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Advances in rapid diagnosis of tuberculosis disease and anti-tuberculous drug resistance

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A B S T R A C T

Rapid diagnosis of tuberculosis (TB) and multidrug-resistant (resistance to at least rifampin and isoniazid) *Mycobacterium tuberculosis* (MDR-TB) is one of the cornerstones for global TB control as it allows early epidemiological and therapeutic interventions. The slow growth of the tubercle bacillus is the greatest obstacle to rapid diagnosis of the disease. However, considerable progress has recently been made in developing novel diagnostic tools, especially molecular methods (commercial and 'in-house'), for direct detection in clinical specimens. These methods, based on nucleic acid amplification (NAA) of different targets, aim to identify the *M. tuberculosis* complex and detect the specific chromosome mutations that are most frequently associated with phenotypic resistance to multiple drugs. In general, commercial methods are recommended since they have a better level of standardization, reproducibility and automation. Although some aspects such as cost-efficiency and the appropriate setting for the implementation of these techniques are not yet well established, organizations such as the WHO are strongly supporting the implementation and universal use of these new molecular methods. This chapter summarizes current knowledge and the available molecular methods for rapid diagnosis of TB and anti-tuberculous drug resistance in clinical microbiology laboratories.

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Avances en el diagnóstico rápido de la enfermedad tuberculosa y de la resistencia a los fármacos antituberculosos

Palabras clave:
Tuberculosis
Diagnóstico molecular
Detección rápida de la resistencia

R E S U M E N

El diagnóstico rápido de la enfermedad tuberculosa y la resistencia múltiple a los fármacos antituberculosos (al menos isoniazida y rifampicina) en *Mycobacterium tuberculosis* complex (MDR-TB) es una de las piedras angulares en el control de esta enfermedad, ya que permite una acción epidemiológica y terapéutica precoz. El crecimiento lento del bacilo tuberculoso es uno de los mayores impedimentos para un diagnóstico rápido. En los últimos años ha existido un importante avance en el desarrollo de nuevas herramientas diagnósticas, sobre todo moleculares (comerciales y caseras), para el diagnóstico directo de muestra clínica. Estos métodos se basan en la amplificación de diversas dianas de ácidos nucleicos (AAN), para la identificación de *M. tuberculosis* complex y la detección de las mutaciones cromosómicas más frecuentemente relacionadas con la resistencia fenotípica a diversos fármacos. En general, entre las múltiples técnicas existentes, se recomiendan los métodos comerciales por su mayor estandarización, reproducibilidad y automatización. A pesar de que aspectos como el coste-efectividad y las indicaciones para la adecuada implementación de estas técnicas no están del todo bien establecidos, organizaciones como la OMS están apoyando de forma firme la aplicación y utilización universal de estos nuevos métodos moleculares. Este capítulo resume el conocimiento actual y los métodos moleculares disponibles para el diagnóstico rápido de la TB y la resistencia a los fármacos en los laboratorios de microbiología clínica.

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Introduction

Delayed diagnosis of tuberculosis (TB) and multi-drug resistant forms of the disease constitute one of the biggest obstacles to effective control of TB worldwide.[1] Recently, the Stop TB Partnership, a network of concerned governments, organizations and donors led by the World Health Organization (WHO) (http://www.stoptb.org/stop_tb_initiative/), outlined a global plan to halve TB prevalence and mortality by 2015 and eliminate the disease as a public health problem by 2050. Multidisciplinary approaches, including studies of TB epidemiology, comparative genomics, evolution and host-pathogen interaction, will be necessary in order to develop better tools and strategies to control and eliminate TB.[2] In this context, several rapid and accurate diagnostic methods have recently appeared, and they will be briefly discussed in this chapter.

Rapid detection of *Mycobacterium tuberculosis*

Although the presumptive diagnosis of TB is often based on clinical suspicion and radiological data, a definitive diagnosis of disease and drug resistance requires microbiological assays. Laboratory diagnosis of TB has traditionally been based on smear microscopy, culture and phenotypic identification. While the quickest, easiest and cheapest method available is acid-fast staining, its low sensitivity (45%-80% of positive cultures) has limited its usefulness, especially in geographical areas of lower incidence, in extrapulmonary forms (paucibacillary) of TB, and in HIV-infected patients. It should also be noted that a significant percentage (17%) of transmission occurs from smear-negative pulmonary tuberculosis patients.[3] A further point is that despite having good overall specificity the smear has a low positive predictive value (50%-80%) in areas of higher incidence of non-tuberculous mycobacteria (NTM) clinical isolates.[4-6]

By contrast, the culture technique is still regarded as the reference method due to its sensitivity and the fact that further studies can be conducted with the isolated mycobacteria (identification, sensitivity and epidemiological typing).[5,6] However, the slow growth of the tubercle bacillus is a major obstacle to rapid disease diagnosis. Indeed, while the last two decades have witnessed spectacular improvements to the culture method through the use of new media and automated systems such as Bactec 460TB (Becton Dickinson Diagnostics, Sparks, USA), MB/BacT ALERT (bioMérieux, Marcy-l'Etoile, France), MGIT 960 (Becton Dickinson Diagnostics) and VersaTREK (Trek Diagnostic System, Westlake, USA), several weeks are still required to obtain the final laboratory confirmation, and even longer in the case of conventional phenotypic identification procedures.[4-7] Therefore, in recent years new methods have been developed for the rapid diagnosis of active TB, the best alternative being the molecular or genotypic techniques.

Chromatographic methods

Direct *M. tuberculosis* identification from clinical samples has been attempted by using different chromatography methods to detect tuberculostearic acid (TBSA) alone or in combination with other structural components of the mycobacterial cell wall.[8,9] Several fairly fast and sensitive methods have been developed so far,[9] one of the most interesting of which is fast gas chromatography mass spectrometry (GC-MS).[10,11] However, because TBSA is not specific to species and its detection requires a differential diagnosis between *Mycobacterium* and *Nocardia* species and other Gram-positive bacilli, which also contain the same acid, other lipids have been studied and proposed. Among these, hexacosanoic acid in combination with TBSA appears to be quite specific for the presence of *M. tuberculosis.*[11] However, although chromatographic methods may have some utility for mycobacterial identification from positive cultures, such as in the case of the new immunochromatographic assays based on the MPT-64 antigen,[12] they do not yet represent a significant alternative for the rapid diagnosis of tuberculosis from clinical specimens.

Phagotypic methods

During the last decade a number of bacteriophages with specific affinity for mycobacteria have appeared for the rapid diagnosis of tuberculosis. Since 1947, over 250 different types of mycobacteriophages have been isolated and described, and they have constituted important tools for the genetic manipulation of mycobacteria. However, a degree of clinical utility has only been shown by two of the phage-based approaches developed to date, namely the Luciferase Reporter Phage Assay (LRP) and the Phage Amplified Assay (PhaB or MAB).[13-15] The most important difference between these methods concerns the detection of phage-infected mycobacterial cells. LRP relies on the emitted light that is encoded by the gene for luciferase (*fflux*), which is inserted into the phage genome. By contrast, PhaB or MAB is based on the presence of viable *M. tuberculosis* complex cells after phage amplification (Mycobacteriophage D29) in *Mycobacterium smegmatis*.

LRP has proven useful in differentiating *M. tuberculosis* and NTM from culture and, especially, in susceptibility tests to isoniazid and rifampin.[13] PhaB or MAB has been commercialized (FASTPlaque-TB or the variant PhageTeK MB, Biotec Laboratories Ltd, Ipswich, Suffolk, UK) for diagnosing tuberculosis in respiratory specimens,[14] and has also been studied for antimicrobial susceptibility testing in *M. tuberculosis.*[16] Both techniques are generally quick and simple, requiring little training and technical equipment, and they are relatively inexpensive. However, although they have demonstrated good specificity, several problems of sensitivity have been encountered in most studies.[14,15,17] Their routine application has therefore been somewhat delayed, and it remains to be seen what their real usefulness will be in the diagnosis of tuberculosis or the detection of resistance to anti-tuberculosis drugs

Genotypic methods

Numerous molecular techniques (commercial and 'in-house') and various applications of them are now available for the microbiological diagnosis of mycobacterial infections.[7,18-20] Although DNA probes were the first major innovation in the molecular diagnosis of tuberculosis, the direct detection from clinical samples of *M. tuberculosis* and specific mutations correlating with resistance (see below) requires methods based on amplifying specific sequences of nucleic acids (NAA). These techniques have several advantages, such as a fast turnaround time and feasibility for automation. However, a number of disadvantages emerge when applying these methods directly to clinical specimens, for example, problems with inhibitors, sensitivity in smear-negative samples and DNA extraction.

Although the clinical utility of these methods has been widely discussed, solid evidence and a global consensus regarding their implementation has yet to be definitively achieved.[21] This is due in part to the wide variety of techniques available, as well as to the lack of standardization between studies, most of which use culture as the *gold standard*, which theoretically has a lower sensitivity than nucleic acid amplification (NAA) tests. Furthermore, the lack of assessment of clinical aspects in most studies has led to some confusion regarding how, with whom and when to use this technology. Nevertheless, the current findings regarding the use of NAA tests to diagnose tuberculosis suggest that: *a)* they can quickly detect the presence of *M. tuberculosis* in 50%-85% of acid-fast bacillus (AFB) smear-negative and culture-positive specimens; *b)* the positive predictive value in AFB smear-positive specimens is higher (>95%) than that of microscopy in geographical areas with a large number of NTM isolates; and *c)* in general, these molecular methods can diagnose TB

Table 1
Comparison of different commercial nucleic acid amplification (NAA) tests for direct detection of *Mycobacterium tuberculosis* complex from clinical samples

Assay	Amplification method	Target	Detection	Sample vol (µl)	Turnaround Time (h)	Automation	IAC
Cobas Amplicor	PCR	16S rRNA	Colorimetric	100	6-7	Yes	Yes
AMTD	TMA	16S rRNA	Chemiluminiscent	450	2.5	No	No
LCx	LCR	PAB	Fluorimetric	500	6	Yes	No
BD Probe Tec	SDA	IS*6110* – 16S rRNA	Fluorimetric	500	3.5-4	Yes	Yes
Inno-Lipa	Nested-PCR	*rpoB* gene	Colorimetric	500	12	Yes	No
GenoType MD	NASBA	23S RNA	Colorimetric	500	5.5	Yes	Yes
RT-PCR*	Real-time PCR	16S rRNA	Fluorimetric	10-100	2-3	Yes	Yes
GeneXpert	Real-time PCR	*rpoB* gene	Fluorimetric	1,000	2	Yes	Yes
GenoQuick	PCR	IS*6110*	Colorimetric	500	2.5	No	Yes

IAC: internal amplification control; LCR: ligase chain reaction; NASBA: nucleic acid sequence-based amplification; PCR: polymerase chain reaction; SDA: strand displacement amplification; TMA: transcription-mediated amplification.
*RT-PCR: several commercial techniques of real-time PCR.

several weeks earlier than culture in 80%-90% of patients with a high level of TB suspicion.[21-23]

NAA tests include a wide variety of 'in house' methods with multiple protocols of nucleic acid extraction and amplification (PCR) of different genetic targets (IS*6110*, *rpo*B, *hsp*65, 16S rDNA or MBP64). However, although these 'in house' amplification tests have generally improved in recent years, the recommendation is to use commercial tests that have a greater level of standardization and reproducibility.[20,23,24]

All NAA methods require further post-amplification analysis by electrophoretic observation of the amplified fragment or hybridization, restriction or sequencing.[18,20,25] The analysis based on sequencing (16S rDNA) or restriction (such as PCR-RFLP of the *hsp*65 gene or 16S-23S spacer region) could theoretically be used on clinical specimens. However, for the diagnosis of tuberculosis the most developed and commercialized methods are based on hybridization assays, and these are briefly described below, especially the newly marketed systems (Table 1).

Conventional DNA amplification by PCR. The Amplicor *Mycobacterium tuberculosis* test (Roche Diagnostic System Inc., Basel, Switzerland) is one of the oldest marketed techniques to rely on standard PCR. It is a DNA-based test that amplifies a specific segment of the 16S rRNA gene, followed by hybridization and colorimetric detection. This method may be automated (Cobas Amplicor) and was approved in 1996 by the US Food and Drug Administration (FDA) for use in respiratory samples that have positive AFB smears.[23] Numerous studies have reported high sensitivity in smear-positive respiratory specimens (87%-100%), the figure being lower in smear-negative cases (40%-73%) and extrapulmonary samples (27%-98%). The specificity of this method ranges from 91% to 100%.[20,24,26,27]

Transcription-mediated amplification (TMA). The commercial Amplified *M. tuberculosis* Direct Test (AMTD; Gen-Probe Inc., San Diego, CA, USA) is a rapid isothermal (42 ºC) method based on the amplification of 16S rRNA. Reverse transcriptase is used to copy rRNA to a cDNA-RNA hybrid, and the chemiluminiscent method is then applied to detect the *M. tuberculosis* complex by specific DNA probes. The AMTD was the first test to be approved by the FDA (1995) for smear-positive respiratory specimens, and in 2000 the FDA recommendation was extended to smear-negative samples.[23] There is now evidence that AMTD shows high specificity (95%-100%) and high sensitivity (91%-100%) for smear-positive respiratory samples, although the latter is lower for smear-negative (65%-93%) and extrapulmonary samples (63%-100%). The most important disadvantages are the lack of internal amplification control (IAC) and no possibility of automation.[20,24,26,27]

Ligase chain reaction (LCR). The LCX *M. tuberculosis* assay (Abbot Laboratories, Chicago, IL, USA) is a semi-automated DNA amplification method using LCR for direct detection, from clinical samples, of chromosomal gene encoding the *M. tuberculosis* protein antigen b. However, although good specificity (90%-100%) and sensitivity (65%-90%) were reported in several studies from respiratory specimens, this product was withdrawn from the European market in 2002.[20,26,27,28]

Strand displacement amplification (SDA). The BD ProbeTec ET Direct TB System (DTB; Becton Dickinson) was introduced in 1998 as a semi-automated technique for rapid detection of MTBC in respiratory samples. It is an isothermal (52.5 ºC) enzymatic amplification process for generating multiple copies of target sequences of the IS*6110* and 16S rRNA genes, whose amplification product is detected by the fluorescent method. Evaluations in respiratory samples have shown a sensitivity of 90%-100% in smear-positive samples and 30%-85% in smear-negative ones, with high specificity (90%-100%).[20,24,26,27]

Solid-phase hybridization assays. Three line probe assays are commercially available: the INNO-LiPA Rif. TB kit (Innogenetics, Gent, Belgium), the GenoType MTBDR*plus* assay and the GenoType Mycobacterium Direct (MD) assay (Hain Lifescience, Nehren, Germany). Although the first two systems can detect and identify *M. tuberculosis* complex from clinical samples their major use is with positive cultures and for the detection of rifampin and isoniazid (by GenoType MTBDR*plus* only) resistance (see below). However, the GenoType MD assay is specific for the direct detection of RNA in clinical specimens of *M. tuberculosis* complex and other common NTM (*Mycobacterium avium*, *Mycobacterium intracellulare*, *Mycobacterium kansasii* and *Mycobacterium malmoense*) by the NASBA amplification method. The limited data available to date indicate good sensitivity and specificity in respiratory specimens, with the potential advantage that this assay can detect five clinically common mycobacterial species.[29-31]

Real-time PCR (RT-PCR). These techniques are based on simultaneous amplification of different DNA targets and fluorimetric detection by labelled probes (for example, TaqMan, molecular beacons, bioprobes or FRET). These tests have a number of important advantages, especially their rapidity and fewer cross-contamination problems; this is because the processes, after DNA extraction, occur in a single tube. In recent years, numerous commercial techniques, such as the Cobas TaqMan MTB test (Roche Diagnostic System), have been developed with high overall sensitivity and specificity, especially in smear-positive respiratory samples (Table 1).[19,20,24,32] Among these, the GeneXpert MTB/RIF (Cepheid, Sunnyvale, CA, USA and FIND Diagnostics, Geneva, Switzerland) has recently been introduced as a semi-quantitative nested RT-PCR *in vitro*

diagnostic test, one that integrates and automates sample processing (DNA extraction) and offers simultaneous detection of *M. tuberculosis* complex and rifampin resistance (see below) within single-use disposable cartridges. The time to result is less than two hours and minimal training is required to use the test. Preliminary studies suggest good sensitivity and specificity in pulmonary samples.[33-36] Although further research is needed, the WHO has recently supported the use of this system as an initial diagnostic test in respiratory specimens of patients with high clinical suspicion of having tuberculosis or who could be multidrug resistant (see below).[37]

Other new methods. The loop mediated isothermal amplification (LAMP; Eiken Chemical Co. Japan and FIND Diagnostics, Geneva, Switzerland) is a relatively new isothermal (64-65 °C) amplification DNA technique.[38] The LAMP assay can synthesize large numbers of DNA targets (*gyrB* or IS*6110*) in a single tube, and the amplification product may be detected by turbidity or colorimetric and fluorimetric methods. Despite limited testing in the context of tuberculosis the early data are promising and the assay has the advantage of being rapid (2 hours) and relatively inexpensive, which could be useful in resource-limited settings.[24,39] Another new commercial NAA assay for rapid diagnosis of tuberculosis in respiratory samples is the GenoQuick MTB test (Hain Lifescience), which is based on PCR and subsequent hybridization. The complex obtained binds selectively to a dipstick and is detected by a colorimetric method (gold labelling). No studies have been published to date, but the preliminary data are promising.

Rapid diagnosis of anti-tuberculous drug resistance

The rapid emergence of multidrug-resistant *Mycobacterium tuberculosis* (MDR-TB: resistance to at least rifampin and isoniazid) and extensively drug-resistant tuberculosis (XDR-TB: MDR plus resistance to fluoroquinolone and one of the three injectable second-line drugs, amikacin, kanamycin and capreomycin) poses a serious threat to the treatment of tuberculosis. The World Health Organization estimates that 500,000 new cases of MDR-TB occur globally every year and more than 45 countries have reported XDR cases.

Because genetic resistance to an anti-tuberculosis drug is due to spontaneous chromosomal mutations the MDR/XDR phenotype is caused by sequential accumulation of mutations in different genes involved in individual drug resistance. This drug resistance may be attributable to direct transmission of drug-resistant strains (primary resistance) or to *de novo* acquisition of resistance during individual patient treatment (secondary resistance), i.e. due to inappropriate treatment or poor adherence to treatment.

A delay in diagnosing MDR-TB associated with standard drug susceptibility testing methods is likely to contribute to the acquisition of further drug resistance, as well as to the dissemination of drug-resistant strains through person-to-person transmission. By contrast, the rapid detection of drug-resistant strains facilitates early access to the appropriate therapy, reduces transmission rates and improves treatment outcomes. The long turnaround time and laboriousness of drug susceptibility testing methods has therefore stimulated the search for alternative and faster techniques. New genotypic methods search for the genetic determinants of resistance rather than the resistance phenotype. In this regard, the WHO has recommended the worldwide use of rapid genotypic assays for the rapid diagnosis of MDR-TB. Those genotypic assays should be able to detect the mutations responsible for isoniazide (INH) and rifampin (RMP) resistance. Moreover, if XDR-TB is to be ruled out, then the mutations responsible for resistance to streptomycin (STR), amikacin (AMK), kanamycin (KAN), capreomycin (CM) and fluoroquinolones should also be screened for.

Mutations confined to a short 81bp DNA region of the *rpoB* gene, encoding the β-subunit of RNA polymerase and spanning codons 507-533, have been found in ~95% of RMP-resistant strains. Mutations in rpoB generally result in high-level resistance to RMP and cross resistance to all rifamycins. However, specific mutations in codons 511, 516, 518 and 522 result in a phenotype of lower-level resistance to RMP and rifapentin, and retained susceptibility to rifabutin and rifalazil.[40] As RMP monoresistance is relatively rare, molecular detection of mutations in this region (RRDR, rifampin resistance determining region) is a good indicator of MDR-TB. It should be noted that a recent paper by Zaczek et al[41] reported that direct molecular identification for RMP-resistant *M. tuberculosis* clinical isolates is only possible for strains carrying selected mutations in RpoB. The identification of other mutations suggests that investigated strains might be resistant to this drug, in other words, these mutations require a specific genetic background to develop resistance.

The molecular mechanisms of resistance to INH are more complex. They have been associated with a variety of mutations which affect one or several genes involved in mycolic acid biosynthesis or that are overexpressed as a response to the build-up or cellular toxicity of INH. Mutations in the *katG* gene are responsible for 60%-70% of INH-resistant strains, with the most frequent mutation occurring at codon 315 (S315T, serine-to-threonine substitution). The S315T alteration located within the active site of *katG* prevents KatG-mediated activation of INH and results in a high level of resistance. Mutations in the *mabA-inhA* regulatory region that exhibit both low-level INH resistance and ethionamide resistance account for 8%-20% of INH resistance. A C-to-T substitution at nucleotide -15 results in the overexpression of InhA, an NADH-dependent enoyl-acyl reductase involved in mycolic acid synthesis, and INH resistance arises as a result of drug titration.[42] In our experience, a rapid genotypic assay including the 315-*katG* codon and -15 nt of the *mabA-inA* regulatory region would cover 62% of isoniazid-resistant strains in Barcelona.[43]

STR acts on the ribosome, inhibiting the translation of mRNA and, therefore, disrupting protein synthesis. Mutations associated with STR resistance in *M. tuberculosis* have been identified in the *rpsL* and *rrs* genes, which encode the ribosomal proteins S12 and 16S rRNA, respectively. More than half the STR-resistant clinical isolates present mutations associated with these genes.[44] The most common mutations in the *rpsl* gene have been detected in codons 43 and 48, and in two specific regions (the 530 loop and 912 regions) of the *rrs* gene.[45] In our experience, mutations in the *rpsL* and *rrs* genes are detected in 37.7% of STR-resistant *M. tuberculosis* isolates[46]. There is also a strong correlation between the level of resistance and the type and position of mutations.[46,47] High-level resistance has mainly been associated with *rpsL* gene alterations, whereas intermediate and low levels of resistance have been linked with mutations in the *rrs* gene and wild-type patterns.[46,47]

Only a few studies have investigated the genetic background of AMK, KAN and CM resistance.[48-50] Resistance to AMK and CM is associated with mutations in the *rrs* gene, especially in the region between nucleotides 1.400 and 1.500, each of which are responsible for a specific resistance pattern. Mutations G1484T and A1401G were found to cause high-level resistance to all drugs, whereas C1402T only causes resistance to CM and KAN. Resistance to CM is thought to be additionally mediated by mutations located anywhere in the *tlyA* gene, which encodes a 2'-O-methyltransferase.

Resistance to fluoroquinolones is mediated mainly by mutations in *gyrA* (around 85%) and less frequently by those in *gyrB* (around 10%),[51] which are genes that encode the respective subunits of the DNA topoisomerase gyrase.[52] Most mutations accumulate in a short discrete region known as the quinolone resistance-determining region (QRDR). It has been observed that certain isolates show a mixture of wild-type and multiple mutant alleles of *gyrA* (heteroresistant isolates).[53] Heteroresistance is considered a preliminary stage of full resistance.

In summary, a genotypic method to detect INH resistance should be based on the analysis of the 315-*katG* codon and -15 nt of the *mabA-inA* regulatory region. For RMP resistance the method should explore the short 81bp DNA region of the *rpoB* gene. In this context, it has to be stressed that RMP resistance is a surrogate marker for

Table 2
Description of studies that have evaluated line probe assays*

Author (year)	Country	Reference test	Sample	Susceptible/resistant	Sensitivity	Specificity
Genotype MTBDRplus assay (Hain Lifescience, Nehren, Germany)						
Isoniazid						
Lacoma (2008)	Spain	Bactec460TB	Isolate	14/48	73	100
Mohito (2008)	Italy	Bactec460TB	Isolate	0/173	79	100
Hillemann (2007)	Germany	Bactec460TB	Isolate	50/75	92	100
Evans (2009)	South Africa	DNA sequencing	Isolate	90/123	83.8	98.9
Lacoma (2008)	Spain	Bactec460TB	Clinical specimen	21/30	93	100
Causse (2008)	Spain	MGIT	Clinical specimen	22/37	94.6	
Hillemann (2007)	Germany	Bactec460TB	Clinical specimen	31/41	90.2	100
Rifampin						
Lacoma (2008)	Spain	Bactec460TB	Isolate	50/12	91.7	100
Hillemann (2007)	Germany	Bactec460TB	Isolate	50/75	98.7	100
Evans (2009)	South Africa	DNA sequencing	Isolate	131/92	90.8	100
Lacoma (2008)	Spain	Bactec460TB	Clinical specimen	22/29	100	95.4
Causse (2008)	Spain	MGIT	Clinical specimen	23/36	100	
Hillemann (2007)	Germany	Bactec460TB	Clinical specimen	41/31	96.8	100
INNO-LiPA Rif. TB kit (Innogenetics, Gent, Belgium)						
Ahmad (2002)	Kuwait	Bactec460TB	Isolate	29/12	97	100
De Oliveira (1998)	Brazil	Proportion	Isolate	113/15	97	100
Gamboa (1998)	Spain	Bactec460TB	Isolate	46/13	100	100
Hirano (1999)	Japan	Proportion	Isolate	90/26	92	100
Johansen (2003)	Denmark	Bactec460TB	Isolate	35/24	97	100
Jureen (2004)	Sweden	Bactec460TB	Isolate	27/26	100	92
Lemus (2004)	Belgium	Bactec460TB	Isolate	10/10	100	100
Rossau (1997)	Belgium	Proportion	Isolate	203/61	98	100
Sintchenko (1999)	Australia	Bactec460TB	Isolate	22/11	96	100
Somoskovi (2003)	USA	Proportion	Isolate	64/37	95	100
Srivastava (2004)	India	MIC	Isolate	45/10	82	100
Tracevska (2002)	Latvia	Bactec460TB	Isolate	34/19	100	100
Traore (2000)	Belgium	Proportion	Isolate	266/145	99	100
Watterson (1998)	England	Bactec460TB	Isolate	16/16	100	94
De Beenhouwer (1995)	Belgium	Proportion	Clinical specimen	21/46	91	100
Gamboa (1998)	Spain	Bactec460TB	Clinical specimen	46/13	98	100
Johansen (2003)	Denmark	Bactec460TB	Clinical specimen	26/21	100	100
Watterson (1998)	England	Bactec460TB	Clinical specimen	10/24	80	100

*Modified from references 56 and 57.

MDR-TB. Finally, detection of XDR-TB would be based on the study of *rpsL*, *rrs* and *tlyA*, for resistance to STR, AMK, KAN and CM, and of *gyrA* and *gyrB*, for fluoroquinolone resistance.

Several molecular methods have been proposed to detect the specific mutations correlating with resistance in the amplified products: DNA sequencing, PCR-single-strand conformation polymorphism, PCR-heteroduplex formation, RT-PCR or solid-phase hybridization assays.[20,54] As solid-phase hybridization assays and RT-PCR have been commercialized and are widely used in clinical laboratories, they will be reviewed in some detail.

Solid-phase hybridization assays

Line probe assays. Line probe assays are a family of novel DNA strip tests that use PCR and reverse hybridization methods. Results are determined by colorimetric development. They have been designed to identify *M. tuberculosis* complex and simultaneously detect genetic mutations related to drug resistance. Amplified DNA can be obtained from cultured strains or clinical samples. Commercially available kits include the INNO-LiPA Rif. TB kit (Innogenetics, Gent, Belgium), the GenoType MTBDR*plus* assay and the GenoType MTBDR*sl* assay (Hain Lifescience).[54] The INNO-LiPA Rif. TB kit hybridizes the amplified DNA to ten oligonucleotide probes (one specific for the *M. tuberculosis* complex, and nine encompassing the core region of the *rpoB* gene: five overlapping wild-type S probes and four R probes for detecting specific mutations) that are immobilized on a nitrocellulose strip.[55] A number of studies have evaluated the diagnostic accuracy of LiPA for detecting resistance in several settings (Table 2). A recent metaanalysis[56] suggests that the LiPA assay is highly sensitive and specific for detecting rifampin-resistant *M. tuberculosis* in culture

and, to a slightly lesser degree, in clinical specimens. The Genotype MTBDR*plus* assay detects mutations in the *rpoB* gene for rifampin resistance, in the *katG* gene (S315T) for high-level INH resistance and in the promoter region of the *inhA* gene (nucleotides -8, -15, and -16) for low-level INH resistance. Various studies on the kit's accuracy have been performed and summarized in a recent metaanalysis.[57] Sensitivities for the detection of rifampin resistance range from 91% to 100%, whereas in the case of INH they range from 73% to 94%. The main limitation for the detection of INH resistance is that the molecular mechanisms behind some INH-resistant *M. tuberculosis* isolates are not known. Differences in the observed sensitivity could be due to the distribution of resistance-associated mutations in the different studies.[58] The GenoType *Mycobacterium tuberculosis* second line (MTBDR*sl*) assay was developed with a specific focus on the most prevalent *gyrA*, *rrs* and *embB* mutations. Although few studies have been published to date, this new assay may represent a reliable tool for the detection of fluoroquinolone and amikacin/capreomycin resistance, and, to a lesser extent, ethambutol resistance. In combination with a molecular test for the detection of RMP and INH resistance, the potential to detect XDR-TB can also be postulated.[53]

LCD microarrays. A low cost and density microarray (LCD) to detect RMP and INH resistance has been developed by Chipron GmbH (Berlin, Germany). Owing to high costs, complex protocols and the need for substantial additional laboratory equipment, microarrays have yet to become part of routine molecular diagnostics. However, LCD arrays do not need special equipment and the working protocols are similar to those used with line probe assays. Moreover, the LCD array offers increased throughput (eight samples per chip). The LCD array has been tested with *M. tuberculosis* clinical isolates[59] and a good correlation with sequencing data was obtained for *katG* S315T and S315N, for -8, -15, -17 nt of the *mabA-inA* regulatory region, and for *rpoB* core region mutations. Additional studies based on clinical samples are now needed.

Real-time PCR (RT-PCR)

As mentioned above, the GeneXpert MTB/RIF system (Cepheid) has recently been introduced. In addition to the high sensitivity and specificity obtained for the detection of *M. tuberculosis*, the few studies performed to date have also observed a good response as regards resistance to rifampin.[33-36] Although these results are promising, they obviously require further validation.

It is widely accepted that the extent of any future MDR or XDR tuberculosis epidemic will largely depend on the transmission efficiency or relative fitness of drug-resistant *M. tuberculosis* compared to drug-susceptible strains. For infectious pathogens, fitness is a composite measure of an organism's ability to survive, reproduce and be transmitted. However, the fitness cost associated with drug-resistance, in terms of reduced virulence and transmissibility, remains largely unknown.[60] Although INH-resistant strains were, in general, less often transmitted between humans in recent years, several studies have shown that the *katG* S315T mutation is associated with INH resistance without diminishing the virulence or transmissibility of *M. tuberculosis* strains.[61] This lack of attenuation, its high frequency among INH-resistant clinical isolates and the association between *katG* S315T and the Haarlem strain family (which may partly explain the successful spread of Haarlem strains in South America) suggests that the majority of these isolates will be virulent. In other words, it can therefore be considered a 'no-cost' mutation. Similarly, several studies have shown that different mutations conferring resistance to RMP varied in their effects on bacterial fitness.[62] It is important to highlight that a strain's genetic background could also influence the fitness effects of particular mutations. In light of these data, it may become necessary to provide information not only about the molecular mechanisms of resistance, but also about the particular clone that harbours them.

Conflict of interest

The authors declare they have not any conflict of interest.

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