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Review

# Molecular diagnostic tools in mycobacteriology

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ABSTRACT

Although the diagnosis of mycobacteriosis and susceptibility testing are still primarily based on conventional methods (staining, culture, biochemical analysis, proportional method), a series of molecular assays are increasingly introduced and incorporated in the workflow of clinical mycobacteriology laboratories worldwide. These assays are rapid and offer high sensitivities and specificities. In the present review, we describe the molecular assays concerning the early detection of Mycobacteria in clinical specimens, the identification of mycobacterial species, the detection of drug resistance and the typing for epidemiological investigations.



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## 1. Introduction

Tuberculosis (TB) remains a major public health challenge worldwide as one third of the world population is exposed at some stage to *Mycobacterium tuberculosis* and TB is the first cause of death due to a single infectious agent in adults (Raviglione et al., 1995). It is estimated that nearly 1 billion people will be newly infected with TB between 2000 and 2020 and, furthermore, two hundred million people will develop disease and 35 million will die from TB within this period (WHO, 2001; Amdekar, 2005). Increasing movement of populations towards Europe and the United States in the last two decades has brought TB to the foreground of public health concern (CDC, 2006). Early diagnosis, together with adequate therapy and prevention measures against further transmission are essential for TB control. In addition, the incidence of infections by nontuberculous mycobacteria (NTM) has increased significantly over the same period, mainly due to the AIDS epidemic and the increase in the size of immunodeficient population. As treatment and infection control measures vary according to the aetiologic species, rapid and accurate identification to the species level is highly relevant.

The conventional methodology, which includes specimen treatment, microscopic examination for acid-fast bacilli, isolation with the use of solid and/or liquid culture, and the classic differentiation with biochemical tests (Fig. 1), is slow and takes several weeks. Over the last few years, new molecular methods have been introduced, including PCR-Restriction Fragment Length Polymorphism, real-time PCR, DNA sequencing, DNA strip assays as mycobacterial diagnostic tools (Fig. 1), leading to considerable improvement of both speed and accuracy of identification. Moreover, new species have been detected, the medical importance of which is under constant evaluation.

The prevalence of TB is further complicated by the appearance of strains with multidrug resistance (MDR) in almost 3% of all newly diagnosed patients (Dye et al., 2002). The conventional phenotypic methods for assessing drug resistance are slow and in order to avoid delays in both therapy and prevention of MDR transmission, various genotypic methods based on line probe assays, DNA sequencing or real-time PCR, have been proposed for detection of the mutations associated with resistance to anti-tuberculosis drugs.

The aim of the present report is to review the molecular methods used in mycobacterial diagnostics and to assess their diagnostic usefulness in a modern clinical mycobacteriology laboratory.

## 2. Direct detection of mycobacteria in clinical specimens

Several molecular techniques have been developed for direct detection of mycobacteria from clinical samples. These are based on amplification of unique mycobacterial DNA or RNA target fragments by PCR. The available in-house and commercial assays include:

### *2.1. In-house PCR for detection of mycobacteria from clinical specimens*

Since the '90s, a series of in-house assays have been described. Each laboratory uses its own protocol for pretreatment, DNA extraction and detection of amplification products. The insertion element IS 6110 and the 16S rDNA are the most common targets used (Thierry et al., 1990;

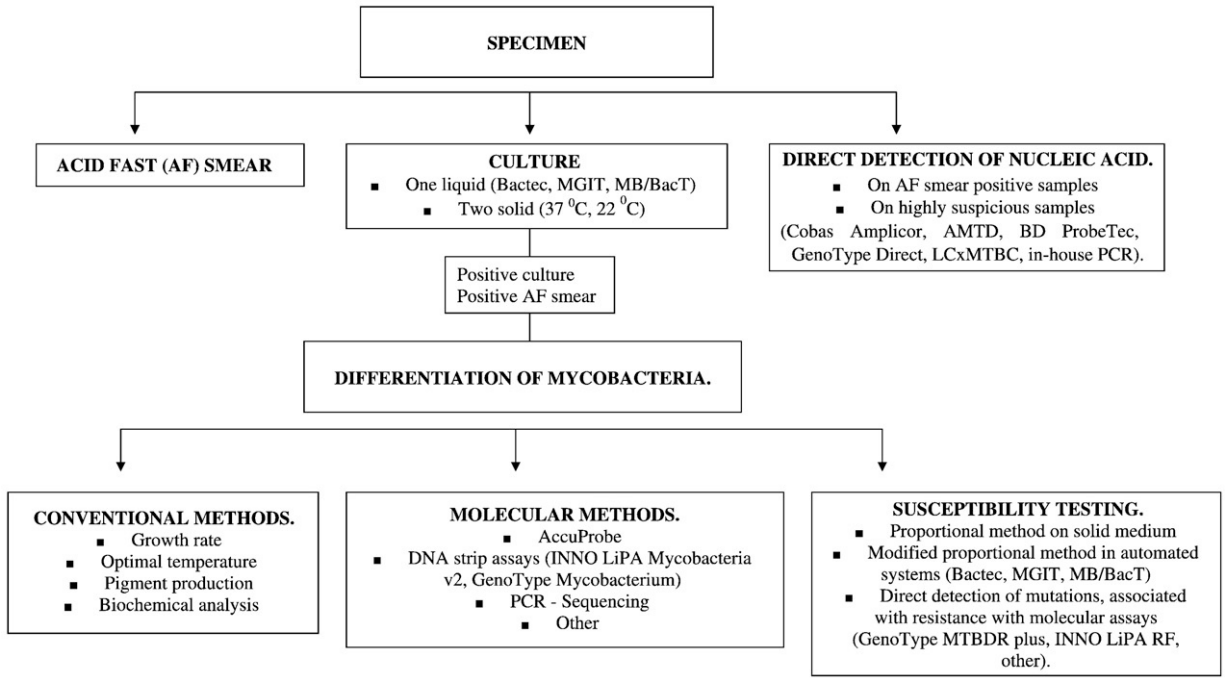


**Fig. 1.** Algorithm for the handling of mycobacterial specimens.

Kox et al., 1995; Springer et al., 1996; Kirschner et al., 1996; Garcia-Quintanilla et al., 2002; Tortoli, 2003). Other regions used for amplification include the *rpoB* gene encoding the β-subunit of the RNA polymerase (Adekambi et al., 2003; Kim et al., 1999; Chaves et al., 2000), the gene coding for the 32 kD protein (Soini et al., 1992; Del Portillo et al., 1996), the *recA* gene (Blackwood et al., 2000), the *hsp65* gene (Ringuet et al., 1999; Telenti et al., 1993; Steingrube et al., 1995; Mun et al., 2007), the *dnaJ* gene (Inyaku et al., 1993), the *sodA* gene (Domenech et al., 1997) and the 16S-23S rRNA internal transcriber spacer (Roth et al., 1998).

In a large-scale investigation (5000 samples) performed by Clarridge et al. (1993) using the 317-bp fragment of IS 6110 for PCR amplification, the overall sensitivity, specificity and positive predictive value compared with culture were reported to be 83.5%, 99.0% and 94.2%, respectively. According to the authors, the reasons for discrepant results depended on the presence of reaction inhibitors in the specimen, nucleic acid contamination and possible low-level non-specific hybridization. In another study, Nolte et al. (1993) using the same aforementioned target reported 95% and 57% positive PCR results for smear-positive and smear-negative pulmonary specimens respectively, as compared with culture results. There were no false-positive PCR results.

The need for increased sensitivity led to the use of nested PCR methodology, where the amplification of a large region of DNA is followed by a second amplification step, targeting a shorter interval and more specific region within the amplicon. This technique resulted in high sensitivities, although false-positive results due to cross-contamination of the amplified products, were often detected. In order to improve specificity, Garcia-Quintanilla et al. (2000) developed a new method, named Balanced Heminested PCR, which avoided asymmetric amplification. This was achieved by replacing the outer primer that participated in both steps of amplification in the standard heminested procedure by another primer, containing the sequence of the inner primer, attached at its 5' end. The results showed 75% sensitivity and 100% specificity, when compared with smear-negative culture-positive sputum samples.

Over the last few years, real-time PCR systems have been increasingly used in routine mycobacteriology laboratories. The technique allows real-time monitoring of a DNA amplification reaction by measuring an accumulating fluorescence signal. Real time PCR provided improved sensitivity and specificity, reducing turnaround time and avoiding the use of ethidium bromide-stained gels. Different real-time instruments are now available in the market.

Real-time PCR detection technology has been widely evaluated. According to Drosten et al. (2003) in a study for detection of *M. tuberculosis* in various samples [twenty two sputa; two BAL specimens; two tissue biopsy specimens; two gastric and one urine sample], the sensitivity of real-time PCR in culture positive samples was 92% and a similar value was found when comparing the Amplicor assay (see later). The time required for real-time PCR was significantly reduced in comparison with the Amplicor assay (2.5 h and 5 h respectively), and moreover, the cost was significantly lower than that with the commercial assay. Burggraf et al. (2005) compared an internally controlled, large-volume LightCycler assay (Roche Applied Science, Penzberg, Germany) for detection of *M. tuberculosis* in clinical samples with the Cobas Amplicor assay and reported 100% sensitivity and 98.6% specificity.

Ruiz et al. (2004) evaluated a real-time PCR method, using a LightCycler system to identify *M. tuberculosis* in auramine-rodamine-positive sputum samples. The sensitivity was 97.5%. In the same study, 97.5% sensitivity was also reported for the detection of *rpoB* and *inhA* genes [conferring resistance to rifampicin (RMP) and isoniazid (INH), respectively] and 96.5% sensitivity when the *katG* gene (conferring resistance to INH) was used as a target. These data were based on a comparison with the BACTEC MIGIT 960 (Becton Dickinson, Sparks, MD) method.

Finally, it should be noted that, over the last few years, mycobacteriophage-based techniques have been reported as potential useful tools for rapid detection of *M. tuberculosis*. The phage-based assay relies on the ability of *M. tuberculosis* to support the growth of an infected mycobacteriophage. The number of phages observed represents the number of viable bacteria detected in a plate with agar containing growing *Mycobacterium smegmatis*. The commercially available FASTPlaque TB assay (Biotec Laboratories Ltd., Ipswich, UK) is based on this technology for the early detection of *M. tuberculosis*, directly from sputum specimens. The turnaround time is 48–72 h. According to Muzaffar et al., 2002, the sensitivity and specificity of the assay for smear-positive samples, in comparison with culture, was 87.4% and 88.2%, respectively and for smear-negative samples it was 67.1% and 98.4% respectively (Muzaffar et al., 2002). For better evaluation of the assay, more extensive studies are needed. The low cost of the test is a great advantage for application in developing countries.

### 2.2. Commercially available assays

#### 2.2.1. Cobas Amplicor M. tuberculosis assay (Amplicor; Roche Diagnostic Systems, Branchburg, NJ )

Cobas Amplicor *M. tuberculosis* assay is based on PCR amplification of a 584-bp segment of the 16S rRNA gene followed by hybridization of the biotin-labeled amplified products to a *M. tuberculosis* complex-specific oligonucleotide probe, coated to microtiter plates. The assay includes an internal PCR control. The turnaround time is approximately 6.5 h.

The assay was reported to show high sensitivity when evaluated with smear-positive respiratory specimens (87.5–100%), but the sensitivity was much lower for extrapulmonary cases (27.3 to 85%) and smear-negative respiratory samples (17.2–70.8%) (Piersimoni and Scarparo, 2003). In the same study, the overall specificity was found to range between 91.3% and 100%. According to a series of other studies, the overall sensitivity (when compared to culture) for respiratory specimens ranged between 79.4% and 91.9%, and between 40.0% and 73.1% for smear-negative specimens (Bergmann and Woods, 1996; Stauffer et al., 1995; Tevere et al., 1996; Soini and Musser, 2001). The specificity was reported between 99.6% and 99.8%. (Bergmann and Woods, 1996; Stauffer et al., 1995; Tevere et al., 1996; Soini and Musser, 2001).

#### 2.2.2. Amplified M. tuberculosis direct test (AMTD; bio Merieux, Genprobe, Inc., San Diego, Calif.)

The AMTD test is based on amplification of the released ribosomal RNA sequences (amplicons) from the target cell. Their detection is achieved by nucleic acid hybridization. An *acridinium* ester-labeled DNA probe combines with the amplicon to form a stable hybrid and the labeled hybrids are measured in a luminometer. The test takes approximately 3.5 h. Specimens that are bloody are not suitable for testing with AMTD. The lack of internal amplification control is, also, a serious drawback.

In evaluation studies, the overall sensitivity for respiratory specimens was found in the range between 90.9% and 95.2% and the specificity between 97.6% and 100% (Soini and Musser, 2001; Bergmann et al., 1999; Pfyffer et al., 1994; Gamboa et al., 1998). According to Gamboa et al. (1998) AMDT showed 86.8% sensitivity and 100% specificity for nonrespiratory specimens.

#### 2.2.3. DProbe Tec ET (energy transfer) M. tuberculosis Direct Detection Assay (DTB), (BDProbe Tec; Becton Dickinson Bioscience, Sparks, Md.)

The technique is based on homogeneous Strand Displacement Amplification (SDA) and fluorescent energy transfer detection on an instrumental system. The SDA is a novel DNA amplification method (Barrett et al., 2002; Piersimoni et al., 2002). Target sequences of a 95-bp region in IS*6110*, a highly specific insertion element to

*M. tuberculosis* complex, and 16S rRNA gene, common to most mycobacteria, are coamplified isothermally. The process is based on the nicking of a modified recognition sequence in double-stranded DNA, by the restriction endonuclease *BsoB1*, and the extension and repair of that site by the DNA polymerase *Bst*, which synthesizes a new strand of DNA while displacing the existing strand. The displaced strand can then serve as a template for further amplification. The entire process occurs at 52.2 °C. Fluorescent energy transfer detection is performed on the same instrumented system. An internal amplification control is run with each sample and is designed to verify that no inhibition of the SDA reaction is detected in the specimen. The assay is recommended by the manufacturer for use with respiratory specimens and is completed within 4 h.

According to Barrett et al. (2002) the BDProbe Tec ET system offers a reliable molecular biological approach for the detection of *M. tuberculosis* complex (MTBC) in respiratory samples in a semi-automated format with sensitivity and specificity of 92.7% and 96.0%, respectively. Cerebrospinal fluid samples gave sensitivity and specificity of 100 and 95%, respectively, when compared to culture, while pleural fluid samples had poor sensitivity (30%) (McHugh et al., 2004).

Comparing BDProbeTec with AMTD, Piersimoni et al. (2002) reported that the sensitivities for ProbeTec were 94.5% and 92.3% vs. 88.0% and 74.3% for AMTD when respiratory and extrapulmonary samples were used, respectively. These differences were associated with the presence of reaction inhibitors, which AMTD could not detect due to lack of internal amplification control (Piersimoni et al., 2002). Comparing BD ProbeTec ET with Amplicor, Goessens, 2005, reported sensitivities of 86.2% and 78.0%, respectively and identical specificities (99.9%) for both assays (Goessens et al., 2005).

*2.2.4. Genotype mycobacteria direct assay for detection of M. tuberculosis complex and four atypical mycobacteria (Hain Lifescience, Nehren, Germany)*

This novel assay is based on the nucleic acid sequence-based amplification (NASBA) applied to DNA strip technology. According to the manufacturer, the assay has three steps. The first step consists of isolation of 23S rRNA, the second step includes amplification of RNA by NASBA method, and the third step involves the reverse hybridization of the amplified products on membrane strips using an automated system. The assay has the ability for simultaneous detection of *M. avium*, *M. intracellulare*, *M. kansasii*, *M. malmoense* and MTBC. Isolation of highly specific RNA is achieved by the use of the "magnetic bead capturing" method. According to Franco-Alvarez de Luna et al. (2006) the assay is useful, reliable and rapid, with sensitivity and specificity of 92% and 100%, respectively.

*2.2.5. LCx MTBC assay (Abbott Laboratories, Diagnostic Division, Chicago, USA)*

The assay uses the ligase chain reaction for amplification of a target sequence within the chromosomal gene that codes for protein antigen b, which is specific for members of the MTBC (Andersen and Hansen, 1989; Sjöbring et al., 1990). The whole process takes approximately 6 h. According to Moore and Curry (1998) comparing LCx with cultures, the overall sensitivity and specificity of the assay was 74% and 98%, respectively. For smear-positive samples the sensitivity reached 100%, but for smear-negative it was only 57% (Moore and Curry, 1998). In a multicenter evaluation of Amplicor and LCx, Tortoli et al. (1999) reported that the sensitivity of both methods was significantly better when only respiratory specimens were considered (78% and 88%, respectively). When non-respiratory samples were used, the sensitivity was reduced to 59% for Amlicor and 65% for LCx.

A brief presentation of the available commercial tests is shown in Table 1.

*2.2.5.1. Remarks.* In conclusion, it should be noted that, although the traditional methods for diagnosis of tuberculosis, such as microscopy and culture, cannot be replaced by direct amplification tests, these assays provide a major improvement in terms of speed. They could be used for rapid confirmation in patients with smear-positive samples. In smear-negative patients, the amplification tests are recommended only when suspicion for TB is high and always in relation to clinical data (Pfyffer, 2003). For extrapulmonary specimens, the use of the amplification methods is advocated, since rapid and accurate laboratory diagnosis is critical (e.g, tuberculous meningitis). Although the use of IS*6110* as an amplification target and the use of nested PCR methods have improved the sensitivity of the in-house tests, the commercially available tests have the advantage that they are optimized and validated systems that use simplified protocols and are often supported with automation. The specificities of amplification methods are very high, whereas, the sensitivities vary greatly. Multiple specimens from the same patient, proper decontamination procedures, improved extraction methods and use of internal controls decrease the frequency of false-negative results.

## 3. Identification of mycobacterial species from culture by molecular methods

For many decades, the identification of mycobacterial isolates was performed on the basis of biochemical reactions and phenotypic characteristics, which are time-consuming and often give ambiguous results. The molecular methods for mycobacterial identification are now providing rapid and accurate results. Several methodologies have been used.

### 3.1. PCR-based sequencing

This methodology is considered the "gold" standard for identification of mycobacteria. Initially, a PCR amplification takes place followed by sequencing of the amplicons in an automatic sequencer. The identification of an unknown strain is completed by comparison of the nucleotide sequence with a library of known sequences. The databases for this purpose are available in the internet. Such databases are the GenBank (GenBank, 2008), the Ribosomal Differentiation of Medical Microsystems database (RIDOM) (Harmsen et al., 2002) and that of the European Molecular Biology Laboratory (EMBL) (EMBL, 2008). Several target genes have been used for the procedure but the most common is the 16S rRNA gene (Kox et al., 1995; Kirschner et al., 1996). This gene has been widely sequenced because it contains both highly conserved

**Table 1**
Commercial tests for direct detection of Mycobacteria from clinical samples

| Assay | Cobas Amplicor (Roche) | AMTD (GenProbe) | BD (ProbeTec) (BD) | GenoType MDA Hain Lifescience) | LCHX (Abbot) |
|---|---|---|---|---|---|
| Amplification technology | PCR | TMA | SDA | NASBA | Legase chain reaction |
| Target | 16SrDNA | rRNA | IS*6110* | 23SrRNA | PAB (protein antigen b) |
| Detection | Colorimetric | Chemiluminescent | Fluorimetric | Colorimetric | Fluorimetric |
| Turnaround time (h) | 6.5 | 3.5 | 4 | 4 | 5–6 |
| Instrumental use | Thermocycler, photometer | Heat block, luminometer | ProbeTec instrument | Twin cubator thermocycler | LCX fluorimetric analyzer |

TMA: transcription-mediated amplification; SDA: strand displacement amplification; NASBA: nucleic acid sequence based amplification.

and variable regions. It consists of more than 1500 bp but usually the first 500 bp are adequate for identification of a common *Mycobacterium* species. As previously mentioned, other important target genes are those encoding for the 65-kDa heat shock protein, the 32 kDa protein, the 16S-23S rRNA internal transcribed spacer (ITS) and the *recA* gene. Determination of 16S-23S rDNA ITS sequences was performed by Roth et al. (1998). The results showed that spacer sequences can differentiate slowly growing mycobacteria which are identical or closely related on the basis of their 16S rDNA sequences. The occurrence of conserved primary and secondary structure elements in ITS sequences indicates their potential utility in mycobacterial identification. The MicroSeq System (Applied Biosystems, CA) is a commercial 16S ribosomal DNA sequencing system (Patel et al., 2000). Evaluations of the MicroSeq System for routine use were performed by Cloud et al. (2002) and Hall et al. (2003) with good results. The system offers the ability to mycobacteriology laboratories to identify many of the recently described mycobacteria.

### 3.2. DNA probe technology

The DNA probe technology for identification of bacteria is one of the most successful molecular methods. The AccuProbe (Gen-Probe, San Diego, CA, USA) is the assay based on this technology that is used by the majority of clinical mycobacterial laboratories worldwide. It has the ability to identify a series of clinically important mycobacteria. These are *M. tuberculosis* complex, *M. avium* complex, *M. avium*, *M. kansasii*, and *M. gordonae.* The DNA probes are single-stranded DNA oligonucleotides labeled with acridinum ester that are complementary to the target, which is the rRNA. After sonication, the probes are added to the broken mycobacterial cells, to form a stable DNA-RNA complex. Following separation of the labeled complex from unhybridized DNA, the hybridization is detected by light emission in a luminometer. The AccuProbe can be used for both solid and liquid cultures. The method is easy to perform and only a sonicator and luminometer are required as equipment. The method has been widely evaluated with good results (Musial et al., 1988; Drake et al., 1987; Goto et al., 1991; Tortoli et al., 1996; Badak et al., 1999; Reisner et al., 1994). The AccuProbe kits are rapid, highly sensitive and specific. The procedure can be completed in less than two hours.

### 3.3. Line probe technology (hybridization in strips)

The line probe technology includes PCR (with biotinylated primers), reverse hybridization with different specific DNA probes, immobilized in parallel lines on a strip and colorimetric detection in an automated instrument. The banding pattern is indicative of the species of the isolate. The turnaround time is approximately five hours. Two systems of line probe assay are commercially available: (a) the Inno LiPA Mycobacteria v2 and (b) the GenoType *Mycobacterium*:

#### 3.3.1. Inno LiPA Mycobacteria v2, (Innogenetics, Ghent, Belgium)

This assay is based on the amplification of the mycobacterial spacer region 16S-23S rRNA for the simultaneous identification, in just one strip test, of the 17 most frequently isolated mycobacterial species: *M. tuberculosis* complex, *M. avium*, *M. intracellulare*, *M. scrofulaceum*, *M. kansasii*, *M. xenopi*, *M. chelonae*, *M. gordonae*, *M. fortuitum* complex, *M. malmoense*, *M. genavense*, *M. simiae*, *M. smegmatis*, *M. haemophilum*, *M. marinum*/*M. ulcerans* and *M. celatum*. Moreover, it has the ability to discriminate subgroups within *M. kansasii* and *M. chelonae* on the same strip. Mixed populations are easily identified.

Mijs et al. (2002) evaluated the test, comparing the results of the assay with the results obtained using biochemical and molecular tests. The accuracy of the assay was 99.2% after discrepancy analysis (636/641 mycobacterial isolates) and both sensitivity and specificity were 100% (all 641 mycobacterial isolates reacted with the *Mycobacterium* species probe and all 27 non-*Mycobacterium* species scored negative). Tortoli et al. (2003) reported that the overall sensitivity and specificity was 100% and 94.4%, respectively. The probes specific for *M. fortuitum* complex, for *M. avium-intracellular-scrofulaceum* group and for *M. intracellulare* type 2 cross-reacted with several mycobacteria rarely isolated from clinical specimens (Tortoli, 2003).

#### 3.3.2. GenoType Mycobacterium (Hain Lifescience, Nehren, Germany)

The procedure includes a multiplex PCR, followed by reverse hybridization and line probe technology. There are three kits: (a) the GenoType MTBC for distinguishing members of the *M. tuberculosis* complex, and (b) the GenoType *Mycobacterium* CM (Common Mycobacteria), and GenoType *Mycobacterium* AS (Additional Species) for NTM. The GenoType MTBC is based on the *gyrB* gene polymorphism (Richter et al., 2003). The AS and CM assays use 23S rDNA as their target, thus the amplicon generated in the CM assay can be used for the AS assay without the need to perform a second PCR. The combined use of CM and AS can distinguish almost 30 different NTM including the following species: *M. avium*, *M. chelonae*, *M. abscessus*, *M. fortuitum*, *M. gordonae*, *M. intracellulare*, *M. scrofulaceum*, *M. interjectum*, *M. kansasii*, *M. malmoense*, *M. marinum*, *M. ulcerans*, *M. peregrinum*, *M. xenopi*, *M. simiae*, *M. mucogenicum*, *M. goodii*, *M. celatum*, *M. smegmatis*, *M. genovense*, *M. lentiflavum*, *M. heckeshornense*, *M. szulgai*, *M. phlei*, *M. hemophilum*, *M. gastri*, *M. asiaticum* and *M. shimoidei.* The GenoType assays are rapid, easy-to-perform and easy-to-interpret (Gitti et al., 2006). They have allowed clinical mycobacteriology laboratories to detect infrequent mycobacterial species, without the need of sophisticated techniques (Neonakis et al., 2007b). Richter et al. (2003) evaluated the ability of the GenoType MTBC to differentiate *M. tuberculosis* complex species and demonstrated that all the *M. tuberculosis* complex species can be unambiguously identified, with the exception of *M. tuberculosis*, *M. africanum* subtype II and *M. canetti* that have identical hybridization patterns. Neonakis et al. (2007c) analyzed 120 clinical *M. tuberculosis* complex isolates with GenoType MTBC assay and found full agreement with previous identification with gene probes and biochemical analysis. According to Russo et al. (2006) the sensitivity and the specificity of the GenoType *Mycobacterium*, compared with 16S rRNA gene sequencing, were 97.9% and 92.4% for CM and 99.3% and 99.4% for AS, respectively. Richter et al. (2006) found 92.6% and 89.9% concordant results with CM and AS assays respectively, when they were compared with sequencing data.

### 3.4. PRA method. [polymerase chain reaction and restriction enzyme analysis for identification of mycobacteria from culture]

Telenti et al. (1993) developed a rapid method, based on the amplification of the gene encoding the 65-kDa heat shock protein, followed by restriction-fragment-length polymorphism, using two restriction enzymes *BstEII* and *HaeIII*. Isolates from both solid and liquid cultures can be used. The fragments of the restriction enzyme digestion are analyzed by agarose gel electophoresis and compared. The test can be completed within a day. It is a cost-effective and reliable assay that can be used by low-budget laboratories as well.

### 3.5. Pyrosequencing

Pyrosequencing™ (Biotage, Uppsala, Sweden) technology is a novel method of nucleic acid sequencing-by-synthesis that is based on the detection of released pyrophosphate (PPi) during DNA synthesis (Ronaghi, 2001; Tuohy et al., 2005). The cascade of enzymatic reactions generates visible light. The generated light is proportional to the number of incorporated nucleotides. The method is optimal for determining short sequences (typically 20–30 bases of a DNA) rapidly and in a semi-automated format (Ronaghi, 2001). The ability of this technology to accurately characterize common strains of mycobacteria was evaluated by Tuohy et al. (2005). Pyrosequencing

targeted a 30-bp sequence of the hypervariable A region of the 16S rRNA gene. It provided an acceptable identification for 179 of 189 (94.7%) isolates tested. Although the short sequences generated by Pyrosequencing are not as discriminating as the 300 to 500-bp sequences that can be generated by traditional sequencing, the authors demonstrated that abundant clinically useful information may be obtained.

### 3.6. DNA microarrays (DNA chips)

The method is based on hybridization of fluorescently labeled PCR amplicons of an unknown strain to a DNA array, containing nucleotide probes for 16S ribosomal RNA gene. The hybridization pattern and intensity is determined by scanning the chip using laser confocal fluorescence microscopy. The process of generating the target, its hybridization and reading on the chip requires approximately two hours. It allows the identification of a large number of strains in one reaction (Gingeras et al., 1998). Gingeras et al. (1998) designed an array to determine the specific nucleotide sequence of 705 bp of the *rpoB* gene of *M. tuberculosis*. It accurately detected RMP resistance associated with mutations of 44 clinical isolates of *M. tuberculosis*. (Gingeras, 1998). Troesch et al. (1999) investigated this probe array strategy focusing on mycobacterial diseases (Affymetrix, GeneChip technology, Santa Clara, Calif). Sequences of regions from the 16S rRNA and *rpoB* loci had been developed. Unique hybridization patterns allowed for the identification of *Mycobacterium* species and the RMP-resistant alleles (Troesch et al., 1999). Seventy mycobacterial isolates from 27 different species and 15 RMP-resistant *M. tuberculosis* strains were tested. A total of 26 of 27 species were correctly identified as well as all of the *rpoB* mutants alleles (Troesch et al., 1999).

A great disadvantage is, however, the current high cost of the required equipment.

## 4. Molecular methods for detecting drug resistance in mycobacterial strains

Over the last few years, increasing resistance rates of *M. tuberculosis* have been observed in many parts of the world (Dye et al., 2002; WHO, 2000; Neonakis et al., 2007a). Problems with inadequate treatment and compliance are the usual causes of drug resistance development (Sharma and Mohan, 2004). Knowledge of the susceptibility pattern of the isolate is crucial for successful therapy. Although novel, alternative methods for phenotypic drug susceptibility testing have been proposed [MODS- Microscopic observation broth drug susceptibility assay (Caviedes et al., 2000; Moore et al., 2006), TK-Medium (Baylan et al., 2004), etc], the standard conventional method for susceptibility testing is the proportional method, which is performed on solid media such as Löwenstein-Jensen slants or Middlebrook agar (Canetti et al., 1963). In this method, the ratio of the number of colonies growing on drug containing medium to the number of colonies growing on drug-free medium indicates the proportion of drug resistant mycobacteria within the bacterial population. Below a certain proportion, named critical proportion, the isolate is classified as sensitive. The method usually takes more than 3 weeks and if performed using automated detection systems with liquid media such as BACTEC TB-460 (Becton Dikinson, Sparks, MD), BACTEC MGIT 960, or Bact/Allert 3D (bioMerieux, Durham, NC)], the turnaround period may be shortened to 1–2 weeks.

Resistance to anti-tuberculosis drugs is primarily due to mutations in a series of genes. The most frequently found mutations in RMP resistant isolates (96%) are mutations in an 81-bp segment of the *rpoB* gene that encodes the β-subunit of RNA polymerase (Miller et al., 1994; Alcaide and Telenti, 1997; Ramaswamy and Musser, 1998). In 75–85% of INH resistant *M. tuberculosis* strains there are mutations in two genes, *katG* encoding catalase-peroxidase and *inhA* that takes part in fatty acid elongation (Telenti et al., 1997a; Cardoso et al., 2004; Kim et al., 2003; Baker et al., 2005; Zhang et al., 2005). Mutations in the *embB* gene, which plays a role in the synthesis of lipoarabinomanan and arabinogalactam, are connected with ethambutol resistance (Telenti et al., 1997b; Alcaide et al., 1997; Sreevatsan et al., 1997). More than 70% of pyrazinamide resistance is due to mutations in the *pncA* gene, which encodes for pyrazinamidase that converts pyrazinamide to its active form (Scorpio and Zhang, 1996). Mutations in the 16S rRNA gene or the *rpsL* gene that encodes for the ribosomal protein 12S cause approximately 65–75% of resistance to streptomycin (Honore and Cole, 1994; Meier et al., 1994; Cooksey et al., 1996; Fukuda et al., 1999). Molecular assays have the ability to detect these mutations and reveal the underlying resistance mechanism within hours.

### 4.1. PCR-DNA sequencing

This is considered as the reference method for detection of drug resistance mutations (Kapur et al., 1994; Kim et al., 2001). Initially, the region that is most frequently associated with resistance mutations is amplified. Then, the amplicons are sequenced in order to determine the presence or absence of a specific mutation. The expensive equipment and the expertise needed are probably the most serious drawbacks of the method.

### 4.2. Hybridization-based techniques

#### 4.2.1. Line probe technology

There are two commercially available assays: the Inno-LiPA Rifotuberculosis (Inno-LiPA RFTB; Innogenetics, Belgium) and the GenoType MTBDR plus, (HAIN, Lifescience; Nehren, Germany). These assays are variations of the previously described INNO LiPA Mycobacteria v2, and GenoType *Mycobacterium*, respectively. Instead of using species-specific probes, these assays use probes specific only for the *M. tuberculosis* complex and additionally for the detection of the mutations responsible for drug resistance.

*4.2.1.1. Inno-LiPA RifTB (Innogenetics, Ghent, Belgium).* The kit contains 10 oligonucleotide probes: one specific for *M. tuberculosis* complex, five wild type probes (S1-S5), and four probes (R) for the detection of the most frequent mutations that cause resistance to RMP. More than 95% of the RMP-resistant strains have mutations within an 81-bp hot spot region (codons 507–533) of the *rpoB* gene. The R probes used are: R2: Asp516Val, R4: His526Tyr, R4b: His526Asp, R5: Ser531Leu. All the probes are immobilized on a nitrocellulose strip. A *M. tuberculosis* isolate is considered susceptible to RMP, if all the wild type probes give a positive signal and all the probes for resistance are negative. The absence of hybridization of one or more of the S probes is indicative of a mutation that may be identified by one of the R probes.

An evaluation of the assay was performed by Rossau et al. (1997) and the results indicated that *M. tuberculosis* complex probe was 100% specific when compared to the results of nucleotide sequencing (Rossau et al., 1997). All strains sensitive by in vitro susceptibility testing were correctly identified. Among the resistant strains only 2% yielded conflicting results. Sharma et al. (2003) found Inno-LiPA Rif TB to be a reliable, simple and informative tool with absolute correlation (100%) between its results and those obtained by the classic susceptibility testing, and the *M. tuberculosis* probe to be completely specific (Sharma et al., 2003). In a meta-analysis by Morgan et al. (2005) it is reported that 12 of 14 studies that applied LiPA to isolates had sensitivity greater than 95% and 12 of 14 had specificity of 100%.

Although the assay is recommended for use only on isolates where the amount of DNA is large, it can be used directly on clinical specimens after modifications of the protocol (nested PCR). Studies evaluating the line probe assay directly to clinical samples are limited. For Tortoli and Marcelli (2007) the sensitivity and specificity of the system was 69.5% and 98.4%, respectively, when compared to cultures. The sensitivity was significantly higher for smear-positive (92%) than for smear-negative specimens (47%), and for respiratory (77%) more

than for non-respiratory specimens (49%) (Tortoli and Marcelli, 2007). The assay is, therefore, insufficiently sensitive to be used directly on unselected specimens, but it may represent an alternative choice to other amplification methods on smear-positive samples, as it can additionally detect RMP-resistance. For Johansen et al. (2003) the Inno LiPA assay provides a rapid and reliable detection of RMP resistance in 78.3% of clinical specimens, compared to Bactec 460 and to *rpoB* gene sequencing. Traore et al. (2006) used the assay to detect *M. tuberculosis* complex DNA and RMP resistance in 420 sputum samples from untreated ($n=160$) and previously treated ($n=260$) patients from 11 countries. The Inno LiPA test was positive for *M. tuberculosis* complex DNA in 389 (92.9%) specimens. Of these, 30.6% were RMP resistant (Traore et al., 2006).

*4.2.1.2. GenoType MTBDR plus (Hain Lifescience, Germany).* This assay offers the simultaneous identification of *M. tuberculosis* complex and detection of the most common resistance mutations in *rpoB* (RMP resistance), *katG* and *inhA* gene (INH resistance). This assay is the newer version of the GenoType MTBDR assay, which did not have the ability to detect INH resistance, caused by mutation in *inhA*. According to Hillemann et al. (2007) both the previous and the new version of the assay could correctly identify rifampicin-resistance in 98.7% of the cases, when compared to conventional susceptibility testing. Furthermore, the new GenoType MTBDR plus achieved better sensitivity for INH resistance (92% vs. 88.0% of the previous version). According to the authors, GenoType MTBDR plus is a reliable tool for the detection of INH and RMP resistance either in strains or directly in smear-positive specimens. In another study the GenoType MTBDR assay was also used directly on smear-positive specimens (Somoskovi et al., 2006). High sensitivities were reported: 94.4% for the detection of *M. tubrculosis* complex, and 96.2% for mutations in *rpoB* and 84.2% for mutations in *katG* gene. As the test relies on the occurrence of only certain INH resistance-related mutations, the authors pointed some weakness of the test when compared with the reference BACTEC system and DNA sequencing analysis.

### 4.3. Hybridization on DNA chips

The DNA microarrays can also be used for rapid detection of mutations responsible for drug resistance. It can simultaneously detect different drug resistant mutations of *M. tuberculosis*. The DNA chip technology seems to be the most promising method for future investigation on drug resistance. As it has been previously described, Troesch et al. (1999) used this probe array strategy for the detection of *rpoB* mutants alleles. All 15 RMP-resistant *M. tuberculosis* strains were correctly identified (Troesch et al., 1999). Kim et al. (2006) evaluated a drug resistance detection DNA chip (CombiChip Mycobacteria, Geneln, Pusan, South Korea), for identifying mutations associated with resistance to INH and RMP (*katG*, *inhA* and *rpoB* genes). It is an oligonucleotide microchip coupled to PCR for the detection of mutations. The results were compared to DNA sequencing and culture based drug susceptibility tests. The CombiChip detected all RMP resistant isolates by screening 7 codons in the *rpoB* hot spot region and it correctly identified 84.1% of INH resistant isolates by screening the *katG* codon 315 and *inhA*.

### 4.4. PCR-SSCP (single-strand-conformation-polymorphisms)

SSCP is based on the conformational distortion that a nucleotide substitution can cause in a single strand DNA fragment. This conformational change leads to an electrophoretic mobility different to that of the wild-type single-strand fragment. The procedure involves amplification of a DNA fragment including the region of interest by PCR, denaturation and running of this fragment in a polyacrylamide gel together with a denaturated wild-type reference sample. Mobility shifts in the clinical sample indicate presence of mutation.

The usefulness of the method has been assessed by Telenti et al. (1997a) with complete specificity for RMP and INH resistance and sensitivity for RMP>96% and for INH 87%, using four genetic regions (*rpoB*, *katG*, *inhA*, *ahpC*,). Kim et al. (2001) developed a nested PCR-linked SSCP analysis, directly on sputum samples, to detect *M. tuberculosis* and determine RMP susceptibility. In this study, the target was a 157 bp portion of *rpoB* gene, which has been widely used for PCR-SSCP. The results were concordant with those of conventional drug susceptibility testing and DNA sequencing of culture isolates. Furthermore, the nested PCR-SSCP method enabled the direct detection of RMP resistance from primary clinical specimens. However, it should be noted that the assay does not identify the precise mutation and, consequently, the method is significantly less precise than sequencing. Its usefulness is restricted by extensive labour required and high level of technical skills.

### 4.5. Pyrosequencing

Jureen et al. (2006) developed a new assay for rapid detection of rifampicin resistance using Pyrosequencing technology. The target was an 180-bp region of the *rpoB* gene, amplified by PCR and subjected to Pyrosequencing analysis, using four different sequencing primers in four overlapping reactions. The results were compared to other molecular methods (line probe assay and cycle sequencing) and the phenotypic BACTEC 460 method. There was full agreement with the molecular methods showing that Pyrosequecing analysis offers high accuracy.

### 4.6. Real-time PCR methodology

Real-time PCR has been used for detection of mutations responsible for INH and RMP resistance. Piatek et al. (2000) developed a method that exhibited 85% and 98% sensitivity for the detection of mutations responsible for INH and RMP resistance respectively and complete specificity for both antibiotics. Garcia de Viedma et al. (2002) managed to design a method that efficiently detect 12 different mutations associated with either RMP or INH resistance using one single reaction tube.

### 4.7. Mycobacteriophage D29-based assay

Although this method is not a molecular one, it is of value to mention it in this point. It is a low-cost method, thus it can be valuable to many laboratories especially in the developing world. The method is based on the ability of resistant mycobacteria to support mycobacteriophage D29 infection, once exposed to RMP or INH, while sensitive mycobacteria will not be able to support phage replication. An in-house mycobacteriophage-based assay in a microtiter plate format was standardized by Gali et al. (2003). The results were compared with the Bactec 460 method with excellent sensitivity and specificity, indicating the test as an alternative to conventional drug susceptibility methods. Chauca et al. (2007) using the mycobacteriophage D29 to determine the susceptibility of *M. tuberculosis* to RMP and INH, reported complete sensitivity and 98% specificity for RMP, whereas sensitivity and specificity for INH were 80.4% and 80.8% respectively. A meta-analysis of studies on bacteriophage-based assays for the detection of RMP resistance in *M. tuberculosis* was performed by Pai et al. (2005). In this meta-analysis, 11 of 19 (58%) studies reported sensitivity and specificity estimates>or=95% and 13 of 19 studies (68%) studies reported>or=95% agreement with reference standard results.

## 5. Molecular epidemiological methods

Regarding mycobacteria, molecular epidemiology is of critical significance. A series of new molecular methods try to associate

specific genetic markers with the virulence of the strains, the underlying resistance mechanisms, the pathogenesis and the transmission dynamics. All this information is of great relevance for disease control efforts. The major methods used today are:

### 5.1. IS6110-RFLP method

The identification of IS*6110* in the early 90s was a major development for the epidemiology of tuberculosis (van Soolingen et al., 1991). IS*6110* is an insertion sequence, which consists of 1355 bps and its distribution in the *M. tuberculosis* chromosome is highly variable among different strains. The analysis of IS*6110* by restriction fragment length polymorphism (RLFP) has become the gold standard method for genotyping *M. tuberculosis*, and for understanding TB transmission dynamics. The polymorphism of the IS*6110* RFLP patterns is based on the number of copies and their locations on the bacterial genome (Thierry et al., 1990; Burgos and Pym, 2002). Usually, there are 8 to 18 copies per strain, but the number can vary from 0 to 25. Standardized methodology for strain identification by DNA fingerprinting, using *PvuIII* digestion of IS*6110*-containing genomic DNA is available (van Embden et al., 1993). In order to compare their results, a series of laboratories worldwide use the recommendations proposed in this study. The RFLP images can be scanned and digitized for computer analysis. The sizes of the bands in the image are calculated by comparison with size standards run on the gel. Systematic RFLP analysis using hospital-based sampling can detect the spread of TB in specific environments in the community (Lemaitre et al., 1998).

### 5.2. Spoligotyping (spacer oligotyping)

Spoligotyping is a PCR-based method for simultaneous detection and typing of *M. tuberculosis* complex using one particular chromosomal locus with high polymorphism, named “Direct Repeat” (DR) region. The direct repeat sequences consist of 36 bp that are separated by 43 nonrepetitive DNA spacers, each of which contains 36 to 41 bp in length. Most of *M. tuberculosis* strains have at least one IS*6110* element in the DR region of the genome. PCR amplification of the DR locus is performed where the polymorphism is considerable in a small part of the chromosome. A biotin-labeled reverse primer is used, so that all the reverse strands to be biotin labeled. PCR products are hybridized perpendicular to the oligolines on an activated membrane. The membrane is incubated in streptavidin-peroxidase conjugate that binds to the biotin label of the PCR products. Detection of hybridized signals is performed by enhanced chemiluminescence detection system (ECL). When the DR regions of several strains are compared, the order of the spacers is almost the same in all strains but deletions and insertions of spacers can be found in the DR region of 43 spacers of known sequence by hybridization of the amplicon to a set of immobilized oligonucleotides, representing each of the unique spacer DNA sequences. A new, automated spoligotyping assay that codes and reports the results to TB programs, has been developed in order to compare the spoligotypes with published results. This is achieved by converting the banding pattern results to a final octal code designation.

Spoligotyping is particularly helpful when the *M. tuberculosis* strains have few IS*6110* bands. Bauer et al. (1999) evaluated spoligotyping as a technique for further characterizing IS*6110* low-copy number *M. tuberculosis* complex strains in comparison to RFLP. The discriminatory power of spoligotyping was higher than that of IS*6110* fingerprinting. Based solely on RFLP typing, 83% of the low-copy number *M. tuberculosis* complex strains were found to form part of a cluster and, on the basis of spoligotyping, 75% were found to form a cluster. When the two techniques were combined, the amount of clustering decreased to 55%. Spoligotyping can be applied directly on sputum samples. Kamerbeek et al. (1997) developed a spoligotype method that simultaneously examines the clinical samples for *M. tuberculosis* strains, differentiates *M. bovis* from *M. tuberculosis*, and types *M. tuberculosis* isolates. Most of the clinical isolates tested showed unique hybridization patterns, while outbreak strains shared the same spoligotype. The types obtained from direct examination of clinical samples were identical to those obtained by using DNA from cultured *M. tuberculosis* strains. van der Zanden et al. (2002) proposed the use of a second generation spoligotyping membrane using 51 new spacer oligonucleotides, when extended discrimination is required.

### 5.3. MIRU-VNTR method

MIRUs (Mycobacterial Interspersed Repetitive Units) are loci in the *M. tuberculosis* genome that contain variable numbers of tandem repeats (VNTRs). Supply et al. (2000) have identified 41 such loci in the *M. tuberculosis* genome. Among them, 12 loci have been shown to vary in tandem repeat numbers and, in most, sequence between repeat units. The MIRU-VNTR is a PCR-based method that uses these 12 different intergenic regions for genotyping. Calculation of the number of repeats is performed, on the basis of the amplicon size. MIRU results are reported as 12 character designations, each of them corresponds to the number of repeats at one of the 12 loci. The discriminatory power of the 12 MIRU-VNTR regions is much higher than that of spoligotyping and close to IS*6110* RFLP for typing of *M. tuberculosis* strains. Recently, a MIRU-VNTR typing system with 24 loci has been proposed (Supply et al., 2006; Oelemann et al., 2007).

### 5.4. Repetitive sequence-based PCR method

The repetitive sequence-based PCR (rep-PCR) is a method that uses primers that target noncoding repetitive sequences interspersed throughout the bacterial genome (Healy et al., 2005). When amplified by PCR, these repetitive elements generate highly discriminative genomic fingerprints (Cangelosi et al., 2004). The DiversiLab system (bioMerieux, Marcy l'Etoile, France) is a commercial system based on rep-PCR. The whole procedure requires approximately 5 h and includes DNA extraction, PCR, electrophoretic separation, detection of fluorescently-labeled amplicons on automated microfluidic chips and computer-generated printout of results. The ability of this system to type *M. tuberculosis* and *M. avium* complex isolates was evaluated by Cangelosi et al. (2004). For *M. tuberculosis* as well as *M. avium* subspecies *avium*, the discriminative power of rep-PCR equaled or exceeded that of IS*6110*-RFLP.

### 5.5. Remarks

Molecular methods provide a powerful tool for the epidemiology of *M. tuberculosis*. IS*6110* RFLP analysis has great potential in clonal differentiation of *M. tuberculosis* isolates but is laborious and of limited value for strains containing less than six IS*6110* insertions (Goyal et al., 1997; Burman et al., 1997). On the other hand, spoligotyping has proved to be a reliable method for molecular, epidemiological analysis, especially when rapid results are required but the method is of limited discriminatory power, compared with IS*6110*-RFLP (Kremer et al., 1999). IS*6110*-based RFLP requires much more cultural material than the PCR methods (spoligotyping and MIRU). The stability of the spoligotype is higher than that of the IS*6110* banding pattern and is better suited for large-scale investigations, especially when recurrent TB has to be evaluated (Niemann et al., 1999; Warren et al., 2002). Spoligotyping is cost-effective, reproducible and fast. Results can be obtained within a day after growth in liquid culture. The stability of the MIRU typing allows it to be a suitable tool for investigation of outbreaks, relapses and laboratory contaminations along with long-term epidemiological investigations. According to Cowan et al. (2005) although IS*6110* fingerprinting will continue to be necessary in providing

the greatest level of discrimination, the number of isolates requiring this lengthy and laborious technique can be greatly reduced by a two-step genotyping approach with the combined use of spoligotyping and MIRU typing. IS*6110* fingerprinting can then be used as a secondary typing method to type the clustered isolates when additional discrimination is needed (Cowan et al., 2005). Using the appropriate software, the results of all the aforementioned methods can be analyzed on dendrograms allowing accurate interpretation of evolution among *M. tuberculosis* isolates and for epidemiological studies.

In conclusion, it should be noted that due to their rapidity and accuracy, molecular techniques are of great significance for detection, identification and susceptibility testing of mycobacteria. Although these techniques cannot yet fully replace conventional methods, they should be integrated in the workflow of a routine laboratory. The scale of such integration depends primarily on the available economic resources, on the burden of TB in the region and the intention as far as the level of diagnostic capability of each laboratory is concerned.

At an initial level, all microbiology laboratories worldwide, either state or private ones, should have the ability to perform and evaluate an acid-fast staining. At a second level, laboratories of general or regional hospitals should perform cultivation of the samples using solid or/and liquid media, along with susceptibility testing. At this level in developed countries, the use of nucleic acid amplification tests (NAATs) is advocated for all smear-positive specimens and furthermore for smear-negative, when clinical suspicion of TB is high. A positive NAAT would indicate disease with a very good degree of accuracy, whereas a negative result is less helpful. Commercial NAATs, although expensive, have wide applicability, and they are reliable enough for rapid and accurate diagnosis of TB in routine use. Additionally to the conventional proportional antibiograms a molecular assay, such as a line-probe assay, that can genetically detect the resistance of *M. tuberculosis* to RMP or/and INH should be performed as soon as possible.

At a third level of diagnosis, any reference laboratory should additionally include characterization of mycobacteria with sequencing and, furthermore, should perform molecular epidemiology techniques.

In developing countries, new low-cost TB diagnostic assays are needed. In high burden, low-income countries, rapid alternative tests like the phage system may be useful, due to its simplicity and the no need of sophisticated equipment. In TB-endemic countries, the molecular methods like in-house PCR and RFLP may improve the diagnostic and epidemiologic research. The design and evaluation of new rapid diagnostic tools for *M. tuberculosis* infection for use in low-income countries is a challenge for all researchers.

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