreasing workload and speeding up the diagnosis (Trusov et al., 2009). However, fluorescence microscopy is more expensive due to the maintenance of the fluorescence microscope. To overcome this limitation, in 2011, the WHO recommended the use of light-emitting diodes (LED) fluorescence microscopy for the diagnosis of tuberculosis (World Health

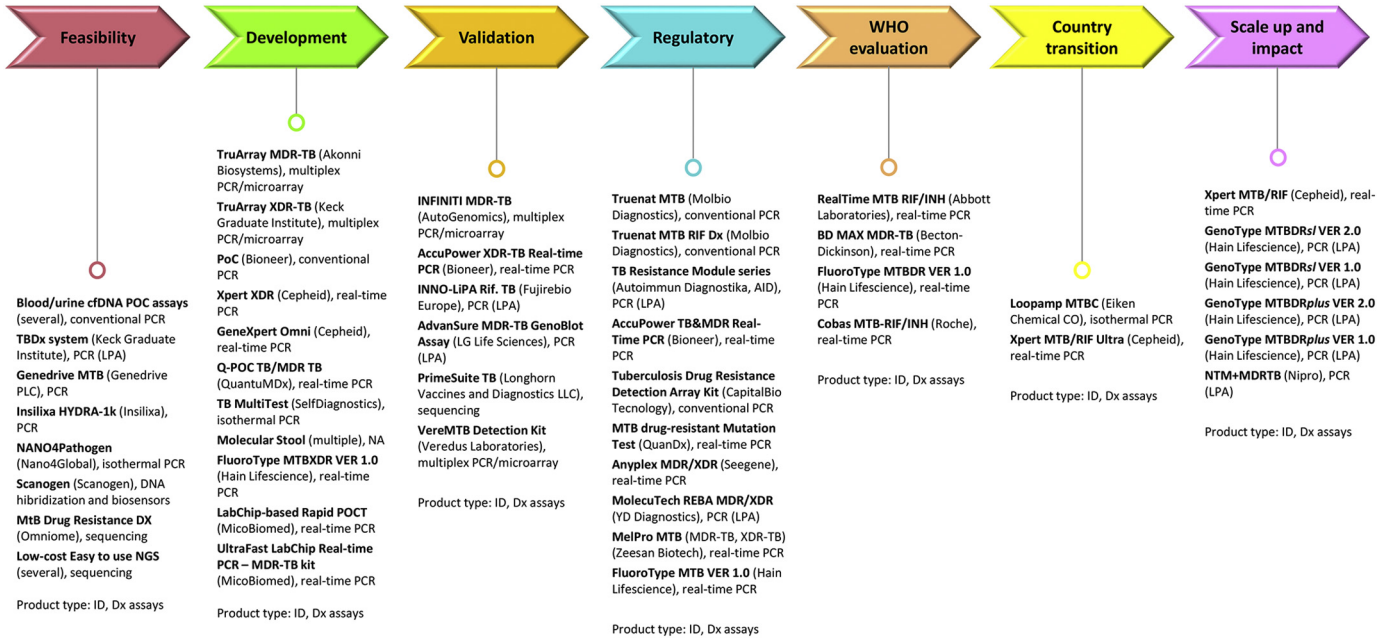

Fig. 1. WHO diagnosis pipeline for tuberculosis (adapted from https://www.finddx.org/dx-pipeline-status/. Accessed 17 September 2018). ID, identification; Dx, drug susceptibility testing.

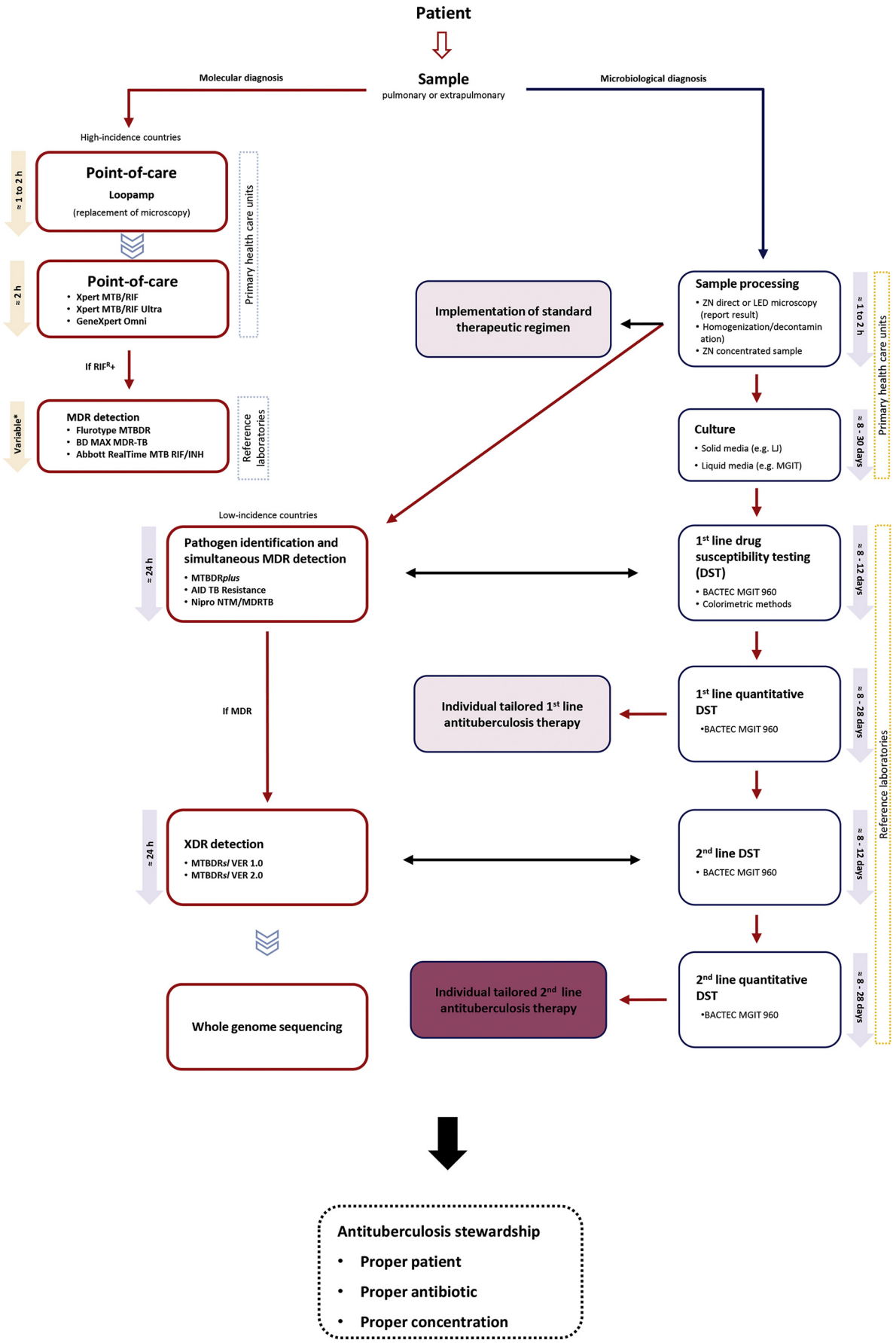

Fig. 2. Overview of the current diagnostic workflow and integration phenotypic methods in the future diagnostic algorithm for tuberculosis. ZN, Ziehl-Neelsen; LJ, Löwenstein-Jensen.

Organization, 2011a). The replacement of the light fluorescence microscopy by LED microscopy allowed the reduction of the costs associated with microscope maintenance, independence on energy as it can run on batteries and does not require a dark room for sample examination. Nevertheless, both methodologies are not able to distinguish between *M. tuberculosis* and nontuberculous mycobacteria (NTM). The early identification of smear-positive patients is of major importance in low-income countries with limited laboratory coverage since it is comparatively inexpensive, easy to preform and directly related with the severity of the disease, preventing transmission, as the patient will then be followed by the health system and start appropriate treatment. LED microscopy is one of the most significant advances for peripheral healthcare settings especially in limited resources countries, where this technology is meant to be used, reaching sensitivities and specificities to detect acid-fast bacilli comparable to many molecular methods (Chang et al., 2016).

Mycobacterial culture, still the gold standard for the laboratory diagnosis of tuberculosis due to its high sensitivity and specificity, especially when combined with molecular techniques, allows species identification, drug susceptibility testing, monitoring the response to drug therapy and epidemiological studies. Culture is an expensive method, since *M. tuberculosis* does not grow in common culture media, demanding the use of specific media (for review, see Viveiros et al., 2013; Procop, 2016). Besides, *M. tuberculosis* slow growth rate delays its detection. For isolation of *M. tuberculosis* from biological samples, three types of culture medium can be employed: egg-based medium (Löwenstein-Jensen and its adaptation for the Ogawa-Kudoh method (Kudoh and Kudoh, 1974), medium with agar (Middlebrook 7H10 and 7H11) and liquid medium (Middlebrook 7H9 and mycobacterial growth indicator tubes – MGITs). Among the solid medium, the Löwenstein-Jensen medium is the most widely used for growth of mycobacteria. The commercial liquid-based culture systems available can be manual, semi-automated or automated using colorimetric or fluorometric detection methods. The liquid media generally used is the Middlebrook 7H9 or the MGIT media, depending on the system and the source of the specimen. Examples of these systems include the BACTEC MB9000, BACTEC MGIT 960 and 320 and the manual MGIT, the Septi-Chek AFB systems (Becton Dickinson, Sparks, MD, USA), the ESP (Extra Sensing Power) Myco-ESPculture System II (Trek Diagnostic Systems, USA) and the BacT/ALERT MB (bioMérieux, Marcy-l'Etoile, France). The most widely used is the BACTEC MGIT 960 system, a fully automated fluorometric system currently used for the isolation of mycobacteria and drug susceptibility testing of *M. tuberculosis*. Despite its importance, several restrictions have been hindered the widespread implementation of mycobacterial cultures as a routine procedure for the laboratory diagnosis of tuberculosis, including the need of specialized infrastructures, equipment and personnel, the maintenance of the appropriate biosafety level 3, uninterrupted power supply, the cost, among others.

Rapid differentiation of *M. tuberculosis* from NTM or other acid-fast bacteria recovered in culture is essential (World Health Organization, 2007). For many years, the identification of *M. tuberculosis* complex was based on the observation of phenotypic characteristics and a battery of biochemical tests - growth rate, growth at different temperatures, colony morphology, pigment production, niacin production, catalase activity, nitrate reduction, susceptibility to thiophene-2-carboxylic acid hydrazide (TCH), among others (David et al., 1989). In spite of their standardization and application in several reference laboratories, these methods were very time consuming, extremely laborious, required trained personnel, and strict biosafety control due to the bacterial load, and were difficult to interpret originating false-results or delaying the final result by several weeks due to repetitions. During the last two decades, the classical biochemical methods have been replaced by immunochomatographic lateral flow assays (LFAs) for the identification of *M. tuberculosis* in culture or by molecular methods for the identification of *M. tuberculosis* from cultures or directly from clinical samples, reducing significantly the time to the final laboratory diagnosis from weeks to hours. The LFAs were developed for the differentiation between the *M. tuberculosis* complex and NTM and use monoclonal antibodies against the *M. tuberculosis* protein MPT64. The commercially available systems include the SD Bioline Ag MPT64 Rapid assay (Standard Diagnostics, Kyonggi-do, Korea), Capilia TB (TAUNS, Numazu, Japan), MGIT TBc Identification Test (Becton and Dickinson) and the TBCheck (Hain Lifescience, Nehren, Germany). These tests have demonstrated to be easy to incorporate in the routine of a mycobacteriology laboratory, are highly sensitive and specific for the detection of the *M. tuberculosis* complex, are simple to use, rapid to perform and inexpensive. They become a major advance for the laboratory differentiation of *M. tuberculosis* from NTMs in laboratories where molecular biology methods cannot be implemented or even as a routine screening method before proceeding for the more expensive molecular identification (Gaillard et al., 2011; Hillemann et al., 2005a; Machado et al., 2014). The old and new nucleic acid based methods will be discussed in the next section.

Finally, susceptibility testing to antituberculosis drugs is essential for the administration of an appropriate therapy. However, if culture is a slow process, drug susceptibility testing is even more time consuming. The phenotypic methods for *M. tuberculosis* drug susceptibility testing consists on the determination of the concentration that completely inhibits growth for that strain or the determination of a proportion of growth ($< 1\%$ of the population) in presence of a fixed critical concentration of the drug. These are as follows: (i) the absolute concentration method; (ii) the resistance ratio method; and (iii) the proportion method (Canetti et al., 1963, 1969). These methods use agar (MB7H10 or MB7H11) or Löwenstein–Jensen medium. The first two methods are very accurate but are cumbersome and lengthy. The proportion method, the most widely used and the reference method for drug susceptibility testing of *M. tuberculosis,* is based on the premise that every wild-type *M. tuberculosis* strain is not a homogenous population but instead contains a mutant subpopulation resistant to the antituberculosis drugs together with a susceptible subpopulation. The difference between a resistant strain and a susceptible strain is the proportion of resistant bacteria among the population that is much higher in the resistant strain than in the susceptible one (Canetti et al., 1963, 1969). The critical concentration of an antituberculosis drug is defined as the concentration that is required to eliminate more than 99% of the population of a control strain that has never been in contact with the drug. The critical concentration is empirically assumed to have correlation with the clinical outcome of the treatment and is inferred from the phenotypic behaviour of the *M. tuberculosis* wild-type strain that has never been exposed to antibiotics (susceptible). The proportion method correlates well with the clinical outcome of the patient and is the gold standard for *M. tuberculosis* drug susceptibility testing. Nowadays, the BACTEC MGIT 960 is the system of choice. The BACTEC MGIT is endorsed by the WHO for drug susceptibility testing of *M. tuberculosis* strains (World Health Organization, 2017a). Colorimetric redox indicator methods are also recommended by the WHO for indirect drug susceptibility testing of *M. tuberculosis* isolates grown in conventional culture suitable for reference laboratories due to biosafety concerns, when other methods cannot be used (World Health Organization, 2011b).

Although the phenotypic methods are the gold standard for *M. tuberculosis* drug susceptibility testing, they are time consuming (may take 4 to 8 weeks in solid media and up to 21 days in liquid media) and require specialized infrastructures and well-trained laboratory staff. The early detection of tuberculosis and its drug-resistant forms affords the effective management of the disease and reduce the frequency of noncompliance. Therefore, since 1990's, the WHO has recommended and supported the development and application of new rapid and accurate diagnostic methods focused on the simultaneous detection of the agent and presence of drug resistance traits, as the best strategy for the advance of tuberculosis laboratory diagnosis (Viveiros et al., 2010;

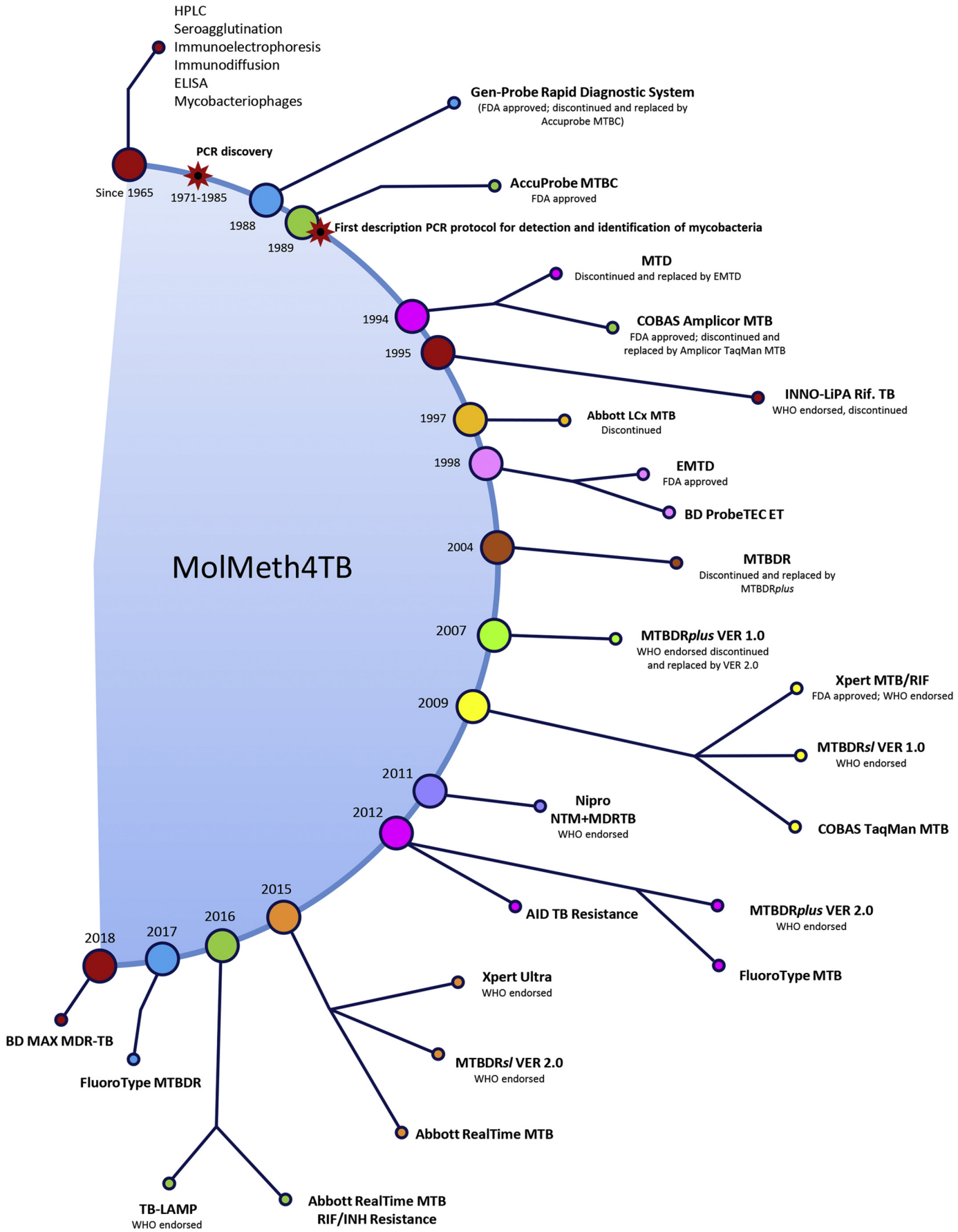

Fig. 3. The landmarks of tuberculosis molecular diagnosis.

Walzl et al., 2018).

3. Major historical landmarks of molecular diagnosis of tuberculosis

The pipeline for new proposed molecular tools for tuberculosis diagnosis has progressed quickly during the last years (Fig. 1). However, this was not always the case (Fig. 3). Until the introduction of the Polymerase Chain Reaction (PCR) in 1985 (Saiki et al., 1985), the identification of mycobacteria isolated in culture relied on methods such as thin-layer, high-performance liquid and gas-liquid chromatographic analysis of mycobacterial cell wall lipids (Brennan et al., 1978; Guerrant et al., 1981; Larsson and Mårdh, 1976; Marks and Szulga, 1965; Ramos, 1994; Tisdall et al., 1979, 1982). Chromatographic methodologies for mycobacterial identification have recently been revisited with the global advent, throughout resource-rich countries, of the matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry. However, as in the previous cases, is able to accurately identify mycobacteria (including *M. tuberculosis* complex) but requires fully-grown pure cultures from solid media (Buckwalter et al., 2016). Other methods used included serodiagnosis using enzyme-linked immunosorbent assay (ELISA) (Daniel and Debanne, 1987), immunoelectrophoresis and immunodiffusion (Daniel et al., 1979; Janicki et al., 1971). Nevertheless, at that time, these methods showed reduced specificity because the antigens used were common to all mycobacterial species as well as other related bacterial genera and presented variable reproducibility, hampering the diagnosis of tuberculosis (Nassau et al., 1976; Narayanan et al., 1986). Others also used mycobacteriophages specific for some members of the *M. tuberculosis* complex for identification and typing (Jones, 1975, 1979). However, phage typing, although highly specific and useful for epidemiological studies, is time-consuming, labour-intensive and required a high-level of technical expertise (Grange et al., 1977; Jones et al., 1982; Snider et al., 1984) becoming difficult to apply on the routine diagnosis of tuberculosis.

Consequently, to respond to the need for rapid and specific diagnostic methods for tuberculosis, during the decade of the 1980´s, methodologies based on restriction endonuclease analysis (Collins and De Lisle, 1984, 1985; Imaeda et al., 1982) and nucleic acid hybridization were developed and applied for the diagnosis of tuberculosis (Eisenach et al., 1986). These techniques provided a new approach for the rapid diagnosis of tuberculosis based on the detection of specific nucleotide sequences of the target organism present in clinical samples using labelled nucleic acid probes (Eisenach et al., 1986, 1988; Reddi et al., 1988; Roberts et al., 1987; Tenover, 1988). In 1987, the US Food and Drug Administration (FDA) approved the Gen-Probe Rapid Diagnostic System (Gen-Probe; currently Hologic, Toronto, Canada) for the identification of *M. tuberculosis* complex clinical isolates based on the hybridization of an I^{125}-labelled DNA probe with ribosomal RNA sequences unique to *M. tuberculosis* complex and later on also for *M. avium* complex (Drake et al., 1987; Gonzalez and Hanna, 1987; Musial et al., 1988; US Food and Drug Administration, 2018a). Although highly sensitive and specific for the identification of these complexes in culture, their laboratory implementation required permission for the use of radioisotopes. This system was latter replaced by a nonradioactive detection system, the Gen-Probe *Accu*Probe *Mycobacterium tuberculosis* complex culture identification test (*Accu*probe MTBC, Hologic) (Lebrun et al., 1992). This method is based on the hybridization protection assay and uses single-stranded DNA probes labelled with acridinium ester, a chemiluminescent compound (Lyle et al., 1989), that are complementary to the mycobacterial rRNA. After a step of sonication and heat to release the rRNA from the mycobacteria, the DNA-labelled probes hybridize with the mycobacterial rRNA forming a stable DNA-RNA complex. The addition of a selection reagent allows the differentiation of non-hybridized and hybridized probe. The hybrids can be detected by light emission on a luminometer (Hologic, 2018a). The method has a turnaround time of 2h, is safe, easy to perform, highly sensitive and specific (Lebrun et al., 1992; Reisner et al., 1994). The *Accu*probe MTBC test have received FDA approval in 1990 for the identification of *M. tuberculosis* complex isolated in culture and is still used nowadays, with one of the highest accuracies ever obtained for molecular methods of mycobacteria identification from culture (Centers for Disease Control and Prevention, 1996; US Food and Drug Administration, 2018b). The test is also commercially available for the identification of *M. avium* complex, *M. avium*, *M. intracellulare*, *M. kansasii* and *M. gordonae* (Hologic, 2018b).

The major limitation of the assays based on DNA probes is their dependence on high amounts of bacteria for detection, limiting their application for the direct detection of *M. tuberculosis* DNA in clinical specimens, a limitation solved by the introduction of nucleic acid amplification-based technologies, providing a huge advance on tuberculosis diagnosis. The detection and identification of mycobacteria by amplification of mycobacterial DNA was described for the first time in 1989 (Hance et al., 1989). Presently, nucleic acid amplification-based techniques have become widely used in routine by many laboratories for species identification from cultures and directly from specimens. Sequencing of PCR amplified specific regions is considered the gold standard for the identification of mycobacteria and the ribosomal 16S rRNA is the most common target (Tortoli, 2003). The 16S rRNA is highly conserved among different species, however, it contains hypervariable regions that are species-specific that can be used for identification at species level (Han et al., 2002). When the analysis of the 16S rRNA is not enough for species identification, other targets such as the 16S-23S rRNA intragenic region, *rpoB*, *gyrB*, *hsp65*, *recA* and *sodA* are used (Adékambi and Drancourt, 2004; Roth et al., 2000).

Among the first commercially available PCR-based methods for the direct detection of *M. tuberculosis* complex isolates are:

(i) The *M. tuberculosis* direct test (MTD) and the Enhanced Amplified *M. tuberculosis* direct test (EMTD), both from Gen-Probe. These were the first FDA-approved *M. tuberculosis* direct tests to be used in smear-positive and negative specimens (Centers for Disease Control and Prevention, 1996, 2000). Both tests target the mycobacterial 16S rRNA using an isothermal transcriptase-mediated amplification assay (Kwoh et al., 1989).

(ii) The COBAS Amplicor *Mycobacterium tuberculosis* test (Roche Diagnostic Systems Inc., Branchburg, NJ, USA), one of the first and oldest molecular methods developed and approved for the detection *M. tuberculosis* DNA from respiratory samples (Centers for Disease Control and Prevention, 1996). The assay target the 16S rDNA gene. For the detection of the amplified PCR products, is used an ELISA-like format assay in which the *M. tuberculosis*-oligonucleotide probe-coated paramagnetic microparticles present in the wells of the microtitre plates capture the biotin-labelled amplification products by hybridization. The colorimetric detection is mediated by the addition of avidin conjugated with horseradish peroxidase (DiDomenico et al., 1996; Soini and Musser, 2001). In 2009, Roche has replaced the COBAS Amplicor MTB by a new real-time PCR assay, the COBAS TaqMan MTB (Roche Diagnostics, 2009).

(iii) The LCx MTB assay (Abbott Laboratories, Abbott Park, IL, USA) uses the ligase chain reaction for amplification of the *pab* gene to detect *M. tuberculosis* complex directly in respiratory specimens (Andersen and Hansen, 1989; Ausina et al., 1997; Viveiros et al., 1999). This assay was withdrawn from the European market in 2002 (Piersimoni and Scarparo, 2003).

(iv) The BD ProbeTec energy transfer (ET) system (Becton and Dickinson) target the IS*6110* for the identification of *M. tuberculosis* complex isolates using isothermal strand displacement for DNA amplification (Becton and Dickinson, 2015; Bergmann and Woods, 1998; McHugh et al., 2004).

None of these methods revealed to be user-friendly and cost-effective to be truly adequate to the particularities and challenges of the tuberculosis laboratory routine diagnosis in the era of drug resistance

Table 1
Main characteristics of the genotypic assays used for the screening of drug-resistance associated mutations in *M. tuberculosis*.

Assay	Type	Target	Drug	Specificities
INNO-LiPA Rif. TB	Line probe assay DNA•STRIP technology	*rpoB* RRDR	RIF	5 WT probes 4 MUT probes - D516V (R2); H526Y (R4a); H526D (R4b); S531L (R5)
Genotype MTBDR	Line probe assay DNA•STRIP technology	*rpoB* RRDR	RIF	5 WT probes 4 MUT probes - D516V (MUT1); D526Y (MUT2A); H526D (MUT2B); S531L (MUT3)
		katG codon 315	INH	1 WT probe 2 MUT probes - S315T1 (ACC); MUT S315T2 (ACA)
Genotype MTBDR*plus* VER 1.0 and VER 2.0	Line probe assay DNA•STRIP technology	*rpoB* RRDR	RIF	8 WT probes 4 MUT probes - D516V (MUT1); D526Y (MUT2A); H526D (MUT2B); S531L (MUT3)
		katG codon 315	INH	1 WT probe 2 MUT probes - S315T1 (ACC); MUT S315T2 (ACA)
		inhA promoter	INH	2 WT probes 4 MUT probes - C-15T (MUT1); A-16G (MUT2); T-8C (MUT3A); T-8A (MUT3B)
Genotype MTBDR*sl* VER 1.0	Line probe assay DNA•STRIP technology	*gyrA* QRDR	FQ	3 WT probes 6 MUT probes - A90V (MUT1); S91P (MUT2); D94A (MUT3A); D94N/Y (MUT3B); D94G (MUT3C); D94H (MUT3D)
		rrs 1400 region	SLID	2 WT probes 2 MUT probes - A1401G (MUT1); G1484T (MUT2) C1402T detected by the simultaneous absence of hybridization with WT1 and MUT1
		embB codon 306	EMB	1 WT probe 2 MUT probes - M306I (MUT1A); M306V (MUT1B)
Genotype MTBDR*sl* VER 2.0	Line probe assay DNA•STRIP technology	*gyrA* QRDR	FQ	Same as Genotype MTBDR*sl* VER 1.0
		rrs 1400 region	SLID	
		eis promoter	KAN	3 WT probes to cover positions G-37, C-12, G-10, C-14 and C-2 1 MUT probe - C-14T (MUT1) G-37T MUT - absence of hybridization with WT1 C-12T and G-10A MUT - absence of hybridization with WT2 C-2A MUT - absence of hybridization with WT3
		gyrB QRDR	FQ	1 WT probe 2 MUT probes - N538D (MUT1); E540V (MUT2)
AID TB Resistance	Line probe assay DNA•STRIP technology	*rpoB* RRDR	RIF	3 WT probes 3 MUT probes - D516V/Y; H526Y/D/R; S531L/W
		katG codon 315	INH	1 WT probe 1 MUT probe - S315T (ACC/A)
		inhA promoter	INH	1 WT probe cover positions -15, -16 and -8 1 MUT probe cover positions -15, -16 and -8
		rpsL	STR	2 WT probes 3 MUT probes - K43R; K88R; K88Q
		rrs 500 region	STR	1 WT probe 4 MUT probes - C513T; A514C; G515C; C517T
		rrs 1400 region	SLID	2 WT probes 3 MUT probes - A1401G; C1402T; G1484C/T
		gyrA QRDR	FQ	1 WT probe 6 WT probes - A90V; S91P; D94A; D94N; D94Y; D94G
		embB codon 306	EMB	1 WT probe 4 MUT probes - M306V; M306I/G918A; M306I/G918C; M306I/G918T
Nipro NTM + MDRTB	Line probe assay DNA•STRIP technology	*rpoB* RRDR	RIF	5 WT probes 4 MUT probes - D516V (R2); H526Y (R4a); H526D (R4b); S531L (R5)
		katG codon 294 to 330	INH	4 WT probes 2 MUT probes - S315T (R9a); S315N (R9b)
		inhA promoter *inhA*	INH	2 WT probes - *inhA* promoter from position -17 to -6 (S6) and codons 6 to 11 on the structural region of the gene (S7) MUT probes - none
Xpert MTB/RIF	Real-time PCR Molecular beacon technology	*rpoB* RRDR	RIF	5 WT molecular beacon probes (A to E) MUT detected by reduced or absence of probe signal (Ct based) Does not specify MUT
Xpert MTB/RIF ULTRA	Real-time PCR Sloppy molecular beacon probes	*rpoB* RRDR	RIF	4 WT sloppy molecular beacon probes (rpoB1 to 4) MUT detected by the shift in T_m compared to WT Does not specify MUT
Abbott RealTime MTB RIF/INH Resistance	Real-time PCR	*rpoB* RRDR	RIF	8 WT probes Does not specify MUT
		katG	INH	1 WT probe 1 MUT probe - S315T1 (ACC)
		inhA promoter	INH	1 WT probe 1 MUT probe – C-15T
Flurotype MTBDR	Real-time PCR Fluorotype technology Linear-After-The-Exponential (LATE)-PCR Lights-on/lights-off Technology	*rpoB*	RIF	MUT detected: T508A; S509T; E510H; L511P; S512K; Q513L; Q513P; Q513R; D516A; D516F; D516V; D516Y; N518I; S522L; S522Q; H526C; H526D; H526G; H526L; H526N; H526P; H526Q; H526R; H526S; H526Y; R529K; S531F; S531L; S531L; S531Q; S531W; L533E; L533P Does not specify MUT
		katG	INH	MUT detected: S315T (ACC/A); S315N; S315R Does not specify MUT
		inhA promoter		MUT detected: G-17T; A-16G; C-15T; G-9A; T-8A; T-8C; T-8G Does not specify MUT

(*continued on next page*)

Table 1 (*continued*)

Assay	Type	Target	Drug	Specificities
BD MAX MDR-TB	Real-time PCR TaqMan probes	*rpoB*; *katG*; *inhA* promoter	RIF INH	Does not specify MUT

ETB, ethambutol; FQ, fluoroquinolones; INH, isoniazid; KAN, kanamycin; MDRTB, multidrug resistant tuberculosis; MTB, M. *tuberculosis*; MUT, mutation; NTM, nontuberculous mycobacteria; QRDR, quinolone resistance determining region; RIF, rifampicin; RRDR, rifampicin resistance determining region; SLID, second line injectable drugs; STR, streptomycin; TB, tuberculosis; WT, wild-type.

(Greco et al., 2006; Salfinger, 2014).

4. Advances in molecular methods for the diagnosis of tuberculosis

The line probe assay technology (hybridization on strips) revolutionized tuberculosis molecular diagnosis. Based on PCR amplification with biotinylated primers, reverse hybridization with species-specific DNA probes that are immobilized on a membrane strip and colorimetric detection of the hybridized probes, these systems allowed the identification of both *M. tuberculosis* complex and other mycobacterial species from cultures. Several assays are commercially available: the INNO-LiPA Mycobacteria v2 and the GenoType assays. The INNO-LiPA Mycobacteria v2 (INNOGENETICS N.V; currently Fujirebio, Ghent, Belgium) is based on the amplification of the 16S-23S rRNA intragenic region and allows the identification of *M. tuberculosis* complex and 16 NTM species (Fujirebio, 2005). The GenoType assays (Hain Lifescience, Nehren, Germany) are based on multiplex PCR. There are currently available three assays for the identification of the *M. tuberculosis* complex, of which two also allow the detection of NTM: (i) the GenoType MTBC for the differentiation within the *M. tuberculosis* complex, (ii) the GenoType Mycobacterium CM (common mycobacteria) and (iii) the Genotype CM*direct*. The GenoType MTBC uses specific fragments of *gyrB* gene, 23S rDNA and the region of deletion 1 (RD1). The analysis of *gyrB* polymorphisms allows the differentiation within the *M. tuberculosis* complex (Niemann et al., 2000). *M. bovis* BCG can be detected by the absence of the RD1 (Talbot et al., 1997). The amplification of the 23S rDNA covers the Gram-positive bacteria with a high content of G + C (amplification control) and all members of the *M. tuberculosis* complex (Richter et al., 2003). The GenoType CM assay target the 23S rDNA gene and is able to identify 14 mycobacterial species (Hain Lifescience, 2016). These tests are rapid, easy-to-perform and interpret, and possess high specificity and sensitivity (Richter et al., 2003, 2004; Couto et al., 2010). Recently, it was launched the Genotype CM*direct* for the identification of *M. tuberculosis* complex and the most common NTM directly from clinical specimens (Hain Lifescience, 2017). According to the manufacturer, the test has a sensitivity and positive predictive value (PPV) of 100% for smear positive samples. For smear negative samples, the sensitivity, specificity, PPV and negative predictive value (NPV) is 63.6%, 93.7%, 53.8% and 95.7%, respectively (Hain Lifescience, 2017). No external evaluation was published so far on the CM*direct* performance. Among the non-commercial assays, the detection of *M. tuberculosis* transrenal DNA (trDNA) from urine specimens is another promising method for the diagnosis tuberculosis focus on identifying short fragments (e.g. 38-bp specific region of the repetitive insertion element IS*6110*) of *M. tuberculosis* DNA in different specimens, including urine (Labugger et al., 2017; Patel et al., 2018). The short fragments of free DNA transverse the renal barrier and are excreted from the body in the urine (Cannas et al., 2008). The assay could be a candidate diagnostic tool for the detection of tuberculosis, especially for particular groups as those with paucibacillary and extrapulmonary disease and paediatric tuberculosis.

Next to the advances in the molecular detection of *M. tuberculosis* complex is the detection of mutations associated with resistance to the main first- and second-line drugs. The methods currently available are based on the evidence that resistance associated mutations occur in specific regions of the genes whose products are targeted by these antibiotics and these are the ones that have been populated the WHO diagnosis pipeline (Fig. 1). The assays included in the three last stages of the WHO diagnosis pipeline will be discussed in detail below. When relevant for contextualization, assays in earlier stages of the pipeline will be also included.

The INNO-LiPA Rif. TB assay (thereafter, LiPA), developed in 1995 by INNOGENETICS, was the first line probe assay developed and commercially available for the detection of *M. tuberculosis* complex DNA and mutations associated with drug resistance, in this case, rifampicin resistance (De Beenhouwer et al., 1995). The LiPA targets the 81 bp rifampicin resistance-determining region (RRDR) of the *rpoB* gene, corresponding to 27 amino acids, where are located the mutations responsible for the great majority of the rifampicin resistance phenotypes (Telenti et al., 1993; Rossau et al., 1997). Initially developed for the use with DNA extracted from full-grown cultured samples, the LiPA was optimized for a nested PCR to increase sensitivity, using outer primers followed by a nested reaction with inner primers, allowing the detection of *M. tuberculosis* complex DNA and mutations associated with rifampicin resistance directly from clinical samples (Viveiros et al., 2005). Both outer LiPA primers and inner LiPA primers are biotinylated at the 5′-end; the outer primers generate a fragment of 410 bp and the inner primers generate a fragment of 260 bp (Viveiros et al., 2005). The S probes hybridize exclusively to the wild-type sequence. If a mutation is present in one of the target regions, the mismatch created will prevent the amplicon from hybridizing with the corresponding probe under the stringent conditions applied. The absence of hybridization with one or more wild-type probes indicates the presence of a mutation that, if one of those particular mutations, they can be detected by the hybridization with one of the mutation probes (Table 1) (Fujirebio, 2006). Exceptions can occur when DNA of more than one strain exists in the sample, when multiple mutations exists or in the presence of heterorresistance. The test contains an additional probe, specific for organisms belonging to the *M. tuberculosis* complex, located approximately 82 bp upstream the RRDR of the *rpoB* gene, and allows the unequivocal identification of *M. tuberculosis* complex isolates (Rossau et al., 1997). LiPA provided results after 24 to 48 h after sample reception in the laboratory.

In 2008, the WHO endorsed LiPA for the rapid screening of patients at risk of MDRTB in smear-positive samples. A meta-analysis on the performance of the LiPA assay conducted by the WHO showed a sensitivity greater than 95% and specificity of 100% for the detection of *M. tuberculosis* complex isolates (World Health Organization, 2008). The pooled sensitivity was 0.97 (95% CI 0.95 - 0.98) and the pooled specificity 0.99 (95% CI 0.98 - 1.00). The overall discriminatory ability of the assay was 99% and overall accuracy 97%. When applied directly to clinical specimens, the LiPA showed 100% specificity and sensitivity ranging from 80% to 100%. Based on these evidences, the WHO Expert Group concluded that LiPA was a highly sensitive and specific assay for the detection of rifampicin resistance in *M. tuberculosis* complex isolates. LiPA appears in the validation stage of the WHO diagnostic pipeline (Fig. 1) however, is no longer commercially available. Despite all laboratory validation and field demonstration data performed worldwide, Fujirebio discontinued the LiPA soon after the introduction of the GeneXpert MTB/RIF.

The GenoType MTBDR (MTBDR) are qualitative *in vitro* assays

developed by Hain Lifescience for the detection of *M. tuberculosis* complex DNA and the most common mutations associated with resistance to the main first- and second-line drugs from cultures as well from clinical specimens and are based on the same principle of the LiPA. The first assay of this series was the MTBDR assay, which was introduced in 2004. The MTBDR assay covers overlapping wild-type sequences of the RRDR of the *rpoB* gene and the *katG* gene and the most frequent mutations in the RRDR of the *rpoB* gene and mutations at codon 315 of the *katG* (Table 1). The results are available within 24 to 48 h from sample reception. Compared to the phenotypic drug susceptibility testing (DST), the sensitivity and specificity of the MTBDR was 99% and 100% for rifampicin resistance and 88.4% and 100% for isoniazid resistance (Hillemann et al., 2005b). Miotto et al. reported a sensitivity and specificity of 91.5%-100% and 100%, respectively, for rifampicin resistance and 67.1%-100% and 100%, respectively for isoniazid resistance (Miotto et al., 2006). The overall low sensitivity for the detection of isoniazid resistance was the main limitation of the test since it only detected the *katG* S315T mutation missing all strains that harboured mutations in the promoter region of the *inhA* gene, mainly the C-15T substitution. Therefore, in order to increase substantially the number of isoniazid resistant strains detected by the test, Hain have developed a second-generation assay, the Genotype MTBDR*plus* VER 1.0 (MTBDR*plus*), which was released in 2007 replacing the MTBDR. It targets all the genes included in the previous version with the addition of codons 505 to 510 in the RRDR of the *rpoB* gene, to improve the detection of rifampicin resistance, and the promoter region of the *inhA* gene, nucleotides -8, -15, and -16, for low-level isoniazid resistance. The WHO approved the MTBDR*plus* VER 1.0 in 2008 (World Health Organization, 2008). In 2012, Hain released the MTBDR*plus* VER 2.0. The differences are related to the composition of the amplification mixtures where in this new version all reagents needed for amplification such as Taq DNA polymerase and the primers are included in the amplification mixes and do not need to be added separately as occurred with the first version of the test. The turnaround-time is similar. The MTBDR*plus* demonstrated excellent accuracy for rifampicin resistance, both on *M. tuberculosis* isolates and clinical samples. The estimated pooled sensitivity was 98.1% (95% CI 95.9-99.1) and pooled specificity was 98.7% (95% CI 97.3-99.4) for rifampicin resistance for both types of samples. The accuracy estimates for isoniazid was variable and the sensitivity highly heterogeneous, ranging from 57% to 100%. The pooled sensitivity was 84.3% (95% CI 0.77-0.90). The pooled specificity estimate was 99.5% (95% CI 0.98-1.00) and sensitivity is around 90% (World Health Organization, 2008). The low sensitivity for isoniazid detection is related to the prevalence of isoniazid resistance due to mutations in *katG* and *inhA,* which was observed to differ considerably between different geographic locations (Hazbón et al., 2006; Köser et al., 2012). Both tests are in the last stage of the WHO diagnostic pipeline (Fig. 1). For rifampicin resistance, the MTBDR*plus* VER 2.0 showed sensitivity of 100%, 92.3% specificity, 96.2% PPV and 100% NPV from smear-positive samples and sensitivity of 96%, 93.3% specificity, 96% PPV and 93.3% NPV from smear-negative samples. For isoniazid resistance, the MTBDR*plus* VER 2.0 has a sensitivity of 96.7%, 87.5% specificity, PPV of 96.7% and NPV of 87.5% from smear-positive samples and sensitivity of 96.7%, 90.0% specificity, PPV of 96.6% and NPV of 90.0% from smear-negative samples (Hain Lifescience, 2012).

The third test of the series is the GenoType MTBDR*sl* (MTBDR*sl*). The test is based on the same principle as the previous ones but for the detection of mutations associated with resistance to second-line drugs: the quinolone resistance determining region (QRDR) of the *gyrA* gene for fluoroquinolone resistance, *rrs* for second-line aminoglycosides and cyclic peptides, and *embB* for ethambutol resistance (Hain Lifescience, 2009). A meta-analysis on the performance of the MTBDR*sl* VER 1.0 showed pooled sensitivity's and specificities of 85.6% and 98.5%, respectively, for fluoroquinolone resistance from cultured material and 86.2% and 98.6%, respectively, for the direct testing. In relation to second-line aminoglycosides detection, the pooled sensitivity and specificity was 76.5% and 99.1%, respectively, for the detection from cultures, and 87% and 99.5% for the direct testing (World Health Organization, 2016b). The MTBDR*sl* VER 1.0 showed poor accuracy for the detection of ethambutol resistance compared to fluoroquinolones and second-line aminoglycosides. Cheng et al. have reported a sensitivity and specificity of 55% and 78%, respectively, and Simons et al. showed 62% sensitivity and 71% specificity for detecting ethambutol resistance (Cheng et al., 2014; Simons et al., 2015).

In order to improve the overall performance of MTBDR*sl*, in particular its sensitivity to detect low-level kanamycin resistance, a new version of the assay, the MTBDR*sl* VER 2.0, was released in 2015. The MTBDR*sl* VER 2.0 contains two new targets, the *eis* promoter region and the QRDR of *gyrB* gene, the *embB* target region for ethambutol resistance has been removed from this version, since it is a first-line drug, and *gyrA* and *rrs* target regions were maintained (Hain Lifescience, 2015). The evaluation studies performed so far showed an improvement in sensitivity and specificity for the determination of the molecular resistance, compared to the MTBDR*sl* VER 1.0, to fluoroquinolones (93-100% and 98.4%, respectively), second-line aminoglycosides (83-91.3% and 91.7-100%) and kanamycin in particular (89.2-96.0% and 92.2-98.5%) (Tagliani et al., 2015; Brossier et al., 2016; Gardee et al., 2017). The WHO endorsed the MTBDR*sl* assay in 2016 for the testing of sputum specimens, irrespective of the smear status, and for cultured isolates of *M. tuberculosis* complex from both pulmonary and extrapulmonary samples. These recommendations apply to the direct testing of sputum specimens from rifampicin-resistant or MDRTB patients, taking in consideration that the indeterminate rate of the assay is higher when testing smear-negative compared to smear-positive sputum specimens, and that the accuracy for the detection of resistance to fluoroquinolones and second-line aminoglycosides differs, reducing the overall accuracy of the diagnosis (World Health Organization, 2016b).

The diagnostic value of the combined use of MTBDR*plus* and MTBDR*sl* VER 2.0 for the rapid detection of drug resistance is the early detection of mutations associated with the M/XDRTB phenotype.

The AID TB Resistance (thereafter, AID TB) are modular line probe assays developed by AutoImmun Diagnostika (AID) GmbH (Strasburg, Germany) and commercially available since 2012. The test is based on a multiplex PCR, using biotinylated primers, followed by reverse hybridization using sequence-specific oligonucleotide probes for the rapid detection of *M. tuberculosis* DNA and mutations associated with resistance to isoniazid and rifampicin (module 1), first- and second-line aminoglycosides and cyclic peptides (module 2), and fluoroquinolones plus ethambutol (module 3). The procedure of the test is similar to that of the LiPA and Genotype assays. The results can be analysed manually, using the evaluation sheet supplied, or automatically, using the AID Scanning System for evaluation and documentation of the data, allowing a better interserial comparison and reproducibility of the results (AutoImmun Diagnostika, 2018).

Each test strips contain specific probes for the detection of the genus *Mycobacterium* and for *M. tuberculosis* as positive controls, a human GAPDH probe as amplification control and several drug-related gene specific probes for the wild-type and for the mutated sequences (Table 1). The AID TB assay covers mutations for isoniazid resistance, specifically the *katG* S315T mutation and *inhA* promoter mutations, resistance to rifampicin can be detected by the presence of mutations in the RRDR of the *rpoB* gene, streptomycin resistance by the analyses of mutations at the *rpsL* gene and the *rrs* gene. Amikacin and capreomycin resistance are detected by the occurrence of mutations in the *rrs* gene. The third module detects mutations associated with resistance to fluoroquinolones and resistance to ethambutol thought the detection of mutations in the *gyrA* and *embB* genes, respectively (Table 1). Notably, this is the only molecular assay commercially available, able to detect mutations associated with streptomycin resistance. These modular line probe assays can be used directly in clinical specimens and cultures. Unfortunately, the assay is not able to discriminate between the

mutations occurring in the *inhA* promoter, in any position of the *rpoB* gene and the G1484C/T on *rrs*, requiring further testing by DNA sequencing for precise identification of the mutation detected.

The evaluation of the three modules for the direct detection of *M. tuberculosis* and mutations associated with resistance on smear positive respiratory and non-respiratory samples showed a high specificity, 100%, 97.1%, and 100%, respectively, compared to the BACTEC MGIT phenotypic DST (Ritter et al., 2014). Molina-Moya et al. reported global concordance rates, between the AID modular assays and the BACTEC MGIT phenotypic DST, of 98.3% for detecting resistance to isoniazid, 100% for rifampicin, 91.5% for fluoroquinolones, 72.9% for ethambutol, 100% for kanamycin/capreomycin and 98.0% for streptomycin (Molina-Moya et al., 2015). Finally, Deggim-Messmer et al. reported an overall accuracy of 98.5% between the molecular DST of clinical specimens and the BACTEC MGIT phenotypic DST on isolates recovered in culture (Deggim-Messmer et al., 2016). The AID TB assays are currently under the WHO regulatory stage for new molecular methods for tuberculosis. Its added diagnostic value is its modular design and the detection of mutations associated with streptomycin resistance.

The line probe assay Nipro NTM+MDRTB Detection kit II (thereafter, Nipro) was developed in 2011 by Nipro Corporation and Home Diagnostics Inc. (Osaka, Japan). The Nipro assay was registered in Japan, in 2012, for the detection of *M. tuberculosis* complex strains and resistance to rifampicin and isoniazid (Foundation for Innovative New Diagnostic, 2015). Besides *M. tuberculosis* complex strains, the assay also identifies three major NTM species: *M. avium*, *M. intracellulare* and *M. kansasii*. The Nipro strips contain 4 species-specific probes and 17 probes for the detection of mutations associated with rifampicin and isoniazid resistance (Table 1). Overall, the Nipro has demonstrated good performance for the detection of resistance to rifampicin and isoniazid when using cultured isolates and directly decontaminated sputum samples. The reported sensitivity and specificity for the detection of mutations associated with rifampicin resistance from *M. tuberculosis* grown on cultures are 92%-100% and 97.3%-98.5%, respectively; for isoniazid, the sensitivity and specificity is 61.5%-91.6% and 96%-100% (Mitarai et al., 2012; Foundation for Innovative New Diagnostic, 2015; Rienthong et al., 2015; Nathavitharana et al., 2016). When testing decontaminated sputum, the sensitivity and specificity for the detection of mutations associated with rifampicin resistance are 75%-100% and 97.5%-100%; for isoniazid, the sensitivity and specificity is 50%-94.9% and 96.4%-97.8%, respectively (Mitarai et al., 2012; Foundation for Innovative New Diagnostic, 2015; Rienthong et al., 2015; Nathavitharana et al., 2016). A non-inferiority evaluation study of the Nipro assay compared to the Hain MTBDR*plus* VER 1.0 demonstrated the reliability of the assay for the detection of rifampicin and isoniazid resistance in smear-positive samples (Foundation for Innovative New Diagnostic, 2015). Similar to the line probe assays discussed in this Review, the Nipro is user-friendly and evaluation results support the WHO policy recommendations on its use for the rapid detection of MDRTB (Foundation for Innovative New Diagnostic, 2015). It is important to mention that, additionally to the Nipro strips, Nitro Corporation has developed one additional strip for the detection of mutations associated with resistance to pyrazinamide. This is the only assay available so far for the detection of mutations for pyrazinamide resistance. In a near future, another assay might be developed as Hain Lifescience aims to develop an assay for pyrazinamide-resistance detection to be used within the Fluorotype platform (Lessen, 2017). The diagnostic added value of Nipro assays is the detection of mutations associated with pyrazinamide resistance.

The Xpert MTB/RIF, the epitome of direct detection assay for tuberculosis and drug resistance detection, is an *in vitro* diagnostic assay develop in 2009 for the use within the fully automated GeneXpert platform (Cepheid Inc. Sunnyvale, CA, USA) (Cepheid, 2009). The assay is designed to be performed at peripheral laboratories or health care facilities without the need of biosafety cabinets or highly trained laboratory staff (Boehme et al., 2010; Boehme et al., 2011; Helb et al., 2010). The Xpert MTB/RIF assay consists of a single-use multi-chambered cartridge that is supplied preloaded with liquid buffers and lyophilized reagent beads required for sample processing, DNA extraction, and heminested real-time PCR (Helb et al., 2010). Pre-inactivated clinical samples are loaded in the cartridge and bacteria captured with a filter. The filtered microorganisms are lysed using an integrated ultrasonic horn, DNA is eluted and mixed with the PCR lyophilized reagent beads that contain all the regents for the first of the heminested PCR reaction and then moved into the integrated real-time PCR reaction tube. The resulting PCR products are latter on transferred, in an identical process, to the second PCR reaction (Helb et al., 2010). Similar to the LiPA, the Xpert MTB/RIF target only the RRDR of the *rpoB* gene as a marker for multidrug resistance and uses a nested PCR to enhance the sensitivity of target detection. The Xpert MTB/RIF assay simultaneously detects *M. tuberculosis* complex isolates and rifampicin resistance by amplifying a 192 bp *M. tuberculosis*-complex specific sequence of the *rpoB* gene by a heminested PCR encompassing the entire 81 bp RRDR (Blakemore et al., 2010). This region is probed with five molecular beacons (Probes A to E), labelled with different dyes and quenchers, which are designed to differentiate between the wild-type and the mutated sequence that are associated with rifampicin resistance (Table 1) (Helb et al., 2010). The cartridge includes a sample processing control (SPC) that consists of lyophilized *Bacillus globigii* spores, to control for adequate sample processing and to monitor the presence of inhibitors in the PCR reaction. A probe check control (PCC) is also included in the cartridge in order to verify reagent rehydration, PCR tube filling in the cartridge, probe integrity, and dye stability (Cepheid, 2009). The presence of a mutation in the target region inhibits the hybridization with one or more of the *rpoB*-specific molecular beacons, thus reducing or eliminating the signal from the corresponding probes. A delay in the CT of >4 cycles defines rifampicin resistance (Chakravorty et al., 2017). The results are automatically generated by the GeneXpert system, with no operator intervention, and are displayed in tabular and graphic formats within 2 h of sample processing (Helb et al., 2010). The Xpert MTB/RIF can be applied directly to unprocessed clinical samples or to decontaminated respiratory sample pellets, as well with other specimens including cerebrospinal fluid, lymph node tissue or aspirates, pleural fluid, ascetic fluid, urine, dialysis fluid, and pus, however, with varying sensitivities (Denkinger et al., 2014; Scott et al., 2014). Was endorsed by the WHO in 2010 for the direct detection of *M. tuberculosis* and resistance to rifampicin in smear-positive and negative samples in patients with suspected MDRTB and those with HIV and as a follow-on test for smear-negative individuals (World Health Organization, 2011c). In 2013, further recommendations were made for use in individuals with extrapulmonary and paediatric tuberculosis (World Health Organization, 2013a). The Xpert MTB/RIF was approved by the FDA in 2013 (Centers for Disease Control and Prevention, 2013). A Cochrane Review on the accuracy of the Xpert MTB/RIF for the detection of tuberculosis estimated pooled sensitivity and specificity of 89% and 99%, respectively (Steingart et al., 2014). The pooled sensitivity in smear-negative, culture-positive individuals was 67%. For HIV co-infected tuberculosis patients, the sensitivity was lower (79%) compared to those without HIV infection (86%). The Xpert MTB/RIF assay showed a pooled sensitivity and specificity of 95% and 98%, respectively, for the detection of rifampicin resistance (Steingart et al., 2014). The accuracy of the Xpert MTB/RIF for the detection of extrapulmonary tuberculosis estimates pooled sensitivity and specificity of 43.7%-84.9% and 92.5%-99.9%, respectively, and a sensitivity compared to culture in children of 66%, independently of the samples tested (World Health Organization, 2013a). The main diagnostic value of the Xpert MTB/RIF assay is the fully automated, biosafe and the rapid detection of *M. tuberculosis* DNA and rifampicin-resistance associated mutations as a marker for MDRTB at point-of-care.

Several studies reported important limitations regarding the performance Xpert MTB/RIF assay. Among these are: (i) the limited capacity to detect rifampicin-resistance associated mutations in mixed

samples (Blakemore et al., 2010; Zetola et al., 2014), (ii) the reduced capacity to detect the C533G *rpoB* mutation, associated with rifampicin resistance (Rufai et al., 2014), (iii) the production of false-positive resistance results for paucibacillary samples due to delays in the real-time signal generated by assay probes D and E (Williamson et al., 2012) and (iv) the false assignment of the F514F *rpoB* silent mutation as conferring resistance to rifampicin (Köser et al., 2012). Therefore, to improve the sensitivity and specificity of the assay for the detection of tuberculosis and rifampicin resistance, a new version of the assay was developed, the Xpert MTB/RIF Ultra (Table 1) (Alland et al., 2015; Chakravorty et al., 2017). The Xpert Ultra includes (i) two new PCR assays to target the IS*6110* and IS*1081*, (ii) the conversion of the *rpoB* and IS*6110* assays into fully nested PCRs, and (iii) use of a larger PCR tube that doubles the amount of DNA that can be tested allowing for an almost 10-fold increase in analytical sensitivity for the detection (Chakravorty et al., 2017). The inclusion of the IS*1081* reaction guarantees that the assay will detect rare *M. tuberculosis* strains that do not contain the IS*6110* (Lok et al., 2002). To reduce the risk of false resistance, in the Xpert Ultra, the delta C_T approach for mutation detection was replaced by the delta T_m approach, using sloppy molecular beacon probes (Chakravorty et al., 2012). The Xpert Ultra showed enhanced sensitivity for testing smear-negative with culture-positive samples and overall detects more tuberculosis reducing the false negative cases. The accuracy for the detection of rifampicin resistance was comparable between the Xpert MTB/RIF and the Ultra (Chakravorty et al., 2017; Dorman et al., 2018). Xpert Ultra also showed improved: i) detection of resistance in mixed samples, ii) detection of mutations at codon 533; and iii) differentiation of silent mutations at codons 513 and 514 (Chakravorty et al., 2017). The technical procedure required for the Xpert MTB/RIF and Xpert MTB/RIF Ultra are identical and both assays can be run in the same GeneXpert instruments requiring only a software upgrade.

It is expected that the Xpert Ultra will result in increased case detection rates for subjects with lower mycobacterial loads as patients with paucibacillary tuberculosis, such as those with HIV co-infection, paediatric patients and those with extrapulmonary tuberculosis. A multicentre study performed by FIND on the accuracy of the Xpert Ultra showed that, compared to culture, the sensitivity of the assay was higher than Xpert MTB/RIF (87.8% vs 82.9%), but the specificity was lower (94.8% vs 98%) (Foundation for Innovative New Diagnostic, 2017; World Health Organization, 2017b). Focusing on special groups of patients, Zar et al. reported sensitivity and specificity of Ultra of 75.3% and 96.7%, respectively, which was similar in HIV-infected (sensitivity 70.6%; specificity 98.1%) and HIV-uninfected children (sensitivity 76.8%; specificity 96.4%) (Zar et al., 2017). Nicol et al. determined the accuracy of the Xpert Ultra for the diagnosis of pulmonary tuberculosis in children, using banked induced sputum, and found sensitivity and specificity of 75.3% and 96.9% compared with the culture (Nicol et al., 2018). Of those children with microbiologically confirmed tuberculosis (positive culture, Xpert MTB/RIF or Ultra), the Xpert MTB/RIF detected 63.2%, Ultra 73.7% and culture 82.9%. The authors conclude that the Xpert Ultra should not be used as a replacement test for culture in children (Nicol et al., 2018). Bahr et al. evaluated the Xpert Ultra for the diagnosis of tuberculous meningitis in adults and reported a sensitivity of 70% (95% CI, 47–87; 16 of 23 cases) for probable or definite tuberculous meningitis compared with 43% (23–66; 10/23) for Xpert MTB/RIF and 43% (23–66; 10/23) for culture (Bahr et al., 2018). Finally, Dorman et al. reported sensitivities for the Xpert Ultra and Xpert MTB/RIF of 63% and 46%, respectively, for smear-negative culture-positive sputum patients (95% CI, 10 to 24); 90% and 77%, respectively, for HIV-positive participants with culture-positive sputum (6.4 to 21); and 88% and 83%, respectively, across all participants with culture-positive sputum (5.4%, 3.3 to 8.0) (Dorman et al., 2018). Specificities of the Xpert Ultra and Xpert MTB/RIF for case detection were 96% and 98% (–2.7%, –3.9 to –1.7) overall, and 93% and 98% for patients with a history of tuberculosis, revealing that the increased sensitivity of the assay might came at the expense of a reduced specificity and may yield a higher proportion of false positive results (Dorman et al., 2018). Others have stated that this could be due to the detection of non-viable bacilli, a phenomenon previously observed with the Xpert MTB/RIF (Theron et al., 2016; Arend and van Soolingen, 2018) and which can be detected despite successful completion of the tuberculosis treatment.

The diagnostic value of Xpert Ultra assay is its increased sensitivity compared to the Xpert MTB/RIF and for this reason, in March 2017, the WHO recommended the replacement of Xpert MTB/RIF by the Xpert Ultra (World Health Organization, 2017b). However, some caution should be taken due to the reduced specificity, which will require increased precaution in the evaluation of laboratory results, especially among patients in retreatment. The assay is currently in the WHO country transition stage and the specific needs for further implementation are still to be defined.

Next to the Xpert Ultra is the GeneXpert Omni, a platform that is under development and planned to be fully released in the end of 2018/beginning 2019 (Fig. 1). This is a single-cartridge mobile platform, battery-operated, to be used with single Xpert MTB/RIF or Ultra cartridges for point-of-care testing for tuberculosis and rifampicin resistance. GeneXpert Omni is expected to cost about $5315 per device (England, 2018). It will able to the reach primary health posts and centers, particularly in remote settings where very limited infrastructure is available for rapid diagnosis of tuberculosis (Lessen, 2017). Cepheid is also developing another cartridge, the Xpert XDR. The test is being designed for the detection of resistance to isoniazid, fluoroquinolones and aminoglycosides (Denkinger, 2016). A preliminary evaluation study on the Xpert XDR performance showed 98.1% sensitivity and 100% specificity for isoniazid resistance, 95.8% sensitivity and 100% specificity for fluoroquinolone detection, 92.7% sensitivity and 100% specificity for kanamycin, and 96.8% sensitivity and 100% specificity for amikacin resistance compared with DNA sequencing, however, the accuracy decreases when compared to the phenotypic DST (Lessen, 2017).

5. Forthcoming promises in molecular diagnosis for tuberculosis

According to the WHO pipeline for the tuberculosis molecular diagnosis (Fig. 1), new technologies are presently in various stages of development, with the great majority still in the early stages of development (not discussed in this review for the sake of space) while others are currently in evaluation and/or country transition stage by the WHO, and are presented below.

(I) The Loopamp *Mycobacterium tuberculosis* complex detection kit developed by Eiken Chemical Co. (Tokyo, Japan), uses loop-mediated isothermal amplification (LAMP), a nucleic acid amplification method that relies on auto-cycling strand displacement DNA synthesis by the Bst DNA polymerase large fragment (Notomi et al., 2015). It is a simple, rapid, specific, and cost-effective methodology allowing its use with less sophisticated infrastructures and little biosafety requirements. The LAMP method has been employed for the identification of several mycobacterial species, with a turnaround time of 1 to 2 h (Iwamoto et al., 2003; Njiru et al., 2012; Veigas et al., 2013; Joon et al., 2017; Nakiyingi et al., 2018; Van Nguyen et al., 2018). The targets are diverse and include the IS*6110*, *hspX*, *mpb64*, *gyrB*, *rrs*, *rimM*, or *sdaA* genes. Among these, the IS*6110* is the most attractive one, as it demonstrates higher sensitivity and specificity due the multiple copies present in the *M. tuberculosis* genome. The Loopamp assay targets the IS*6110* and the *gyrB* gene thereby increasing the analytical sensitivity of the test and allowing its use on unprocessed sputum samples (World Health Organization, 2013b). The WHO endorsed the TB-LAMP method in 2016 for the diagnosis of pulmonary tuberculosis in adults as a potential rapid alternative to sputum-smear microscopy, which is

the primary diagnostic test for pulmonary tuberculosis in low-resource settings (World Health Organization, 2016c). The TB-LAMP has the potential to be the ideal point-of-care molecular test for diagnosis of tuberculosis but high temperature, humidity, inadequate volume of reagents and cross-contamination between samples have been identified as the major causes of false-positive results (Nagai et al., 2016). A systematic review and meta-analysis on the performance of TB-LAMP from respiratory samples showed summary estimates of 89.6% for sensitivity and 94% for specificity (Nagai et al., 2016). A recent meta-analysis on the performance of TB-LAMP from extrapulmonary tuberculosis samples showed a pooled sensitivity of 77% and specificity of 99% when evaluated against a composite of reference standards and a sensitivity of 93% and a specificity of 77% when compared only to culture (Yu et al., 2018).

(II) The Abbott RealTime MTB RIF/INH Resistance (Abbott Laboratories) is an *in vitro* PCR qualitative assay for the detection of rifampicin and isoniazid resistance-associated mutations in *M. tuberculosis* complex-positive respiratory samples (Table 1) (Kostera et al., 2016; Scott et al., 2017). Designed as a companion assay to the Abbott RealTime MTB assay (Kostera et al., 2016), both were developed for use on the high-throughput automated Abbott *m*2000 system, able to process from three up to 96 samples in one run, including specimens and controls, using the Abbott RealTime MTB assay (Kostera et al., 2016; Tang et al., 2015). The positive *M. tuberculosis* samples can then be reflexed using the Abbott RealTime MTB RIF/INH Resistance and test up to 24 samples simultaneously. The performance of the Abbott Real-Time MTB RIF/INH Resistance assay is equivalent in sensitivity and specificity to the GeneXpert MTB/RIF for the detection of mutations for rifampicin resistance and to the FluoroType MTBDR (discussed below) for isoniazid and rifampicin associated mutations (Hofmann-Thiel et al., 2016; Kostera et al., 2018; Scott et al., 2017). For the direct detection of mutations associated with rifampicin resistance on smear positive and negative respiratory samples it showed a high sensitivity (87.5%-96.3%) and specificity (100%); for isoniazid resistance the reported sensitivities are 78.8%-87.5% with 94.4%-100% specificity (Kostera et al., 2018; Ruiz et al., 2017). Although the assay is designed for the testing of respiratory samples, some studies have shown that it could be also used with extrapulmonary clinical samples (Hofmann-Thiel et al., 2016; Ruiz et al., 2017). The Abbott RealTime MTB RIF/INH Resistance is also suitable for the detection of *M. tuberculosis* in HIV-positive patients presenting sensitivity above 70% and specificity above 90% (Scott et al., 2017). The Abbott RealTime MTB RIF/INH Resistance assay has an advantage over the GeneXpert MTB/RIF as the later does not detect isoniazid-resistance associated mutations and was designed as a fully automated high-throughput method focused on MDRTB early detection.

(III) The FluoroType MTBDR is a semi-automated real-time molecular assay developed by Hain Lifescience for the rapid detection of *M. tuberculosis* complex plus rifampicin and isoniazid resistance associated mutations from respiratory and non-respiratory clinical samples (Table 1) (Hain Lifescience, 2018). The assay is based on the FluoroType technology, performed in a FluoroCycler96 instrument (Hain Lifescience) allowing the amplification and detection of up to 96 samples in a closed system within 3-4 h from DNA extraction. The FluoroType MTBDR combines non-symmetric PCR, also known as Linear-After-The-Exponential (LATE)-PCR (Sanchez et al., 2004), with sets of specific probes using the lights-on/lights-off technology (Rice et al., 2016). Due to the closed characteristics of the system, it provides results independently of the operator reducing the risk of analytic errors (Hillemann et al., 2018). The FluoroType MTBDR assay is marketed in Europe since April 2017, and is presently under WHO evaluation (Figure 1). To date only two studies have evaluated the performance of the FluoroType MTBDR, one from cultures (Hillemann et al., 2018), the other from clinical samples (de Vos et al., 2018). The Fluorotype MTBDR assay has demonstrated a high sensitivity and specificity for the detection of the *M. tuberculosis* complex and rifampicin-resistance associated mutations (97.8%-98.9% and 95.6%-100%, respectively), whereas its sensitivity and specificity for the detection of isoniazid-resistance associated mutations is variable (91.7%-98.8% and 97%-100%) (Hillemann et al., 2018; de Vos et al., 2018).

Similar to the Abbott RealTime MTB RIF/INH Resistance assay, the FluoroType also detects mutations associated with both isoniazid and rifampicin resistance, and focus on MDRTB.

(IV) The BD MAX Multi Drug Resistant Tuberculosis (MDR-TB) assay (Becton and Dickinson) is a fully automated qualitative *in vitro* assay for the direct detection of *M. tuberculosis* complex DNA and mutations conferring rifampicin and isoniazid resistance in respiratory samples, using the BD MAX system (Zimmermann et al., 2018). Specifically, the BD MAX MDR-TB assay was developed for the testing of unprocessed or concentrated sputum prepared from induced or expectorated sputa. The assays utilizes a multiplex real-time PCR and target-specific fluorogenic probes for the detection of *M. tuberculosis* complex DNA and the most common mutations in the *rpoB* and *katG* genes and in the *inhA* promoter region (Table 1). The interpretation of the results is performed automatically by the BD MAX system (Becton and Dickinson, 2018). The BD MAX MDR-TB assay was released in June 2018 (Becton and Dickinson, 2018), and consequently there are no validation studies available. A pre-validation multi-site investigational study of the BD MAX MDR-TB assay have showed good sensitivity and specificity for the detection of *M. tuberculosis* complex DNA and mutations associated with resistance to rifampicin and isoniazid (Zimmermann et al., 2018). Similar to the Abbott RealTime MTB RIF/INH Resistance assay and the FluoroType MTBDR, it has the increased advantage over the GeneXpert MTB/RIF of a turnaround time of 4 h for the testing of 24 samples using one system compared to the GeneXpert MTB/RIF turnaround time of 2 h 15 min for the testing of one sample using one system. The major limitation of the BD MAX MDR-TB system is that, similar to the GeneXpert MTB/RIF, it does not identify the mutations detected and for that, the sample need to be sequenced.

(V) The COBAS TaqMan MTB and MTB-RIF/INH assay for the direct detection of *M. tuberculosis* and drug resistance-associated mutations, developed by Roche Diagnostics Systems Inc. are based upon the previously described real-time PCR assays for the direct detection of *M. tuberculosis* DNA (Roche Diagnostics, 2009). In 2009, Roche has replaced the COBAS Amplicor MTB by a new real-time PCR assay, the COBAS TaqMan MTB (Roche Diagnostics, 2009) which is also the first commercial TaqMan-based assay developed for tuberculosis diagnosis. The diagnostic accuracy of COBAS TaqMan MTB is excellent for respiratory samples with an overall estimate sensitivity and specificity of 80.8% and 99.4%, respectively (Horita et al., 2015). For smear-positive respiratory samples, the estimated sensitivity is 95.2% and the estimated specificity is 91.6%. For smear-negative respiratory samples, the estimated sensitivity is 60% and the estimated specificity is 98.9%. The test is validated for respiratory samples only; however, it has been evaluated for the use with non-respiratory samples due to the lack of alternatives for this kind of samples. For non-respiratory samples, the diagnostic accuracy is lower, however is within acceptable ranges. The estimate sensitivity and specificity were 58.6% and 98.4%, respectively (Horita et al., 2015). Roche is currently developing two new high-throughput real-time PCR assays: the COBAS MTB Test and the COBAS MTB-RIF/INH assays for their use

with the COBAS 6800 (https://diagnostics.roche.com/global/en/products/systems/cobas-6800-system.html) and the COBAS 8800 systems (https://diagnostics.roche.com/global/en/products/systems/cobas-8800-system.html). These are fully automated very high-throughput systems, that can process 384 and 960 samples, respectively, in an 8 h working day. Both systems use a universal sample preparation process to isolate, purify and extract the total nucleic acids. The WHO is currently evaluating the COBAS MTB-RIF/INH assay for the detection of rifampicin and isoniazid resistance-associated mutations (Fig. 1). It is not yet commercially available and, to our knowledge, there are no prevision date for its release.

6. Whole-genome sequencing

Whole-genome sequencing (WGS) techniques, through automated genetic sequencing platforms, have been proposed as the ultimate molecular diagnostic test for *M. tuberculosis* and detection of all molecular determinants for resistance (Pankhurst et al., 2016; Phelan et al., 2016; Papaventsis et al., 2017). Continued advances in WGS techniques and bioinformatics analytic platforms expanded the opportunities for monitoring tuberculosis drug resistance, allowing a fast and accurate prediction of bacteriological resistance to most antituberculosis drugs used for treatment in 1 to 3 days after sample collection, depending on the sequencing platform and the quality and quantity of the extracted mycobacterial DNA. Significant progress has already been achieved, as there are several automated bioinformatics platforms to rapidly predict antimicrobial resistance that can be used and implemented in routine diagnostic settings (Coll et al., 2015; Miotto et al., 2017; McNerney et al., 2018). Several studies described a high sensitivity and specificity using WGS for the first-line drugs isoniazid and rifampicin; however, a substantial variation in WGS accuracy was observed for the remaining first- and second-line drugs (Satta et al., 2017; Walker et al., 2017). Contrary to isoniazid and rifampicin, where the majority of the target-mutations and respective levels of resistance caused are well studied, mutations for the other drugs still have to be correlated to the respective levels of resistance attributed and for that, epidemiological cut-off studies for each mutation and each drug are still needed (Schön et al., 2017; Domínguez et al., 2016). Besides this limitation, there are still other challenges to overcome, such as: (i) the lack of standardization in WGS analysis directly from direct sputum samples, including specific procedures to remove non-mycobacterial genetic material and enrich the genome of *M. tuberculosis*, (ii) the need of consensus guidelines to validate the information obtained with WGS for the laboratory diagnosis of tuberculosis, and (iii) to determine the clinical and economic impact of the incorporation of such technologies into the cascade of diagnosis and treatment of MDRTB in high-burden countries under routine conditions, in order to translate its potential to the clinical practice (Cirillo et al., 2017). Recently, Colman et al. evaluated the performance of direct WGS and automated data analysis for drug resistance prediction in sputum samples of tuberculosis (the Next Gen-RDST assay) (Colman et al., 2016). One hundred and sixty six of the 176 (94.3%) of the sputum samples obtained from patients treated in the Republic of Moldova produced complete Next Gen-RDST profiles for 7 drugs of interest. The assay showed high sensitivity and specificity (above 95%) for rifampicin, isoniazid, and kanamycin, and low sensitivity in detecting resistance to amikacin, capreomycin and fluoroquinolones, compared to the phenotypic drug susceptibility test (Colman et al., 2016). Important scientific, technical and logistical challenges need to be solved before WGS fulfils its potential in tuberculosis and antimicrobial resistance diagnostics as in other infectious diseases (Balloux et al., 2018).

7. Concluding remarks and perspectives

Unfortunately, tuberculosis still remains a global health concern in spite of the successes achieved by the STOP TB program and now the END TB program, under the WHO guidance and support. Molecular diagnostic tests for tuberculosis have been a major R&D innovation and experienced significant advances in the last decade and will continue evolving. The molecular diagnostics landscape appears promising with a pipeline of new methods in several stages of development, improving its efficacy and accuracy based upon the filed experience gathered in the last 20 years (Fig. 1). These technologies have the capacity to provide accurate results coupled with cost-effectiveness, rapidity, scalability, high-throughput capacity, reproducibility, and sensitivity. Unquestionably, the reference method for direct or culture identification of *M. tuberculosis* and NTMs.

Timely and accurate identification and determination of the antibiotic susceptibility profile of the infecting strain is nowadays crucial for proper management of the disease. The emergence of multi- and extensively drug resistant *M. tuberculosis* strains is driving the need for the expeditious diagnosis and prudent use of antibiotics in tuberculosis. In Fig. 2 is presented an overview of the laboratorial diagnostic workflow showing the role of molecular methods in tuberculosis diagnosis and how their use should be complemented with phenotypic drug susceptibility testing. Currently, using the traditional approach, it can take several weeks for identification and susceptibility testing of *M. tuberculosis*. The ability of the molecular methods to shrink the diagnostic window has the potential to provide earlier and effective antimicrobial therapy and to decrease the administration of drugs to which the bacteria is already resistant. These techniques offer increased diagnostic resolution while at the same time shorten the time-to-result, and are thus of obvious importance for antimicrobial stewardship (Miotto et al., 2018). Yet, these technologies do not provide comprehensive information on antimicrobial susceptibility and many times miss important information for the clinical outcome of the treatment, if used alone.

Molecular diagnostic techniques are based on the association between specific gene mutations and phenotypic resistance to certain drugs and offer the opportunity to rapidly ascertain whether drug resistance will be present. However, it is known that the clinical resistance in tuberculosis is a balance between drug-resistant persistent bacterial populations and drug susceptible populations coexisting in the patient (Mitchison and Davies, 2012; Heifets and Cangelosi, 1999). At the course of the disease and treatment follow-up, this balance needs to be monitored by phenotypic methods to assess and quantify the number of drug resistant bacteria in the population, since the presence of one single mutated target in a population (easily detectable by PCR) does not guarantee the outburst of clinical resistance to treatment, although increases substantially its probability, depending mainly on patient-related and treatment-related factors, e.g. comorbidities, side-effects, pharmacogenetics, nutritional state, compliance and effectiveness of the health-care system in providing timely detection and monitoring of treatment (Dheda et al., 2017). Furthermore, the mutational pattern of a strain varies according to geographical regions. The host-bacterial population genetics and the variability of drug-resistance related mutations according to the genotype of the strain constitutes also major drawback for the single application of molecular assays for the detection of drug resistance on a routine basis and the phenotypic methods still have to be performed at least in the reference laboratories, especially focused on the suspected drug-resistant cases (Böttger, 2011). Quality assured microscopy and culture still has an important role in limited-resource countries alongside with the new advances on the molecular detection of mycobacterial agents (Noor et al., 2015).

Molecular methods have undoubtedly a huge impact on patient care and was the most important advance in the last decades for the laboratory diagnosis of mycobacterial related infections, in particular tuberculosis, but it's not the only and ultimate solution for the mycobacteriology laboratory, and we need to be aware of their limitations. These include (i) technical problems (e.g, amplification inhibition, cross-contamination, reduced portability, etc.), (ii) require expensive

Table 2
Genotype-phenotype correlation of the most common mutations identified by the line probe assays determined using the BACTEC 460TB or MGIT 960.

Drug	CC (μg/ml)	Target	Mutation	Resistance level (μg/ml)	Selected references
Rifampicin	1	*rpoB*	D516V	Usually result in low (R at CC; S at 4) to intermediate (R at 4; S at 20) level resistance Rarely associated with high-level resistance (R ≥ 20)	Springer et al., 2009 Cambau et al., 2015
			H526Y	Usually result in intermediate to high-level resistance Rarely associated with low-level resistance (R at CC; S at 4)	Kambli et al., 2015b Berrada et al., 2016
			H526D	Usually result in high-level resistance (R ≥ 20) Rarely associated with low or intermediate-level resistance	
			S531L	High-level resistance (R ≥ 20)	
Isoniazid	0.1	*katG*	S315T	Usually result in intermediate (R at 1; S at 10) to high (R ≥ 10) level resistance Rarely associated with low-level resistance (R at 0.1; S at 1)	Springer et al., 2009 Machado et al., 2013
		inhA promoter	C-15T	Usually result in low-level resistance (R at CC; S at 1) Rarely associated with intermediate or high-level resistance (R ≥ 1)	Cambau et al., 2015 Kambli et al., 2015b
Streptomycin	1	*rrs* region 500	a523c	Usually result in intermediate (R at 4; S at 20) to high (R ≥ 20) level resistance Rarely associated with low-level resistance (R at 1; S at 4)	Meier et al., 1996 Cambau et al., 2015
			c526t	Usually result in low (R at CC; S at 4) to intermediate (R at 4; S at 20) level resistance Rarely associated with high-level resistance (R ≥ 20)	Springer et al., 2009
		rpsL	K43R	Usually result in intermediate (R at 4; S at 20) to high (R ≥ 20) level resistance Rarely associated with low-level resistance (R at 1; S at 4)	Cambau et al., 2015 Springer et al., 2009
			K88R	Usually result in intermediate (R at 4; S at 20) to high (R ≥ 20) level resistance Rarely associated with low-level resistance (R at 1; S at 4)	Nhu et al., 2012
Ethambutol	5	*embB*	M306I	Usually result in low (R at CC; S at 12.5) to intermediate (R at 12.5; S at 50) level resistance Rarely associated with high-level resistance (R ≥ 50)	Springer et al., 2009 Cambau et al., 2015
			M306V	Usually result in low (R at CC; S at 12.5) to intermediate (R at 12.5; S at 50) level resistance Rarely associated with high-level resistance (R ≥ 50)	
Amikacin	1	*rrs* region 1400	A1401G	High-level resistance (R ≥ 20)	Springer et al., 2009 Cambau et al., 2015 Tessema et al., 2017 Heyckendorf et al., 2017 Zimenkov et al., 2013 Kambli et al., 2016 Sirgel et al., 2012
		eis promoter	G-10A	Usually result in borderline (I at CC) or low-level resistance (R at CC; S at 4)	Machado et al., 2017
Capreomycin	2.5	*rrs* region 1400	A1401G	Usually result in low (R at CC; S at 5) to intermediate (R at 5; S at 25) level resistance	Cambau et al., 2015 Tessema et al., 2017 Heyckendorf et al., 2017 Zimenkov et al., 2013 Kambli et al., 2016 Sirgel et al., 2012a Pietersen et al., 2015
Kanamycin	2.5	*rrs* region 1400	A1401G	High-level resistance (R ≥ 20)	Tessema et al., 2017 Heyckendorf et al., 2017 Kambli et al., 2016 Zimenkov et al., 2013
		eis promoter	G-10A C-12T C-14T G-37T	Usually result in low (R at CC; S at 5) to intermediate (R at 5; S at 25) level resistance Rarely associated with high-level resistance (R ≥ 25)	Tessema et al., 2017 Kambli et al., 2016 Zimenkov et al., 2013
Ofloxacin	2	*gyrA*	A90V S91P D94A/G/H	Usually result in low to intermediate level resistance (R at CC; S at 10) Occasionally high-level resistance (R ≥ 10)	Cambau et al., 2015 Alvarez et al., 2014 Sirgel et al., 2012b Kam et al., 2006
Levofloxacin[a]	1	*gyrA*	A90V S91P D94A/G/H	Usually result in low to intermediate level resistance (R at CC; S at 10) Occasionally high-level resistance (R ≥ 10)	Nosova et al., 2013 Alvarez et al., 2014 Kambli et al., 2015a
Moxifloxacin	0.25	*gyrA*	A90V S91P D94A/G/H	Usually result in low (R at CC; S at 2.5) to intermediate level resistance (R at 2.5; S at 7.5) Occasionally high-level resistance (R ≥ 7.5)	Cambau et al., 2015 Alvarez et al., 2014 Sirgel et al., 2012a, 2012b Kam et al., 2006 van Ingen et al., 2010 Kambli et al., 2015c Isaeva et al., 2013; Nosova et al., 2013
Gatifloxacin[a]	0.25	*gyrA*	A90V S91P D94A/G/H	Usually result in low to intermediate level resistance (R at CC; S at 10) Occasionally high-level resistance (R ≥ 10)	Isaeva et al., 2013; Nosova et al., 2013 Zimenkov et al., 2013

Only mutations specified by the assays are shown.
Low-level defined as resistance at CC and susceptible at intermediate concentration (Cambau et al., 2015).
Intermediate resistance defined as resistance at intermediate concentration and susceptible at the highest concentration tested (Cambau et al., 2015).
High-level resistance defined as resistant at the highest concentration tested (Cambau et al., 2015).
[a] Levels of resistance not described to date. Here were used those of ofloxacin for comparison purposes only.

reagents and labour costs, (iii) limited multiplexing capacity, and (iv) relatively long turnaround time especially for DNA extraction, (v) technical skills requirement, (vi) and the requirement for specialized facilities. Phenotypic DST allows predicting not only drug resistance but also drug susceptibility and it permits to quantify the level of susceptibility of a given isolate to individual antimicrobials (quantitative DST) (Figure 2; Table 2). Quantitative DST is of major importance as a clear correlation between the molecular resistance result and the drug susceptibility phenotype obtained is not always possible (Cambau et al., 2015). The implementation of these methods on a routine basis should be done carefully and the best results are those obtained when these methods are combined with the drug susceptibility testing.

To ensure that the diagnostic molecular method selected is appropriate for a particular clinical setting, it is important to consider testing capacity, diagnostic yield, and the feasibility of performing the test in the laboratory setting. For example, Xpert MTB/RIF has a major role at point-of-care testing in high-burden countries, however, for low-incidence countries the diagnostic value is surpassed by the line probe assays. Another example, independently of the cost and type of technology used, BD MAX, FluoroType, Amplicor and COBAS varied in the number of samples evaluated at a time, from 24 up to 960 samples in certain commercially available assays. Therefore, before implementing a particular tuberculosis related diagnostic test, the potential utility with regard to medical decision-making in that particular setting should be carefully evaluated by cost effectiveness studies in close collaboration with the national tuberculosis control programs, and according the international guidelines.

Much has been achieved since the first use of nucleic acid probes to identify mycobacteria in the late 1980's (Fig. 3), with a tremendous added value for the early laboratory detection of tuberculosis and other mycobacterial infections reflected in the huge increase of global case detection since then and the steady global decrease of the tuberculosis incidence. However, a long path still has to be followed until we can rely exclusively on the genotypic information for the global struggle against all forms of tuberculosis.

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