MINIREVIEW

Molecular detection, identification and drug resistance detection in *Mycobacterium tuberculosis*

Juan Carlos Palomino

Mycobacteriology Unit, Institute of Tropical Medicine, Antwerp, Belgium

Correspondence: Juan Carlos Palomino, Mycobacteriology Unit, Institute of Tropical Medicine, Nationalestraat 155, 2000 Antwerp, Belgium. Tel.: +32 3 247 6334; fax: +32 3 247 6333; e-mail: jcpalomino@itg.be

Received 22 December 2008; revised 22 February 2009; accepted 22 February 2009.

DOI:10.1111/j.1574-695X.2009.00555.x

Editor: Willem van Leeuwen

Keywords
diagnosis; molecular tests; rapid methods; *Mycobacterium tuberculosis*.

Abstract

This minireview presents recent developments in molecular methods for the diagnosis of tuberculosis, including detection, identification and determination of drug resistance of *Mycobacterium tuberculosis*. Tuberculosis remains one of the major causes of global death from a single infectious agent. This situation is worsened by the HIV/AIDS pandemic because one-third of HIV/AIDS patients are coinfected with *M. tuberculosis*. Also of great concern is the emergence of drug-resistant tuberculosis because there are almost no treatment options available for patients affected by highly resistant strains of *M. tuberculosis*. Advances in molecular biology techniques and a better knowledge of the molecular mechanisms of drug resistance have provided new tools for the rapid diagnosis of tuberculosis. Several nucleic acid amplification technologies have been developed and evaluated. New molecular approaches are being introduced continuously. This minireview will also comment on the future perspectives for the molecular diagnosis of tuberculosis and the feasibility for the implementation of these newer techniques in the clinical diagnostic laboratory.

Introduction

More than a century after the discovery of the tubercle bacillus by Robert Koch, tuberculosis remains one of the major causes of global death from a single infectious agent and constitutes a serious public health problem worldwide. Approximately one-third of the world's population is considered to be latently infected with *Mycobacterium tuberculosis* and 10% of these persons will develop active disease at some point in their lifetimes. According to the last report of the World Health Organization (WHO), there were > 9 million new cases of tuberculosis in 2006 and 1.7 million deaths were attributed to the disease (WHO, 2008a). Moreover, tuberculosis is the only disease ever declared as a global emergency by the WHO. Its association with the HIV/AIDS pandemic forms a lethal combination, introducing further complications for the proper control and management of the disease; in 2006, there were 200 000 deaths in tuberculosis patients coinfected with HIV/AIDS. The emergence of drug-resistant tuberculosis in all its forms, such as the recently described extensively drug resistant tuberculosis (XDR-TB), resistant to almost all first- and second-line antibiotics poses an additional threat for the control of the disease (Gandhi *et al.*, 2006).

Laboratory diagnosis of pulmonary tuberculosis is performed by culture-based conventional methods performed after sputum samples are digested and decontaminated. These specimens are then inoculated into liquid or solid media and controlled for several weeks until detection of mycobacterial growth. In many low-resource high-burden countries, tuberculosis diagnosis is performed solely on the basis of microscopical examination of stained sputum smears to detect acid-fast bacilli; however, sputum smear microscopy is not sensitive and specific enough, requiring 5000–10 000 bacilli mL^{-1} to become positive (Yeager *et al.*, 1967). Nevertheless, bacterial culture and isolation of *M. tuberculosis* is still required for drug-susceptibility testing (DST), but due to the slow growth of the tubercle bacillus these procedures require several weeks to yield results.

Major advances in molecular biology and the availability of new information generated after deciphering the complete genome sequence of *M. tuberculosis* (Cole *et al.*, 1998; Camus *et al.*, 2002) stimulated the development of new tools for the rapid diagnosis of tuberculosis, differentiation of *M. tuberculosis* from nontuberculous mycobacteria, and for the rapid detection of drug resistance (Shamputa *et al.*, 2004; Palomino, 2005; Pai *et al.*, 2006). In this minireview,

I will present recent developments in molecular techniques for the detection, differentiation and determination of drug resistance of *M. tuberculosis* and will comment on the future perspectives of these methods for the rapid diagnosis of tuberculosis and the feasibility for their implementation in tuberculosis diagnostic laboratories.

Molecular methods for detection of *M. tuberculosis*

Because of the slow growth rate of *M. tuberculosis*, conventional methods for its detection, based on solid culture media, take several weeks to yield results. The introduction of liquid culture-based techniques was a great improvement for diagnosis, shortening the time to detection to about 10–14 days instead of weeks that are needed with the conventional media (Idigoras *et al.*, 2000; Scarparo *et al.*, 2002). With the purpose of obtaining faster results and early diagnosis of tuberculosis, several molecular detection methods were introduced and have been evaluated in numerous studies.

Nucleic acid amplification (NAA)-based techniques

All those procedures based on NAA techniques mostly using the PCR to amplify short sequences of DNA or RNA that are specific for the *M. tuberculosis* complex are considered under this category. Many 'in-house' PCR methods have been proposed and tested and many studies have been published describing their application for tuberculosis diagnosis (Kivihya -Ndugga *et al.*, 2004; Sperhacke *et al.*, 2004). Initial studies had, as a limitation, the lack of specificity, as was demonstrated in a multicentre evaluation performed in several laboratories in middle- and low-income countries. This study also found that many laboratories did not use adequate quality controls (Suffys *et al.*, 2000). More recent evaluations, however, have shown that, in general, the performance of in-house NAA tests has improved (Noordhoek *et al.*, 2004).

In-house NAA tests use different targets, either DNA or RNA genus or species specific, followed by a detection step performed on agarose or acrylamide gels, or hybridization in various formats. The most commonly used target for identification of *M. tuberculosis* is the insertion sequence IS*6110*. Overall, the reported sensitivity and specificity are in the range of 84–100% and 83–100%, respectively, for respiratory specimens; lower sensitivity and specificity have been obtained with nonrespiratory specimens (Shamputa *et al.*, 2004). A recent meta-analysis and metaregression of the multiple studies published in the literature found that the use of IS*6110* as the amplification target together with nested-PCR techniques were associated with a higher diagnostic accuracy, but due to the heterogeneity in sensitivity and specificity obtained in many of the studies, useful estimates of their accuracy in the clinic were difficult to conclude (Flores *et al.*, 2005).

NAA tests are also commercially available from different sources. The first two and oldest in the market are the Amplicor Mycobacterium Tuberculosis Test (Amplicor) (Roche Diagnostic Systems Inc., NJ) and the Amplified Mycobacterium Tuberculosis (MTB) Direct Test (MTD) (Gen-Probe Inc., San Diego, CA). Both received approval from the US Food and Drug Administration (FDA) several years ago: The Amplicor MTB test is a DNA-based test that amplifies a segment of the 16S rRNA gene using genus-specific primers, which, after hybridization to oligonucleotide probes, is detected in a colorimetric reaction in a microwell plate format (Dalovisio *et al.*, 1996). An automated version of the test, the COBAS Amplicor MTB test together with the COBAS Amplicor analyzer (Roche Diagnostics, Switzerland) allows automation of the amplification and detection steps in one system. More recently, the qualitative COBAS TaqMan MTB test has also been introduced using real-time PCR and hybridization and performed in the COBAS TaqMan 48 analyzer running up to 48 samples simultaneously in 2.5 h. All three tests are meant to be used in decontaminated and concentrated smear-positive respiratory samples from patients without previous treatment. Many studies have evaluated the Amplicor MTB test for the detection of *M. tuberculosis* both in respiratory and extrapulmonary samples (Michos *et al.*, 2006; Ozkutuk *et al.*, 2006). Overall sensitivity has ranged from 83% to 92.4% in respiratory samples, from 90% to 100% in smear-positive samples and from 50% to 95.9% in smear-negative samples. For extrapulmonary samples, the reported sensitivity has been consistently lower. Overall specificity has ranged from 91.3% to 100% (Piersimoni & Scarparo, 2003). The MTD, on the other hand, uses isothermal amplification of 16S ribosomal transcripts, which are detected in a hybridization protection assay with an acridinium ester-labeled *M. tuberculosis* complex-specific DNA probe (Abe *et al.*, 1993). The test is FDA-approved for the direct detection of *M. tuberculosis* in smear-positive and smear-negative respiratory specimens (Centers for Disease Control, 2000) and its interpretation requires the use of a luminometer (Coll *et al.*, 2003). Several studies have also evaluated the amplified MTD test. Overall sensitivity ranged from 77% to 100%, with values of 90–100% in smear-positive samples and 63–100% in smear-negative samples compared with culture and clinical status of the patient (Piersimoni & Scarparo, 2003).

There are other commercially available amplification methods for the direct detection of *M. tuberculosis* in clinical samples. The BD ProbeTec MTB Test (Becton Dickinson, Sparks, MD) was first introduced several years ago as a semi-automated system for the rapid diagnosis of tuberculosis (Bergmann & Woods, 1998). It is based on the strand-

displacement amplification technique that uses enzymatic replication of target sequences in IS*6110* and the 16S rRNA gene. The amplified products are then detected with a luminometer. The method was evaluated in studies with respiratory samples with a reported sensitivity of 100% in smear-positive specimens and 92–100% in smear-negative samples; the overall specificity was 96–99% in the same studies (Bergmann & Woods, 1998; Pfyffer *et al*., 1999). The major drawback was that the sample preparation required at least 2 h. An improved version of this system, the BDProbe Tec ET, which includes an internal amplification control to detect the presence of inhibitors, has been more recently evaluated in respiratory and nonrespiratory specimens in a clinical setting. As with the other NAA tests described above, higher sensitivity and specificity has been found in respiratory smear-positive samples (Rusch-Gerdes & Richter, 2004). The BDProbe Tec ET system is not yet approved by the US FDA.

Simplified isothermal amplification techniques

A recent development based on isothermal amplification is the loop-mediated isothermal amplification termed the LAMP assay (Mori *et al*., 2001), which is based on autocycling strand displacement DNA synthesis using the large fragment of *Bst* DNA polymerase. The main characteristic of LAMP is its ability to synthesize large amounts of DNA. Pyrophosphate, which is produced as a byproduct, yields a white precipitate of magnesium pyrophosphate that can be detected visually in the reaction vial. The presence or absence of this precipitate allows the detection of DNA amplification. Furthermore, the increase in the turbidity of the reaction mixture correlates with the amount of DNA synthesized, allowing real-time monitoring of the LAMP reaction by real-time measurement of the turbidity. Using a slightly modified version of this methodology, adding SYBR Green I to the reaction for easy detection of colour, Iwamoto *et al*. (2003) were able to detect the *M. tuberculosis* complex, *Mycobacterium avium* and *Mycobacterium intracellulare* directly from sputum specimens and in culture isolates grown in the Mycobacterium growth indicator tube (Becton Dickinson) or Ogawa's medium. The whole procedure was carried out in a single tube with the isothermal reaction held at 63 °C. With the exception of a water bath or heating block, no other laboratory equipment was necessary. When compared with the Amplicor test, the LAMP assay showed comparable performance using a very small volume of DNA and a period of 60-min incubation.

An operational study to assess the feasibility of using the LAMP assay for diagnosing pulmonary tuberculosis was conducted in three microscopy centres in low-resource countries. The sensitivity in smear- and culture-positive sputum samples was 97.7%, while in smear-negative, culture-positive specimens it was only 48.8%. The specificity in 11 culture-negative samples was 99% (Boehme *et al*., 2007). Slightly better results were obtained in another recent evaluation performed in Nepal with 100% sensitivity in culture-positive and 94.2% specificity in culture-negative samples (Pandey *et al*., 2008). The simplicity of the assay and the lack of requirement for major equipment render the LAMP assay a promising candidate as a rapid molecular test for the detection of *M. tuberculosis*. Further studies, however, are needed to assess the accuracy and ease of implementation of this technique in resource-limited settings.

A recent meta-analysis assessed the diagnostic accuracy of the commercial NAA tests described above (Ling *et al*., 2008). Overall, a pooled sensitivity of 85% (range 36–100%) and specificity of 97% (range 54–100%) was reported. Both values showed significant heterogeneity, which could not be explained even after analysing the results of each test separately. The major conclusion was that the accuracy of these NAATs with respiratory samples was highly variable, with sensitivity giving lower values than specificity. For these reasons, they probably still do not have enough clinical relevance and could not be recommended to replace the conventional tests for diagnosing pulmonary tuberculosis. Table 1 shows the sensitivity and specificity of the main NAA tests.

Molecular methods for identification of *M. tuberculosis*

Species identification of mycobacteria has traditionally been based on different biochemical tests and phenotypic characteristics, such as growth rate, pigmentation and colonial morphology that allow classification of a particular strain to a group of well-defined mycobacteria. These methods are simple to perform and do not require sophisticated

Table 1. Sensitivity and specificity of NAA tests in clinical specimens

	Smear-positive pulmonary		Smear-negative pulmonary		Extrapulmonary	
	Sensitivity	Specificity	Sensitivity	Specificity	Sensitivity	Specificity
Amplicor	97	> 95	40–73	> 95	27–98	> 95
AMTD	92–100	> 95	40–93	> 95	93	> 95
BDProbe Tec	90–100	92	33–100	83–97	76	> 90
Real-time PCR	78	100	78	100	80	100
LAMP	97.7	99	48.8	99	ND	ND

equipment; they are, however, laborious and cumbersome, delaying in many cases the prompt and correct identification of the bacteria.

Hence, molecular methods have also found a place as rapid procedures for the identification of mycobacteria, especially of the *M. tuberculosis* complex. The first of such methods commercially available was the AccuProbe (Gen-Probe Inc.), based on species-specific DNA probes that hybridize to rRNA for the identification of several important mycobacteria, including the *M. tuberculosis* complex, *M. avium*, *M. intracellulare*, the *M. avium* complex, *Mycobacterium kansasii* and *Mycobacterium gordonae*. Results are obtained after about 2 h from a positive culture. The probes have been extensively evaluated in clinical settings, and have shown sensitivity and specificity $> 90\%$. More recently, other molecular commercial systems have also been introduced for the rapid identification of the *M. tuberculosis* complex: the INNO-LiPA MYCOBACTERIA v2 (Innogenetics NV, Ghent, Belgium), and the GenoType MTBC and GenoType Mycobacterium (Hain Lifesciences, Nehren, Germany), both to be applied on positive cultures.

INNO-LiPA MYCOBACTERIA v2 is a line probe assay that simultaneously detects and identifies the genus *Mycobacterium* and 16 different mycobacterial species. It is based on nucleotide differences in the 16S–23S rRNA gene spacers and can be performed on liquid or solid cultures. Among mycobacteria identified by this assay are the *M. tuberculosis* complex, *M. kansasii*, *Mycobacterium xenopi*, *M. gordonae*, *Mycobacterium genavense*, *Mycobacterium simiae*, *Mycobacterium marinum* and *Mycobacterium ulcerans*, *Mycobacterium celatum*, *M. avium*, *M. intracellulare*, *Mycobacterium scrofulaceum*, *Mycobacterium malmoense*, *Mycobacterium haemophilum*, the *Mycobacterium chelonae* complex, the *Mycobacterium fortuitum* complex and *Mycobacterium smegmatis*. The test has been evaluated with a large variety of mycobacterial species showing an overall sensitivity of 100% and specificity of 94% (Tortoli *et al*., 2003).

The GenoType MTBC and GenoType Mycobacterium are also based on the reverse line probe hybridization assay and are intended for the differentiation of members of the *M. tuberculosis* complex and for the identification of 35 species of mycobacteria including *M. tuberculosis*, respectively. The GenoType MTBC is based on a 23S rRNA gene fragment specific for the *M. tuberculosis* complex, together with *gyrB* sequence polymorphisms, and the RD1 deletion for identification of *Mycobacterium bovis* BCG (Richter *et al*., 2004). As in other reverse hybridization assays, amplified products from a multiplex PCR assay will hybridize to specific oligonucleotides immobilized on a membrane strip. The GenoType Mycobacterium is based on regions of the 23S rRNA gene; after PCR amplification, hybridization is performed on the oligonucleotides immobilized on the membrane strips, and results are interpreted based on the combination of bands that appear. Two kits are offered separately, the GenoType Mycobacterium CM (common mycobacteria) allowing identification of 17 species and the GenoType Mycobacterium AS (additional species) that identifies 18 less common mycobacterial species (Richter *et al*., 2006). The performance of these three line probe assays requires the availability of a thermal cycler and adequate facilities to conduct PCR amplification.

However, not only are commercial systems available for the rapid molecular identification of *M. tuberculosis,* several 'in-house' techniques are also available with sequencing of the 16S rRNA gene as the reference standard to which all other new techniques are generally compared (Rogall *et al*., 1990). In addition to the 16S rRNA gene, the 23S rRNA gene and the internal transcribed sequence, sequencing of other DNA fragments containing conserved and hypervariable regions have also been proposed for molecular identification of mycobacteria. Among them, the 65-kDa heat shock protein genes (McNabb *et al*., 2004), *rpoB* (Somoskovi *et al*., 2003), *sodA* (Bull *et al*., 1995) and *recA* (Blackwood *et al*., 2000), have also been reported. Another approach based on the *hsp65* gene is by PCR-restriction fragment length polymorphism known also as PRA (Telenti *et al*., 1993). In this technique, a 441-bp fragment of the *hsp65* gene is amplified by PCR and followed by two digestions with BstEII and HaeIII. The products of the digestion are then separated and visualized in an agarose gel. The restriction banding pattern thus obtained is used to identify different species of mycobacteria including *M. tuberculosis* (Chimara *et al*., 2008). PRA has also been applied to other genomic regions for identifying mycobacteria including *rpoB* (Lee *et al*., 2000) and *gyrB* (Goh *et al*., 2006).

Molecular methods for detection of drug resistance of *M. tuberculosis*

Detection of drug resistance in *M. tuberculosis* has traditionally been accomplished by culture-based methods that assess growth of the mycobacteria in the presence of the drug as compared with a growth control. The most commonly used proportion method on Löwenstein–Jensen medium or Middlebrook agar requires 3–6 weeks to yield results (Canetti *et al*., 1969; Kent & Kubica, 1985). Most recently, liquid culture-based methods and faster approaches to detect mycobacterial growth have been applied for the detection of drug resistance in *M. tuberculosis* (Palomino *et al*., 2008).

However, the continuous emergence of severe forms of drug resistance, such as the recently described XDR-TB calls for improved and faster methods for its detection. With the purpose of detecting drug resistance in a shorter period of time and for rapid screening of multidrug-resistance markers, such as resistance to rifampicin in certain populations (Traore *et al*., 2000), several molecular approaches have been

proposed in the last years. Molecular tests to detect drug resistance in tuberculosis look for gene mutations known to be associated with resistance to a particular drug (Ramaswamy & Musser, 1998). DNA sequencing of amplified products to look for specific mutations became the reference standard (García de Viedma, 2003). Although, nowadays, DNA sequencing constitutes a straightforward procedure, from a practical point of view, for most tuberculosis diagnostic laboratories, it would be almost impossible to set up facilities to detect resistance to the major first- and second-line drugs by this approach. Although not all mechanisms of drug resistance are fully known for all the drugs involved, simpler procedures based on the detection of mutations are being introduced, as will be described in the following sections.

Solid-phase hybridization assays

This type of assay is based on the reverse hybridization of oligonucleotides on plastic strips to which specific probes have been immobilized; they are also known as line probe assays. Amplified target sequences from the organism under evaluation are then bound to the specific probes, and hybridization is revealed by the development of a coloured reaction on the strip.

There are currently two commercially available solid-phase reverse hybridization assays for the rapid detection of drug resistance in *M. tuberculosis*: the Line Probe Assay (LiPA) (INNO-LiPA Rif TB Assay, Innogenetics) for detecting resistance to rifampicin and the GenoType MTBDR*Plus* (Hain Lifesciences) for the simultaneous detection of resistance to rifampicin and isoniazid.

LiPA was introduced several years ago and is based on reverse hybridization of amplified DNA from cultured isolates or clinical samples to 10 probes covering the core region of the *rpoB* gene of *M. tuberculosis* immobilized on a nitrocellulose strip (De Beenhouwer *et al.*, 1995). The pattern of hybridization obtained indicates the presence or the absence of mutated or wild regions, which is visualized by a colorimetric reaction, and the strain can be considered as resistant or susceptible to rifampicin (Rossau *et al.*, 1997). Several studies have been conducted on the application of LiPA for the detection of rifampicin resistance; most studies were performed on *M. tuberculosis* isolates with just a few performed directly on sputum samples (Juréen *et al.*, 2004; Traore *et al.*, 2006). It has been proposed as a good initial indicator of multidrug resistance with a sensitivity of 98.5% for detecting resistance to rifampicin (Traore *et al.*, 2000). In a systematic review and meta-analysis of studies performed with LiPA, 12 out of 14 identified studies performed LiPA on isolates with sensitivity $> 95\%$ and a specificity of 100% for rifampicin resistance detection. Four studies that performed LiPA on clinical samples showed 100% specificity but the sensitivity ranged from 80% to 100% (Morgan *et al.*, 2005). In a more recent and larger study, not included in the meta-analysis mentioned above, the usefulness of LiPA for detecting rifampicin resistance was assessed in 420 sputum samples originating from different countries (Traore *et al.*, 2006). A 99.6% agreement was obtained between results obtained by culture and LiPA. The study showed that with an adequate DNA extraction method, LiPA allows rapid detection of resistance to rifampicin when performed directly from sputum samples.

The GenoType MTBDR*Plus*, on the other hand, detects resistance to isoniazid and rifampicin in clinical isolates and sputum samples based on the detection of the most common mutations in *katG*, *inhA* and *rpoB* genes (Hillemann *et al.*, 2007). It also uses PCR and reverse-hybridization to probes immobilized on a plastic strip and improves a previous version of the same test, the MTBDR that detected mutations only in *katG* and *rpoB* genes (Makinen *et al.*, 2006). A recent study evaluated the GenoType MTBDRplus assay in 125 *M. tuberculosis* isolates and 72 smear-positive sputum samples, comparing the results with those obtained with the previous version of the test, the MTBDR. Both tests were able to identify resistance to rifampicin with a sensitivity of 98.7% in the clinical isolates and 96.8% in the sputum samples. MTBDRplus had a sensitivity of 92% for isoniazid resistance detection compared with 88% of MTBDR for the clinical isolates. In the sputum samples, these values were 90.2% and 87.8%, respectively (Hillemann *et al.*, 2007). In a more recent large study, MTBDRplus was implemented in a high-volume public health laboratory for the rapid screening of multidrug-resistant tuberculosis (MDR-TB) (Barnard *et al.*, 2008). Overall, 97% of results from smear-positive samples were available within 1–2 days. Sensitivity and specificity for detection of rifampicin resistance was 98.9% and 99.4%, respectively; for isoniazid resistance sensitivity and specificity was 94.2% and 99.7%, respectively, and for detection of multidrug resistance 98.8% and 100% respectively, as compared with conventional DST results.

Both solid-phase hybridization assays are relatively simple to perform; however, basic expertize in molecular biology and PCR techniques are required. As with other genotypic methods, the sensitivity of the test depends on the amount of DNA present in the sample, and the presence of inhibitors could also cause false-negative results (Palomino, 2006).

Real-time PCR techniques

Real-time PCR technology has also been proposed for the rapid detection of drug resistance in *M. tuberculosis*. Real-time PCR is based on hybridization of amplified nucleic acids with fluorescent-labelled probes spanning DNA regions of interest and monitored inside thermal cyclers (Shamputa *et al.*, 2004). The fluorescent signal increases in

direct proportion to the amount of amplified product inside the reaction vial. For the detection of drug resistance different probes have been used, especially rifampicin or isoniazid, such as TaqMan probes (Espasa *et al.*, 2005), fluorescence resonance energy transfer probes (Saribas *et al.*, 2005), molecular beacons (Varma-Basil *et al.*, 2004) and biprobes (Edwards *et al.*, 2001). The main advantage of real-time PCR is the speed in giving results, 1.5–2.0 h after DNA extraction, and the lower risk of contamination because both reaction and detection occur in a single tube. The main disadvantages would be the requirement for expensive equipment and reagents, and the need for skilled technical personnel. Real-time PCR has been evaluated in several studies for detection of *M. tuberculosis* and resistance to antibiotics.

The microarray technology

Back in the 90s when the possibility to spot multiple probes on a solid format opened the possibility to measure the expression levels of many genes in a single reaction, a technology known as microarray or DNA biochip, there was great expectation on the application of this technology for the rapid diagnosis of tuberculosis and the detection of drug-resistant bacilli (Gingeras *et al.*, 1998; Troesch *et al.*, 1999). However, after a decade of several attempts it has not yet become a reality for common use in the tuberculosis diagnostic laboratory (Sougakoff *et al.*, 2004; Vernet *et al.*, 2004). An interesting development has been proposed with the so-called tuberculosis-Biochip that uses oligonucleotides immobilized in polyacrylamide gel pads on a glass slide (Mikhailovich *et al.*, 2001). Initially developed for rapid detection of resistance to rifampicin in *M. tuberculosis* isolates and directly on sputum samples, it has been improved and evaluated for simultaneous detection of resistance to rifampicin and isoniazid (Gryadunov *et al.*, 2005; Caoili *et al.*, 2006). The technique makes use of a multiplex PCR followed by hybridization with an oligonucleotide microarray. More recently, further attempts are being started for rapid detection of resistance to other antituberculosis drugs by microarrays (Antonova *et al.*, 2008; Shimizu *et al.*, 2008). Additional studies are needed to validate these initial reports and to assess the feasibility of implementation of this technology in diagnostic clinical laboratories.

Conclusions

In spite of tuberculosis being one of the main causes of mortality worldwide, its diagnosis in many low-resource high-burden countries, still relies on microscopical examination of sputum smears that lacks sensitivity and specificity. The gold standard for tuberculosis diagnosis by culture of *M. tuberculosis* takes several weeks to become positive with additional tests required for final identification. For these reasons several rapid molecular tests have been proposed for the rapid diagnosis of tuberculosis. Both in-house and commercial assays are available and they have been evaluated in numerous studies performed in different settings. From the methods reviewed in this article, only two commercially available tests, the Amplicor and the MTD, have received clearance from the FDA for use in smear-positive and smear-negative samples, respectively. Higher values of sensitivity and specificity have been obtained when applied to smear-positive sputum samples as compared with smear-negative specimens. More recently, real-time PCR-based methods and the LAMP test have also been proposed. LAMP avoids the use of a thermocycler and relies on visual detection of the amplified product appearing as an interesting alternative for implementation in laboratories with limited resources and equipment. Additional studies performed in target populations are needed to assess the robustness of this methodology.

Other two tests, the LiPA and the GenoType MTBDR*Plus*, aimed at drug resistance detection are licensed for use in Europe and are labelled with the 'CE' (Conformité Européenne) mark. The few published studies applying the latter tests directly on clinical samples have given, however, promising results. This has been reflected in a recent policy statement by the WHO on the use of line-probe assays for the rapid screening of patients at risk of MDR-TB (WHO, 2008b). They remain to be used only in smear-positive samples.

Current recommendations advice that, in general, the use of molecular tests for the diagnosis of tuberculosis should always be interpreted together with patient clinical information. Because of the costs involved, the requirement of equipment, as well as more skilled personnel, molecular tests have not yet been implemented as routine in tuberculosis diagnostic laboratories, especially in low-resource high-burden countries, where the lack of proper laboratory infrastructures may hamper a wider implementation of these techniques.

For rapid molecular tests to have a real impact in the diagnosis and better control of tuberculosis, firstly, they have to be affordable for low-resource countries, where the burden of tuberculosis is more dramatic. Secondly, in their current format, many molecular tests require dedicated equipment and skilled personnel not always easily available in tuberculosis-endemic settings. Further improvements and simplifications should be explored to make them simpler and friendlier so they would have a real chance to be adopted by the laboratory networks of the tuberculosis control programmes. Thirdly, due to the persistence of alarming rates of drug-resistant tuberculosis around the world and the emergence of new categories of drug resistance, such as the recently described XDR-TB, molecular methods for the diagnosis of tuberculosis should not only be directed to the rapid detection of *M. tuberculosis* in clinical samples but also to the simultaneous detection of drug

resistance. In this way, molecular tests should be able to indicate in a short period of time whether a patient is positive for *M. tuberculosis* and whether the strain responsible is already resistant to the main antibiotics used in tuberculosis therapy, allowing one to start the appropriate treatment regimen. With the exception of full-DNA sequencing, the currently available molecular tests are capable of detecting drug-resistant mutations mainly for rifampicin and isoniazid. As long as molecular tests for tuberculosis diagnosis do not offer this possibility and their methodologies remain demanding, their use will be limited to research environments or laboratories in industrialized countries, with the required facilities and skilled personnel able to implement them, and far away from routine use in clinical diagnostic laboratories in tuberculosis-endemic countries where they are urgently needed. In this context, useful recommendations addressing these issues have appeared recently (Drobniewski *et al*., 2006), stressing the need for high accuracy and strict quality control in all procedures to be implemented in tuberculosis diagnostic laboratories

Acknowledgements

Our studies received financial support from the EC LIFE-SCIHEALTH-3 (Contract No. 516028) and EC LSHP-CT-2007-037912.

References

Abe C, Hirano K, Wada M, Kazumi Y, Takahashi M, Fukasawa Y, Yoshimura T, Miyagi C & Goto S (1993) Detection of *Mycobacterium tuberculosis* in clinical specimens by polymerase chain reaction and Gen-Probe Amplified Mycobacterium Tuberculosis Direct Test. *J Clin Microbiol* **31**: 3270–3274.

Antonova OV, Gryadunov DA, Lapa SA *et al.* (2008) Detection of mutations in *Mycobacterium tuberculosis* genome determining resistance to fluoroquinolones by hybridization on biological microchips. *B Exp Biol Med* **145**: 108–113.

Barnard M, Albert H, Coetzee G, O'Brien R & Bosman ME (2008) Rapid molecular screening for multidrug-resistant tuberculosis in a high-volume public health laboratory in South Africa. *Am J Resp Crit Care* **177**: 787–792.

Bergmann JS & Woods GL (1998) Clinical evaluation of the BDProbe-Tec strand displacement amplification assay for rapid diagnosis of tuberculosis. *J Clin Microbiol* **36**: 2766–2768.

Blackwood KS, He C, Gunton J, Turenne CY, Wolfe J & Kabani AM (2000) Evaluation of *recA* sequences for identification of *Mycobacterium* species. *J Clin Microbiol* **38**: 2846–2852.

Boehme CC, Nabeta P, Henostroza G *et al.* (2007) Operational feasibility of using loop-mediated isothermal amplification (LAMP) for the diagnosis of pulmonary TB in microscopy centers of developing countries. *J Clin Microbiol* **45**: 1936–1940.

Bull TJ, Shanson DC & Archard LC (1995) Rapid identification of mycobacteria from AIDS patients by capillary electrophoretic profiling of amplified SOD gene. *Clin Mol Pathol* **48**: M124–M132.

Camus JC, Pryor MJ, Medigue C & Cole ST (2002) Re-annotation of the genome sequence of *Mycobacterium tuberculosis* H37Rv. *Microbiology* **148**: 2967–2973.

Canetti G, Fox W, Khomenko A, Mahler HT, Menon NK, Mitchison DA, Rist N & Smelev NA (1969) Advances in techniques of testing mycobacterial drug sensitivity, and the use of sensitivity tests in tuberculosis control programmes. *B World Health Organ* **41**: 21–43.

Caoili JC, Mayorova A, Sikes D, Hickman L, Plikaytis BB & Shinnick TM (2006) Evaluation of the TB-Biochip oligonucleotide microarray system for rapid detection of rifampin resistance in *Mycobacterium tuberculosis*. *J Clin Microbiol* **44**: 2378–2381.

Centers for Disease Control. (2000) Nucleic acid amplification tests for tuberculosis. *MMWR* **49**: 593–594.

Chimara E, Ferrazoli L, Ueky SY, Martins MC, Durham AM, Arbeit RD & Leão SC (2008) Reliable identification of mycobacterial species by PCR-restriction enzyme analysis (PRA)-hsp65 in a reference laboratory and elaboration of a sequence-based extended algorithm of PRA-hsp65 patterns. *BMC Microbiol* **8**: 48.

Cole ST, Brosch R, Parkhill J *et al.* (1998) Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. *Nature* **393**: 537–544.

Coll P, Garrigo M, Moreno C & Marti N (2003) Routine use of Gen-probe Amplified Mycobacterium Tuberculosis Direct (MTD) test for detection of *Mycobacterium tuberculosis* with smear-positive and smear-negative specimens. *Int J Tuberc Lung D* **7**: 886–891.

Dalovisio JR, Montenegro-James S, Kemmerly SA *et al.* (1996) Comparison of the amplified *Mycobacterium tuberculosis* (MTB) direct test, Amplicor MTB PCR, and IS*6110*-PCR for detection of MTB in respiratory specimens. *Clin Infect Dis* **23**: 1099–1106.

De Beenhouwer H, Lhiang Z, Jannes G, Mijs W, Machtelinckx L, Rossau R, Traore H & Portaels F (1995) Rapid detection of rifampicin resistance in sputum and biopsy specimens from tuberculosis patients by PCR and line probe assay. *Tubercle Lung Dis* **76**: 425–430.

Drobniewski FA, Hoffner S, Rusch-Gerdes S, Skenders G & Thomsen V, and the WHO Laboratory Strengthening Task Force. (2006) Recommended standards for modern tuberculosis laboratory services in Europe. *Eur Respir J* **5**: 903–909.

Edwards KJ, Metherell LA, Yates M & Saunders NA (2001) Detection of *rpoB* mutations in *Mycobacterium tuberculosis* by biprobe analysis. *J Clin Microbiol* **39**: 3350–3352.

Espasa M, González-Martín J, Alcaide F, Aragón LM, Lonca J, Manterola JM, Salvadó M, Tudó G, Orús P & Coll P (2005) Direct detection in clinical samples of multiple gene mutations causing resistance of *Mycobacterium tuberculosis* to isoniazid

and rifampicin using fluorogenic probes. *J Antimicrob Chemoth* **55**: 860–865.

Flores LL, Pai M, Colford JM & Riley LW (2005) In-house nucleic acid amplification tests for the detection of *Mycobacterium tuberculosis* in sputum specimens: meta-analysis and meta-regression. *BMC Microbiol* **5**: 55.

Gandhi NR, Moll A, Sturm AW, Pawinski R, Govender T, Lalloo U, Zeller K, Andrews J & Friedland G (2006) Extensively drug-resistant tuberculosis as a cause of death in patients co-infected with tuberculosis and HIV in a rural area of South Africa. *Lancet* **368**: 1575–1580.

García de Viedma D (2003) Rapid detection of resistance in *Mycobacterium tuberculosis*: a review discussing molecular approaches. *Clin Microbiol Infec* **9**: 349–359.

Gingeras TR, Ghandour G, Wang E, Berno A, Small PM, Drobniewski F, Alland D, Desmond E, Holodniy M & Drenkow J (1998) Simultaneous genotyping and species identification using hybridization pattern recognition analysis of generic Mycobacterium DNA arrays. *Genome Res* **8**: 435–448.

Goh KS, Fabre M, Huard RC, Schmid S, Sola C & Rastogi N (2006) Study of the *gyrB* gene polymorphism as a tool to differentiate among *Mycobacterium tuberculosis* complex subspecies further underlines the older evolutionary age of '*Mycobacterium canettii*'. *Mol Cell Probe* **20**: 182–190.

Gryadunov D, Mikhailovich V, Lapa S *et al.* (2005) Evaluation of hybridisation on oligonucleotide microarrays for analysis of drug-resistant *Mycobacterium tuberculosis*. *Clin Microbiol Infec* **11**: 531–539.

Hillemann D, Rüsch-Gerdes S & Richter E (2007) Evaluation of the GenoType MTBDRplus assay for rifampin and isoniazid susceptibility testing of *Mycobacterium tuberculosis* strains and clinical specimens. *J Clin Microbiol* **45**: 2635–2640.

Idigoras P, Beristain X, Iturzaeta A, Vicente D & Perez-Trallero E (2000) Comparison of the automated non-radiometric Bactec MGIT 960 system and Löwenstein–Jensen, Coletsos, and Middlebrook 7H11 solid media for recovery of mycobacteria. *Eur J Clin Microbiol* **19**: 350–354.

Iwamoto T, Sonobe T & Hayashi K (2003) Loop-mediated isothermal amplification for direct detection of *Mycobacterium tuberculosis* complex, *M. avium*, and *M. intracellulare* in sputum samples. *J Clin Microbiol* **41**: 2616–2622.

Juréen P, Werngren J & Hoffner SE (2004) Evaluation of the line probe assay (LiPA) for rapid detection of rifampicin resistance in *Mycobacterium tuberculosis*. *Tuberculosis* **84**: 311–316.

Kent PT & Kubica GP (1985) *Public Health Mycobacteriology. A Guide for the Level III Laboratory*. US Department of Health and Human Services, Centers for Disease Control and Prevention, Atlanta.

Kivihya-Ndugga L, Van Cleeff M, Juma E *et al.* (2004) Comparison of PCR with the routine procedure for diagnosis of tuberculosis in a population with high prevalence of tuberculosis and human immunodeficiency virus. *J Clin Microbiol* **42**: 1012–1015.

Lee H, Park HJ, Cho SN, Bai GH & Kim SJ (2000) Species identification of mycobacteria by PCR-restriction fragment length polymorphism of the *rpoB* gene. *J Clin Microbiol* **38**: 2966–2971.

Ling DI, Flores LL, Riley LW & Pai M (2008) Commercial nucleic-acid amplification tests for diagnosis of pulmonary tuberculosis in respiratory specimens: meta-analysis and meta-regression. *PLoS One* **2**: e1536.

Makinen J, Marttila HJ, Marjamaki M, Viljanen MK & Soini H (2006) Comparison of two commercially available DNA line probe assays for detection of multidrug-resistant *Mycobacterium tuberculosis*. *J Clin Microbiol* **44**: 350–352.

McNabb A, Eisler D, Adie K, Amos M, Rodrigues M, Stephens G, Black WA & Isaac-Renton J (2004) Assessment of partial sequencing of the 65-kilodalton heat shock protein gene (*hsp65*) for routine identification of *Mycobacterium* species isolated from clinical sources. *J Clin Microbiol* **42**: 3000–3011.

Michos AG, Daikos GL, Tzanetou K, Theodoridou M, Moschovi M, Nicolaidou P, Petrikkos G, Syriopoulos T, Kanavaki S & Syriopoulou VP (2006) First detection of *Mycobacterium tuberculosis* DNA in respiratory and nonrespiratory specimens by the Amplicor MTB PCR. *Diagn Micr Infec Dis* **54**: 121–126.

Mikhailovich V, Lapa S, Gryadunov D *et al.* (2001) Identification of rifampin-resistant *Mycobacterium tuberculosis* strains by hybridization, PCR, and ligase detection reaction on oligonucleotide microchips. *J Clin Microbiol* **39**: 2531–2540.

Morgan M, Kalantri S, Flores L & Pai M (2005) A commercial line probe assay for the rapid detection of rifampicin resistance in *Mycobacterium tuberculosis*: a systematic review and meta-analysis. *BMC Infect Dis* **5**: 62.

Mori Y, Nagamine K, Tomita N & Notomi T (2001) Detection of loop-mediated isothermal amplification reaction by turbidity derived from magnesium pyrophosphate formation. *Biochem Bioph Res Co* **289**: 150–154.

Noordhoek GT, Mulder S, Wallace P & Van Loon AM (2004) Multicentre quality control study for detection of *Mycobacterium tuberculosis* in clinical samples by nucleic amplification methods. *Clin Microbiol Infec* **10**: 295–301.

Ozkutuk A, Kirdar S, Ozden S & Esen N (2006) Evaluation of Cobas Amplicor MTB test to detect *Mycobacterium tuberculosis* in pulmonary and extrapulmonary specimens. *New Microbiol* **29**: 269–273.

Pai M, Kalantri S & Dheda K (2006) New tools and emerging technologies for the diagnosis of tuberculosis: part II. Active tuberculosis and drug resistance. *Expert Rev Mol Diagn* **6**: 423–432.

Palomino JC (2005) Nonconventional and new methods in the diagnosis of tuberculosis: feasibility and applicability in the field. *Eur Respir J* **26**: 339–350.

Palomino JC (2006) Newer diagnostics for tuberculosis and multi-drug resistant tuberculosis. *Curr Opin Pulm Med* **12**: 172–178.

Palomino JC, Martin A, Von Groll A & Portaels F (2008) Rapid culture-based methods for drug-resistance detection in *Mycobacterium tuberculosis*. *J Microbiol Meth* **75**: 161–166.

Pandey BD, Poudel A, Yoda T *et al.* (2008) Development of an in-house loop-mediated isothermal amplification (LAMP) assay for detection of *Mycobacterium tuberculosis* and evaluation in sputum samples of Nepalese patients. *J Med Microbiol* **57**: 439–443.

Pfyffer GE, Funke-Kissling P, Rundler E & Weber R (1999) Performance characteristics of the BDProbeTec system for direct detection of *Mycobacterium tuberculosis* complex in respiratory specimens. *J Clin Microbiol* **37**: 137–140.

Piersimoni C & Scarparo C (2003) Relevance of commercial amplification methods for direct detection of *Mycobacterium tuberculosis* complex in clinical samples. *J Clin Microbiol* **41**: 5355–5365.

Ramaswamy S & Musser JM (1998) Molecular genetic basis of antimicrobial agent resistance in *Mycobacterium tuberculosis*: 1998 update. *Tubercle Lung Dis* **79**: 3–29.

Richter E, Weizenegger M, Fahr AM & Rüsch-Gerdes S (2004) Usefulness of the GenoType MTBC assay for differentiating species of the *Mycobacterium tuberculosis* complex in cultures obtained from clinical specimens. *J Clin Microbiol* **42**: 4303–4306.

Richter E, Rüsch-Gerdes S & Hillemann D (2006) Evaluation of the GenoType Mycobacterium assay for identification of mycobacterial species from cultures. *J Clin Microbiol* **44**: 1769–1775.

Rogall T, Flohr T & Böttger EC (1990) Differentiation of *Mycobacterium* species by direct sequencing of amplified DNA. *J Gen Microbiol* **136**: 1915–1920.

Rossau R, Traore H, De Beenhouwer H, Mijs W, Jannes G, De Rijk P & Portaels F (1997) Evaluation of the INNO-LiPA Rif. TB assay, a reverse hybridization assay for the simultaneous detection of *Mycobacterium tuberculosis* complex and its resistance to rifampin. *Antimicrob Agents Ch* **41**: 2093–2098.

Rusch-Gerdes S & Richter E (2004) Clinical evaluation of the semiautomated BDProbeTec ET System for the detection of *Mycobacterium tuberculosis* in respiratory and non-respiratory specimens. *Diagn Micr Infec Dis* **48**: 265–270.

Saribas Z, Yurdakul P, Alp A & Gunalp A (2005) Use of fluorescence resonance energy transfer for rapid detection of isoniazid resistance in *Mycobacterium tuberculosis* clinical isolates. *Int J Tuberc Lung D* **2**: 181–187.

Scarparo C, Piccoli P, Rigon A, Ruggieron G, Ricordi P & Piersimoni C (2002) Evaluation of the BACTEC MGIT 960 in comparison with BACTEC 460 TB for detection and recovery of mycobacteria from clinical specimens. *Diagn Micr Infec Dis* **44**: 157–161.

Shamputa IC, Rigouts L & Portaels F (2004) Molecular genetic methods for diagnosis and antibiotic resistance detection of mycobacteria from clinical specimens. *APMIS* **112**: 728–752.

Shimizu Y, Dobashi K, Yoshikawa Y *et al.* (2008) Five-antituberculosis drug-resistance genes detection using array system. *J Clin Biochem Nutr* **42**: 228–234.

Somoskovi A, Song Q, Mester J, Tanner C, Hale YM, Parsons LM & Salfinger M (2003) Use of molecular methods to identify the *Mycobacterium tuberculosis* complex (MTBC) and other mycobacterial species and to detect rifampin resistance in MTBC isolates following growth detection with the BACTEC MGIT 960 system. *J Clin Microbiol* **41**: 2822–2826.

Sougakoff W, Rodrigue M, Truffot-Pernot C, Renard M, Durin N, Szpytma M, Vachon R, Troesch A & Jarlier V (2004) Use of a high-density DNA probe array for detecting mutations involved in rifampicin resistance in *Mycobacterium tuberculosis*. *Clin Microbiol Infec* **10**: 289–294.

Sperhacke RD, Mello FC, Zaha A, Kritski A & Rosetti ML (2004) Detection of *Mycobacterium tuberculosis* by a polymerase chain reaction colorimetric dot-blot assay. *Int J Tuberc Lung D* **8**: 312–317.

Suffys P, Palomino JC, Cardoso Leão S *et al.* (2000) Evaluation of the polymerase chain reaction for the detection of *Mycobacterium tuberculosis*. *Int J Tuberc Lung D* **4**: 179–183.

Telenti A, Marchesi F, Balz M, Bally F, Böttger EC & Bodmer T (1993) Rapid identification of mycobacteria to the species level by polymerase chain reaction and restriction enzyme analysis. *J Clin Microbiol* **31**: 175–178.

Tortoli E, Mariottini A & Mazzarelli G (2003) Evaluation of INNO-LiPA MYCOBACTERIA v2: improved reverse hybridization multiple DNA probe assay for mycobacterial identification. *J Clin Microbiol* **41**: 4418–4420.

Traore H, Fissette K, Bastian I, Devleeschouwer M & Portaels F (2000) Detection of rifampicin resistance in *Mycobacterium tuberculosis* isolates from diverse countries by a commercial line probe assay as an initial indicator of multidrug resistance. *Int J Tuberc Lung D* **4**: 481–484.

Traore H, van Deun A, Shamputa IC, Rigouts L & Portaels F (2006) Direct detection of *Mycobacterium tuberculosis* complex DNA and rifampin resistance in clinical specimens from tuberculosis patients by line probe assay. *J Clin Microbiol* **44**: 4384–4388.

Troesch A, Nguyen H, Miyada CG, Desvarenne S, Gingeras TR, Kaplan PM, Cros P & Mabilat C (1999) Mycobacterium species identification and rifampin resistance testing with high-density DNA probe arrays. *J Clin Microbiol* **37**: 49–55.

Varma-Basil M, El-Hajj H, Colangeli R *et al.* (2004) Rapid detection of rifampin resistance in *Mycobacterium tuberculosis* isolates from India and Mexico by a molecular beacon assay. *J Clin Microbiol* **42**: 5512–5516.

Vernet G, Jay C, Rodrigue M & Troesch A (2004) Species differentiation and antibiotic susceptibility testing with DNA microarrays. *J Appl Microbiol* **96**: 59–68.

World Health Organization (WHO). (2008a) Global tuberculosis control: surveillance, planning, financing. WHO Report 2008, Geneva, WHO/HTM/TB/2008.393.

World Health Organization (WHO). (2008b) Molecular line probe assays for rapid screening of patients at risk of multidrug-resistant tuberculosis (MDR-TB). Policy Statement. Available at http://www.who.int/entity/tb/dots/laboratory/lpa_policy.pdf (accessed December 15, 2008).

Yeager H Jr, Lacy J, Smith LR & LeMaistre CA (1967) Quantitative studies of mycobacterial populations in sputum and saliva. *Am Rev Respir Dis* **95**: 998–1004.