

Recent advances in the laboratory diagnosis of tuberculosis

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ABSTRACT

The laboratory plays a decisive role in diagnosing tuberculosis (TB) and the identification and drug sensitivity testing (DST) of *Mycobacterium tuberculosis*. For a long time the laboratories used only microscopy- and culture-based diagnosis, however, due to the slow growth of mycobacteria, these procedures may require 3-4 weeks or longer to yield results. It has been necessary to look for new and rapid diagnostic methods. In the beginning of the 90s, molecular-based diagnosis has become available providing rapid detection, identification and DST of *M. tuberculosis*. The present article will review some of the new methodology that has been introduced in the clinical laboratory. We discuss the LED microscope and PCR-based techniques for the diagnosis of TB, immunological assays for the diagnosis of active TB and latent infection, PCR-based methods and hybridization assays for the identification of mycobacteria and liquid culture methods and line probe assays for fast DST. Although these new techniques are useful for a rapid result, we emphasize that culture-based diagnosis is still the gold standard for the diagnosis and follow up on TB. The newer molecular methods cannot replace

the conventional diagnostic methods but provide preliminary information and improve patient management.

INTRODUCTION

Tuberculosis (TB) is one of the leading infectious diseases in the world and is responsible for more than 2 million deaths and 8 million new cases annually. The disease is caused by a bacterium called *Mycobacterium tuberculosis*. The bacteria usually attack the lungs, but can infect any part of the body such as the kidney, intestine, pleura, spine, and brain. If not treated properly, this infectious disease can be fatal.

The most important control strategy for TB is the early detection and the appropriate treatment of infectious cases. However, globally the case detection rate (CDR) of TB has been estimated in only 64%, which means that about 36% of the incident TB cases are not detected. This leaves a gap of approximately 3.3 million people worldwide with TB who were “missed”, either because they were not diagnosed or because they were diagnosed but not reported (1). For the Americas, the CDR is about 79%, which means that yearly about 33,000 TB patients on our continent are not detected or reported (1).

The laboratories play a central role in TB diagnosis and therefore the strengthening of the laboratory capacity and performance is a priority for TB control. In the majority of the laboratories TB is diagnosed only by smear microscopy. Smear microscopy has suboptimal sensitivity and detects only about 60-70% of the TB cases. In addition, about 25% of all TB cases are extra pulmonary TB and the diagnosis of this presentation of TB is often missed with smear microscopy. The implementation of culture for the diagnosis can improve the TB detection rate of a laboratory by about 30-40%. Culture detects cases with low mycobacterial loads and is needed in cases at risk of drug-resistant TB for drug resistance

testing (DST), or in cases where disease due to another member of the *Mycobacterium* genus is suspected. These two laboratory methods, smear microscopy and culture are still the “gold standards” for the diagnosis of TB and culture is considered as the most sensitive method. Yet, due to the slow growth of mycobacteria, results can take 3-4 weeks or longer and faster and more sensitive diagnostic tests are required to improve patient management.

Since the beginning of the 90s, new laboratory techniques for the diagnosis of TB and DS testing have been developed based on the use of liquid culture medium, nucleic acid amplification techniques (NAATs), DNA hybridization and mutation detection techniques, and antibody and antigen detection. This review is designed to offer some general information about new laboratory technique currently available for the diagnosis of active TB or the detection of latent TB infection. This review is not all-encompassing and will only deal with the most important technical developments. Method comparison is not within the scope of this article and interested readers are referred to the more specialized literature.

IMPROVEMENTS IN SMEAR MICROSCOPY FOR THE DIAGNOSIS OF TB

Microscopic examination of sputum specimens has been the basis of TB case detection for over 100 years and still, in resource-limited settings, the diagnosis of TB relies on Ziehl-Neelsen smear microscopy with the light microscope. Smear microscopy with the light microscope is a relatively insensitive methodology for the diagnosis of TB and only detects about 60-70% of the TB cases. An alternative for the light microscope is the fluorescence microscope, reported to be 10% more sensitive, since the fluorescent bacilli of *M. tuberculosis* can be seen at lower magnification and the smears can be examined

in only 25% of the time taken to read with the light microscope. However, it has been difficult to implement microscope in the diagnosis of TB due to the higher cost associated with purchase of the microscope with a mercury vapor lamp, the need for frequent replacement of the this UV lamp, which lasts only 200–300 h, and the need for a dark room for reading the slides (2).

Recent technical developments of a fluorescence microscope, which uses an illumination system based on a light-emitting diode (LED) with a long life-span of ten thousands of hours, resulted in the LED fluorescence microscopy. Based on LEDs relatively inexpensive fluorescent microscopes are now available. The World Health Organization (WHO) has assessed the efficacy of LED microscopy, and the results showed that diagnosis with this microscope was more sensitive (about 10%) than conventional smear microscopy (2, 3). Based on these findings, WHO recommends that conventional and the fluorescence microscopy will be replaced by LED microscopy.

ALTERNATIVE CULTURE-BASED METHODS FOR THE DIAGNOSIS OF TB

The gold standard test for the diagnosis of TB is the isolation of *M. tuberculosis* on a culture medium. Culture in addition provides isolates for identification (based on biochemical tests or molecular methods) and DST. Until the early nineties culturing was usually done on solid egg-based media like Lowenstein Jensen and Stonebrink medium. A drawback of culturing on these solid media is the slow growth of the bacterium; it can take at least 2 to 4 weeks or even longer before a culture becomes positive.

Liquid medium have an increased sensitivity for the growth of *M. tuberculosis* (up to 20% increase in positivity) and a reduced delay in the detection (10–14 days versus 2–4 weeks). A drawback is the contamination rate of liquid

medium, which seems to be higher in comparison with the solid media (4). WHO now recommends the use of traditional solid media along with liquid media in primary isolation of mycobacteria.

Concerning liquid medium, home-based liquid broth culture can be used for this purpose containing Middlebrook 7H9 broth supplemented with 10% OADC (oleic acid, albumin, dextrose, and catalase) and, to overcome contamination with other microorganisms, PANTA (an antibiotic mixture of polymyxin, amphotericin B, nalidixic acid, trimethoprim, and azlocillin). At present, also a number of elaborate culture systems are available commercially. They range from simple bottles and tubes such as MGIT (BD Diagnostic Systems, USA), Septi-Chek AFB (BD, USA) and MB Redox (Biotest Diagnostics, USA) to semiautomated system (BACTEC 460TB) and fully automated systems (BACTEC 9000 MB and BACTEC MGIT 960) (all BD, USA), ESP Culture System II (Trek Diagnostics, USA) and MB/BacT ALERT 3D System (BioMérieux, NC). For comparison studies of these (semi) automatic systems, the reader is referred to the specialized literature (5, 6, 7).

DNA BASED TOOLS FOR THE DIAGNOSIS OF TB

Classic Nucleic Acid Amplification tests (NAATs)

Since the early 1990, several methodologies have been published for the detection of *M. tuberculosis* with the polymerase chain reaction (PCR) assay, using oligonucleotide primers to amplify a DNA fragment specific for this microorganism. These NAATs can give results in 3–6 hours. Tests include commercial kits and those that are “in-house” and based on a protocol developed in a non-commercial laboratory. Each NAAT uses a different method to amplify specific nucleic-acid regions in the *Mycobacterium tuberculosis* complex.

Several commercial NAATs exist and the U.S. Food and Drug Administration (FDA) has approved the use of select commercial NAATs for respiratory specimens only. These kits include: the GenProbe Amplified *M. tuberculosis* Direct test (AMTD), the Roche Amplicor MTB test, the Cobas Amplicor test, the Abbott LCx test, and the BD-ProbeTec (SDA) test (8). None of these methods has been approved for direct detection of *M. tuberculosis* from extrapulmonary specimens. Although all of these current technologies are rapid and have demonstrated excellent specificity, their performance characteristics can vary and their sensitivity still does not equal that of culture-based methods, especially for smear-negative samples. A recent meta-analysis on the accuracy of commercial NAATs, which examined over 125 studies looking at smear-positive samples, showed a high degree of variability in accuracy across the studies (9). This analysis concludes that there is a need for improvement in diagnostic accuracy of NAATs, particularly sensitivity and commercial NAATs alone cannot be recommended to replace culture and microscopy for diagnosing pulmonary TB (8, 9).

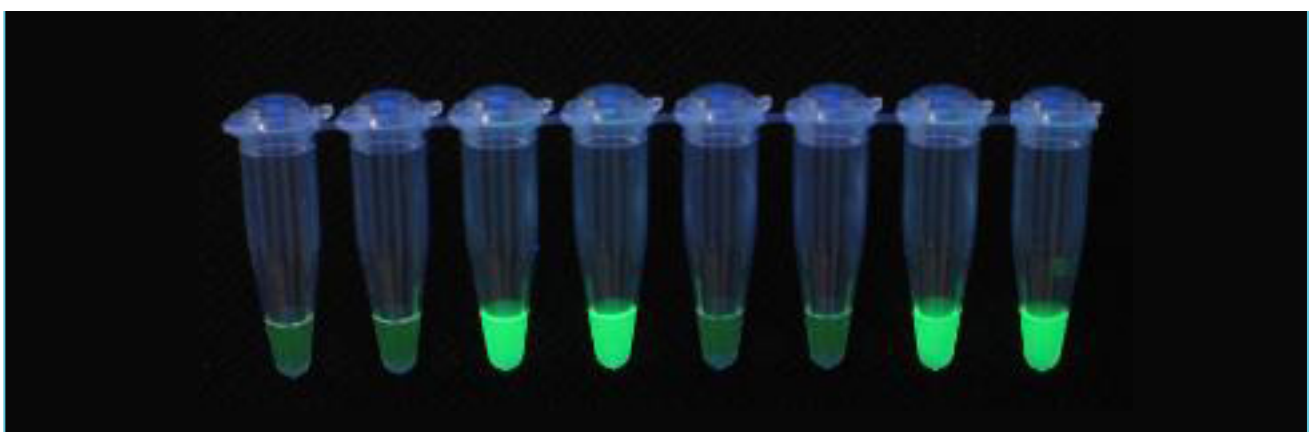
The Loop-mediated Isothermal Amplification test (LAMP)

Another commercial NAAT, which has been developed recently, is the Loop-mediated Isothermal Amplification (LAMP) test. Research experience is limited with this test. The method is based on the novel loop-mediated isothermal amplification (LAMP) platform from Eiken Chemical Co. in Japan. This technology amplifies target DNA under isothermal conditions (about 65°C) and is designed to visually detect DNA directly from clinical samples, in less than two hours and with minimal instrumentation. There is no need for a step to denature double stranded into a single stranded form. Amplification and detection of the DNA takes place in the same microfuge tube. See Figure 1.

The rather complex process of amplification is detailed at the Eiken website, which also includes an animation of the reaction (<http://loopamp.eiken.co.jp/e/lamp/index.html>).

A WHO Expert Group agreed that LAMP technology has potential as a rapid TB diagnostic tool but that the evidence available on the TB-LAMP

Figure 1 LAMP technology as a rapid TB diagnostic tool



Positive LAMP reactions are shown in the two tubes on the right. Amplification and detection of DNA takes place in the same tube. The two tubes on the left are negative reactions. The image was taken under UV illumination and fluorescence is due to addition of PicoGreen to all tubes, a DNA binding dye, which will form a dye-DNA complex with the amplified product, which makes it easy to distinguish between positive and negative reaction. Adapted from: http://www.finddiagnostics.org/programs/tb/find_activities/lamp_assay.html.

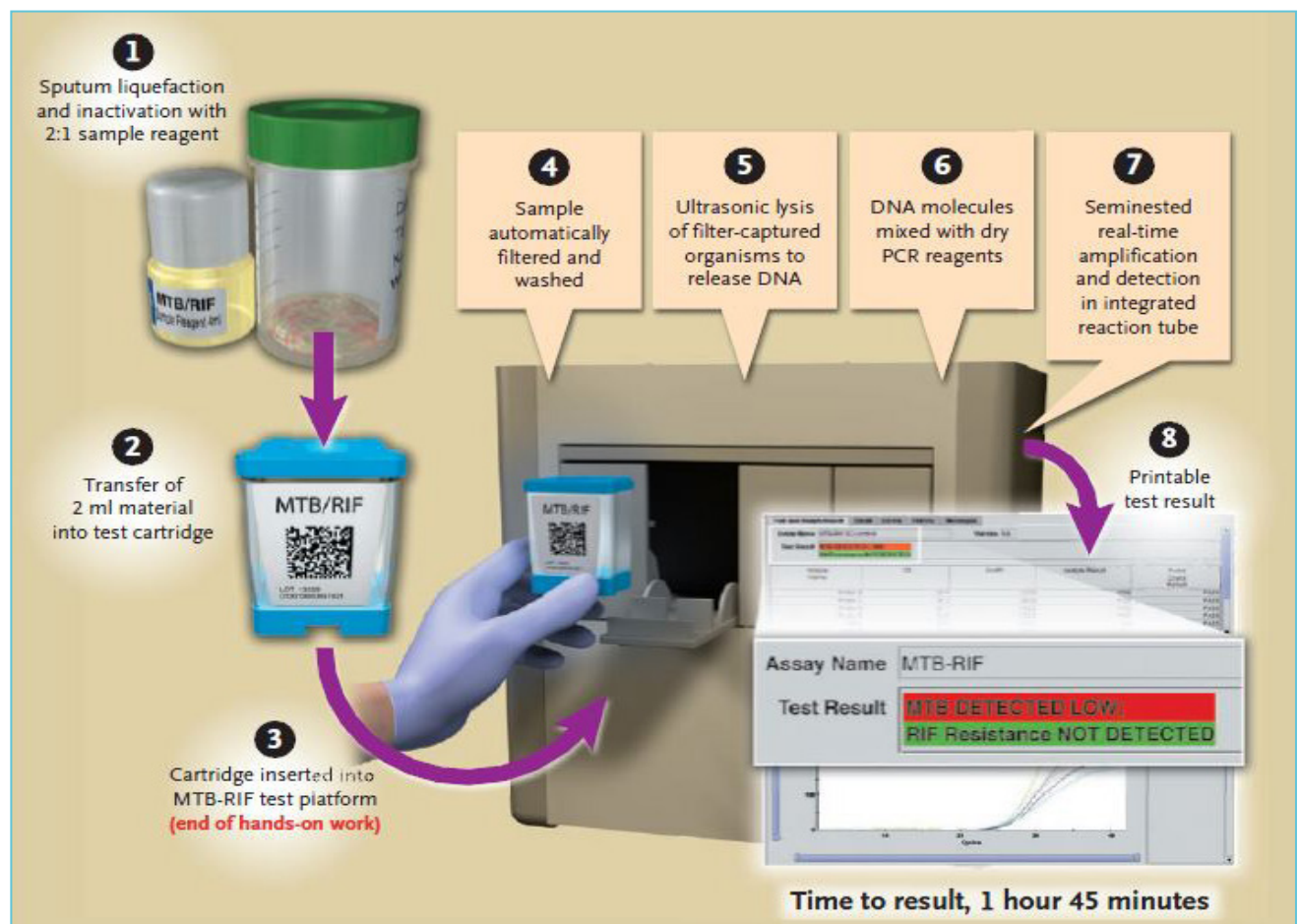
assay is insufficient to make a recommendation either in favour of, or against the use of TB-LAMP as a replacement test for sputum smear microscopy. Actually, at 14 sites, WHO is conducting independent studies to test the assay, assessing the feasibility and cost-effectiveness. Results of this study are expected in the year 2015 (10).

Xpert MTB/RIF nucleic acid amplification tests for diagnosis of drug resistant TB

The current NAAT methods, available for the detection of *M. tuberculosis* DNA, include sputum sample processing and DNA extraction as separated steps. Xpert MTB/RIF integrates sputum processing, the DNA extraction and the

amplification in a one-step sample preparation (see Figure 2). This automated cartridge-based assay detects, directly from sputum in under two hours, simultaneously *M. tuberculosis* complex and rifampicin resistance. The technology is based on the GeneXpert platform (11). The platform enables the detection of rifampicin resistance via the detection of mutations in the *rpoB* gene. The closed system ensures that there is no risk of contamination and no requirement for bio-safety facilities. A diagnostic test accuracy review including 27 unique studies concluded that, in comparison with smear microscopy, Xpert® MTB/RIF increased TB detection among culture-confirmed cases by 23%. For rifampicin resistance detection, the Xpert®

Figure 2 Assay procedure for the MTB/RIF test



From: http://www.finddiagnostics.org/programs/tb/find_activities/automated_naot.html

MTB/RIF pooled sensitivity was 95% and pooled specificity was 98% (12). WHO recommended the use of Xpert® MTB/RIF in December 2010 and is now promoting the global roll-out of the technology. In order to facilitate access to this technology, the public sector in eligible countries can purchase test cartridges at significant price reductions. As of 31 December 2014, a total of nearly 4000 GeneXpert instruments and over 10 million MTB/RIF cartridges had been procured in the public sector of 116 of the 145 countries eligible for concessional pricing (13).

SERODIAGNOSIS OF TB

The detection of antibodies against *M. tuberculosis* in serum, serodiagnosis, could offer low-cost, rapid results. However, recent meta-analyses and systematic reviews concluded that currently available commercial serological tests provided inconsistent results (14) due to cross reactivity and poor sensitivity. The WHO currently recommends against their use for the diagnosis of pulmonary and extrapulmonary TB (15). Further research is needed to develop immune response-based or serodiagnostic tests with appropriate performance.

DIAGNOSIS OF LATENT *M. TUBERCULOSIS* INFECTION

Persons with latent TB infection are infected with *M. tuberculosis*, but do not have TB disease. About 30% of the world population is infected with *M. tuberculosis* and until recently this only could be detected with the tuberculin skin test (TST), also called a Mantoux test. The test is done by injecting intradermally a small amount of a purified protein derivative of *M. tuberculosis* (PPD) into the skin on the inner forearm. If ever been exposed to *M. tuberculosis*, an induration will develop at the injection site within 2 days.

The test does not differentiate between latent infection and active disease and its limitations, including poor sensitivity and specificity, have been well publicized. False-positive TSTs can result from contact with nontuberculous mycobacteria or vaccination with Bacille Calmette-Guerin (BCG), because PPD, a crude protein preparation, contains antigens that are also present in BCG and certain nontuberculous mycobacteria (16, 17). However, skin testing still remains the most widely used method to identify TB infection.

Recognition that interferon gamma (IFN- γ) plays a critical role in regulating cell-mediated immune responses to *M. tuberculosis* infection led to the development of alternative tests for the detection of TB infection; the IFN- γ -release assays (IGRAs). IGRAs are in vitro blood tests of cell-mediated immune response; they measure T cell release of interferon (IFN)-gamma following stimulation of a blood sample from a patient by TB-specific antigens, ESAT-6 and CFP-10, unique to *M. tuberculosis*. There are two commercially available IGRAs; Quantiferon TB Gold tests, Cellestis, Victoria, Australia and T-SPOT. TB, Oxford Immunotec, Abington, UK. Several published studies have demonstrated a better performance of these tests over the TST in the diagnosis of a TB infection (18). Despite these studies, the lack of a reference standard test for LTBI makes it difficult to access the true accuracies of these assays. IGRAs cannot distinguish between latent infection and active tuberculosis (TB) disease and should not be used for diagnosis of active TB.

Mycobacterium species identification

The genus *Mycobacterium* comprises more than 150 species and several newly discovered pathogenic nontuberculous mycobacterial (NTM) species were described in the last 20 years. Although most TB cases worldwide are caused by *M. tuberculosis*, each of the closely

related members of the *M. tuberculosis* complex can cause tuberculosis in humans; for example *M. bovis* transmitted from cattle. The NTM species most frequently associated with pulmonary disease are *M. avium*, *M. kansasii* and *M. abscessus* and in some countries, pulmonary infection with NTM has become more important than TB (19). From an epidemiological point of view and because treatment regimens differ between the mycobacterial species, species identification has become an important additional task for the clinical laboratory and guides therapeutic decision-making.

Traditionally, mycobacteria were identified by phenotypic methods, based on culture, such as morphological characteristics, growth rates, preferred growth temperature, pigmentation and series of biochemical tests for instance niacin accumulation (*M. tuberculosis*), reduction of nitrates, Tween 80 hydrolysis, catalase, aryl-sulfatase and urease activity and iron uptake. Testing is laborious, time-consuming and not without biosecurity risks. In addition, misidentification may occur because different species may have indistinguishable morphological and biochemical profiles.

Identification based on DNA technology

In the last decade, molecular methods have been developed for the rapid and reliable identification of many mycobacterial species. A relative easy “in house” method is based on PCR and restriction enzyme analysis (PRA) of the gene coding for the heat shock protein *hsp65* (20). This gene is present in all the mycobacterial species and restriction enzyme patterns generated with two enzymes (HaeIII and BstEII) of a PCR product of 439 bp of the *hsp65* gene can be compared with patterns available in a database for species identification; the so-called PRA site, with the profiles of almost all mycobacterial species (<http://app.chuv.ch/prasite/index.html>).

Another “in house” method is the sequencing of the 16S ribosomal RNA gene, the reference standard to which all other new identification techniques are generally compared (23). The PCR for the amplification of the 16S rRNA gene can be done with “in house” methods or a commercially available kit: the MicroSeq 500 16S ribosomal DNA (rDNA) bacterial sequencing kit (Applied Biosystems, Foster City, Calif.). This kit is based on PCR and sequencing of the first 500 bp of the bacterial rRNA. Sequencing can be done easily and cheaply with commercial available sequencing service providers (approximately \$5 USD per sequencing). Identification requires a single sequencing reaction, which is efficient and cost-effective. For the final species identification, the obtained sequence can be compared in available public databases; the EzTaxon database (<http://www.ezbiocloud.net/>) or the sequence analysis tools (GenBank) offered by the National Center for Biotechnology Information (NCBI; <http://www.ncbi.nlm.nih.gov/>).

There are several other commercial systems available for the identification of mycobacteria. The first commercially available method was the AccuProbe (Gen-Probe Inc.), based on species-specific DNA probes with a chemiluminescent label that hybridize to the ribosomal RNA of the target organism. The test is easy to perform and makes possible rapid identifications. The results of the test are read with a luminometer. Separated tests for the identification of several important mycobacteria are available, including the *M. tuberculosis* complex, *M. avium*, *M. intracellulare*, the *M. avium* complex, *M. kansasii* and *M. goodii*.

More recently, other molecular commercial systems have been introduced for the rapid identification of *M. tuberculosis* complex and NTM: the INNO-LiPA MYCOBACTERIA v2 (Innogenetics NV, Ghent, Belgium), and the Geno-Type MTBC and GenoType Mycobacterium CM/AS tests (Hain Lifesciences, Nehren, Germany). These tests are

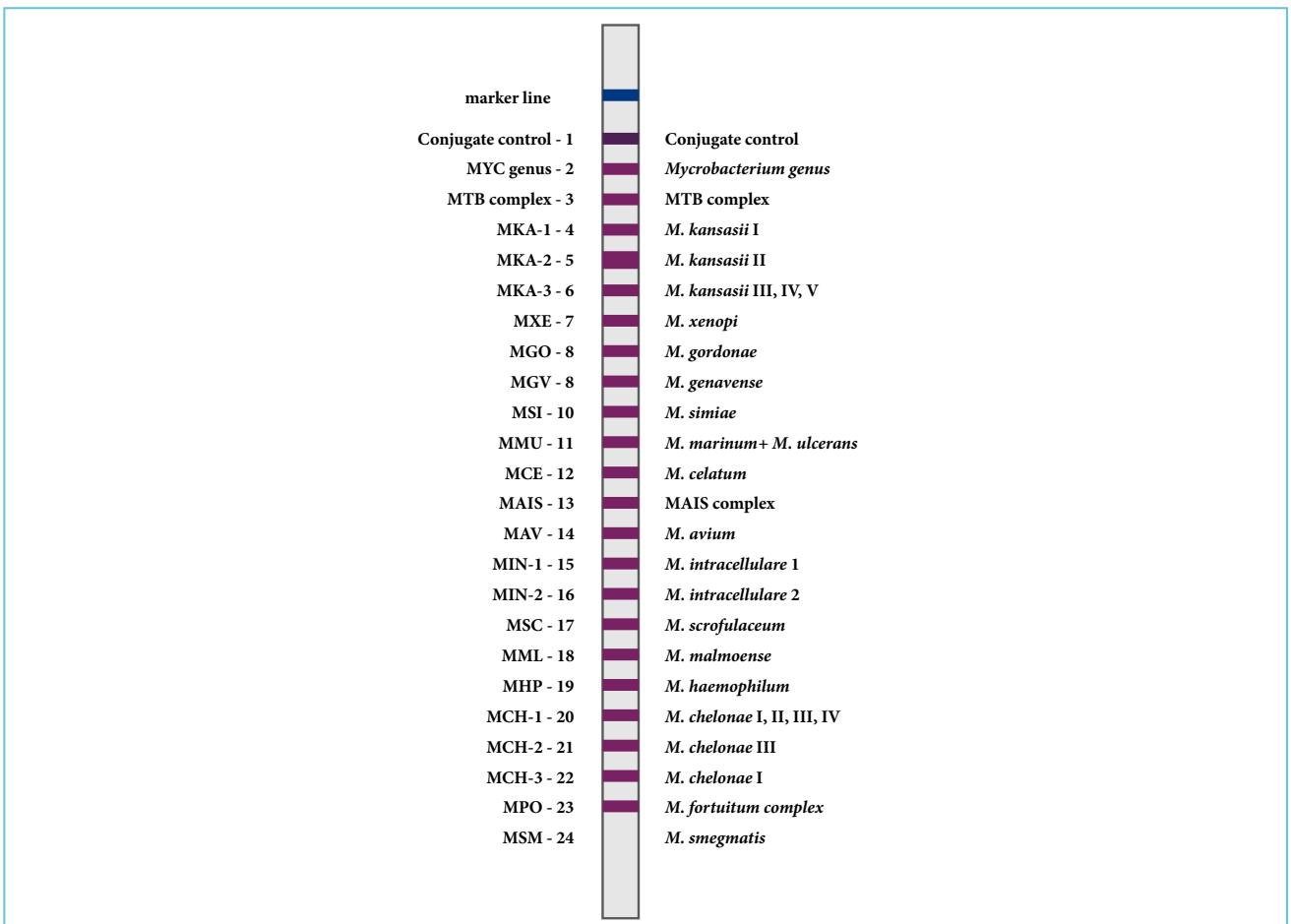
so called “reverse line probe” assays and detect the presence of certain DNA loci, representative for the species, in a hybridization assay with a PCR product of the isolated microorganism. (see Figure 3). INNO-LiPA MYCOBACTERIA v2 is a line probe assay that simultaneously detects and identifies the genus *Mycobacterium* including the *M. tuberculosis* complex and 16 different mycobacterial species.

The test is based on the nucleotide differences in the 16S-23S rRNA spacer region and can be

performed starting from either liquid or solid culture. The GenoType MTBC and GenoType *Mycobacterium* CM and AS assays are also reverse line probe hybridization assays. The GenoType MTBC is intended for the differentiation of members of the *M. tuberculosis* complex, including *M. bovis* BCG.

The GenoType *Mycobacterium* CM and AS assays are for the identification of 40 of the most common NTM species including *M. tuberculosis* complex. The CM tests permits the

Figure 3 Strip design of a reverse line probe assay: The INNO-LiPA® MYCOBACTERIA v2 test



On the strip are covalently attached, in parallel lines, 23 oligonucleotides probes, representing species-specific DNA regions of 16 different NTM species and *M. tuberculosis* complex strains. Some species like *M. kansasii* and *M. chelonae* have more than one probe on the strip because these species have more than one probe sequence type. A labeled PCR product of the tested mycobacterium is incubated with the strip and permitted to hybridize against homologous sequences causing a visible hybridization band on the strip.

simultaneous molecular genetic identification of the *M. tuberculosis* complex and 24 of the most common NTM species while the AS test permits the simultaneous molecular genetic identification of 19 additional NTM species. In general, molecular methods offer several advantages over conventional techniques for the rapid detection and identification of *M. tuberculosis* complex strains and other mycobacteria, such as a short turnaround time for the result (5-48 hours), reliability and reproducibility. The use of these molecular methods improves patient management and has been recommended by the WHO.

Identification based on immunochromatography

Three rapid immunochromatographic assays have been developed to differentiate between *M. tuberculosis* complex strains and MNT: the BD's MGIT TBc ID, the Taun's Capilia TB

(Japan) and the SD Bioline TB Ag MPT64 Rapid Test (Korea).

The three tests are lateral flow immunochromatographic assays (Figure 4). The BD and the SD Bioline assays detect MPT64 antigen, while Capilia detects MPB64 antigen; both *Mycobacterium tuberculosis* complex-specific secretory proteins. Both liquid mediums and solid mediums can be used as samples although the BD assay has been developed for the use with MGIT cultures. For solid mediums, an extraction buffer is required (24). An internal positive control is included to validate proper test performance. Reading time of the tests is 15 min and no special equipment required. These tests have been shown to be highly sensitive (>95%) and specific (> 95%) in a number of studies conducted in clinical settings (24).

Figure 4 An example of a positive lateral flow test for identification of a *M. tuberculosis* strain grown on solid Lowenstein Jensen medium



Adapted from: http://www.finddiagnostics.org/programs/tb/find_activities/rapid_speciation_test.html

DRUG SENSITIVITY TESTING (DST)

The emergence and spread of multidrug-resistant tuberculosis (MDR-TB) and extensively drug-resistant tuberculosis (XDR-TB) are a major medical and public health problem. MDR-TB is TB which is at least resistant to the two first-line anti-TB drugs rifampicin and isoniazid. XDR-TB is defined as TB that is resistant to any fluoroquinolone and at least one of the three injectable second-line drugs (capreomycin, kanamycin and amikacin) in addition to rifampicin and isoniazid. Earlier detection of drug resistance is important and reduces the time from TB diagnosis to the start of a proper TB treatment, improving patient outcomes and helping to control the transmission of resistant strains in the population. Conventional methods for drug susceptibility testing (DST) are slow. The most commonly used method, the standard proportion method, on Löwenstein-Jensen medium or Middlebrook agar, requires 4 – 8 weeks to produce results. This standard proportion is a so-called “indirect method”, requiring a sequential procedure: isolation of mycobacteria from the clinical specimen, identification of *M. tuberculosis* complex, and in vitro testing of strain susceptibility in the presence of anti-TB drugs. In the last 15 years, several other culture- and molecular-based methodology has been developed and some of these methods are “direct methods” using the specimens from the patient and evading the time necessary to isolate *M. tuberculosis* in pure culture from clinical specimens (25, 26)

Non-commercial “in house” culture-based DST methods

In house methods have been proposed for the rapid detection of drug-resistance, aimed at use in low-income settings. Among these methods are microscopic observation of drug susceptibility (MODS), thin layer agar (TLA), colorimetric redox indicator (CRI) methods and the nitrate

reductase assay (NRA) (27-30). These methods can report susceptibility results in 1-2 weeks after inoculation.

In both MODS and TLA testing, drug-free and drug-containing media (liquid medium for MODS, solid for TLA) are directly inoculated with specimens from patients. Thus, no growth is first isolated in pure culture from clinical specimens. Cultures are microscopically examined for early growth or micro-colonies. Growth in drug-free media indicates a positive culture and growth in both drug-free and drug-containing media indicates resistance.

Colorimetric redox indicator (CRI) methods are indirect methods and thus need a pure culture from clinical specimens. These methods are based on the reduction of a colored indicator added to liquid culture medium in a microtiter plate after *M. tuberculosis* has been preincubated for several days in vitro to different antibiotics and different drug concentrations. Resistance is detected by a change in color of the indicator, which is proportional to the number of viable mycobacteria in the medium. Among the different growth indicators used are the redox-indicators Alamar blue and Resazurin.

Nitrate reductase assay (NRA) is a solid culture technique based on the capacity of *M. tuberculosis* to reduce nitrate to nitrite, which is detected by adding a specific reagent (Griess reagent) to conventional Löwenstein-Jensen (LJ) medium into which 1 mg/ml of potassium nitrate (KNO₃) has been incorporated. The NRA test can be used as a direct or indirect test. The reduction of nitrate is detected by a colored reaction. Resistance testing is done by inoculating directly the patient samples or a pure culture of *M. tuberculosis* on media with and without antibiotics. Detection of the colored reaction on the drug-free medium indicates a positive culture and a colored reaction in both drug-free and drug-containing media indicates resistance.

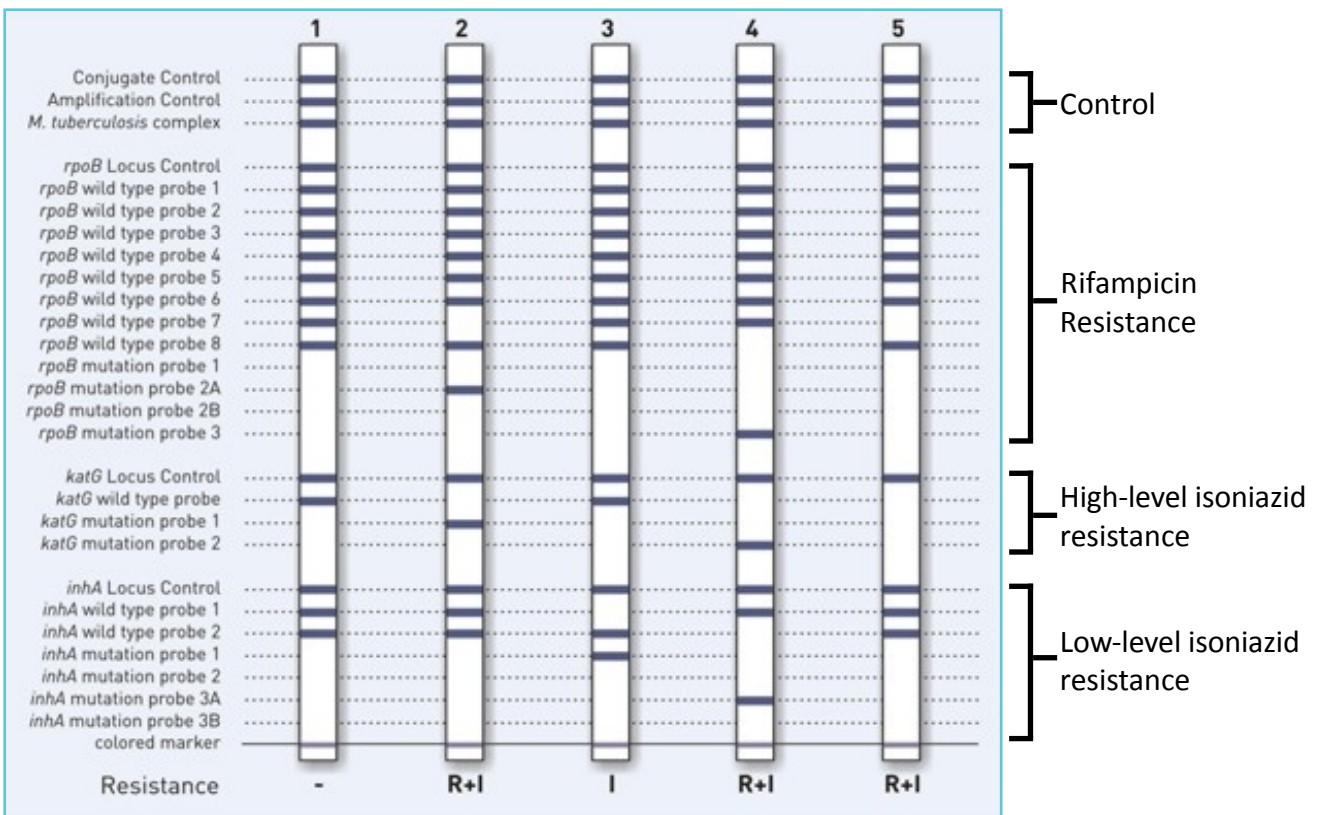
MODS, CRI methods and the NRA, but not TLA testing, have received WHO approval. These methods have similar accuracy to commercial liquid culture systems and can be implemented in high-burden, low-income settings with minimum costs. However, these tests require extensive operator training, standardization and quality assurance before implementation (25, 26).

Commercial liquid culture DST methods

The most commonly used commercially available automated liquid culture DST system is the BACTEC MGIT 960 system with the BACTEC MGIT 960 SIRE kit (Becton Dickinson, Franklin Lakes,

New Jersey, USA). This is an indirect method for the susceptibility testing of first-line antibiotics (isoniazid, rifampin, ethambutol, and pyrazinamide). The test is performed with *M. tuberculosis* complex positive culture inoculated in liquid medium with and without drugs and could report susceptibility results in 1-2 weeks after inoculation. The method has been demonstrated to be equivalent to the proportion method standard and has been FDA approved and endorsed by the WHO. Other automated liquid culture systems capable of DST include the Bact/ALERT MB (bioMerieux Inc., Durham, North Carolina, USA) system and the VersaTREK system (Trek

Figure 5 An example of a reverse line blot assay, the Genotype MTBDRplus strip



Shown here are the results for DST of five strains of the *M. tuberculosis* complex. (1) a susceptible strain; (2) a strain resistant to rifampicin and with high-level resistance to isoniazid; (3) a strain susceptible to rifampicin and low-level isoniazid resistance; (4) a strain resistant to rifampicin and a high- and low-level mutation for isoniazid; (5) a strain resistant to rifampicin and with high-level resistance to isoniazid. The strip contains 27 bands: 21 bands are to detect mutations in regions of genes associated with resistance (eleven bands detect wild type loci and ten bands detect antibiotic resistance loci). Six bands on the strip are control bands: control conjugate, amplification control, *M. tuberculosis* complex control and the amplification controls of the loci of the genes *rpoB*, *katG* and *inhA*.

Diagnostic Systems, West Lake, Ohio, USA). The use of these commercial systems for “direct susceptibility testing”, inoculating smear-positive patient specimens directly in culture medium with drugs would potentially reduce reporting of DST results with 1 to 3 weeks. However, direct testing of clinical specimens is problematic due to the risk of bacterial contamination, resulting in assay failure rates of about 15%. For this reason, most laboratories rely on the indirect method for susceptibility testing.

Rapid molecular DST methods

Much research effort has been put in describing the mutations present in the genes of *M. tuberculosis*, associated with resistance to the anti-TB drugs. This knowledge has enabled the development of rapid, DNA-based, so called molecular line probe assays, which allow for the simultaneous detection of *M. tuberculosis* complex and the detection of mutations associated with rifampicin resistance (alone or in combination with isoniazid). These assays are based on PCR and can be used directly with clinical specimens providing results within 24 to 48 hours; an enormous improvement on the 1 to 2 months needed for culture-based DST. Using a culture or a clinical sample, positive for *M. tuberculosis*, the region of a gene associated with resistance is amplified with PCR followed by a second assay to determine if the sequence contains a mutation associated with resistance. This is done with a hybridization assay. The labeled PCR products are hybridized to oligonucleotide probes immobilized on a nitrocellulose strip. Mutations are detected by lack of binding to wild-type probes or by binding to probes specific for commonly occurring mutations (see Figure 5).

Currently, two commercial line probe assays exist, the INNO-LiPA1 Rif.TB (Innogenetics, Ghent, Belgium) and GenoType MTBDRplus® (Hain LifeScience GmbH, Nehren, Germany). The LiPA

test detects simultaneously Mycobacterium tuberculosis complex and resistance to rifampicin. The GenoType MTBDRplus assay detects resistance to rifampicin and high- and low-level isoniazid resistance (Figure 5). In both tests, the identification of rifampicin resistance is enabled by the detection of the most significant mutations of the *rpoB* gene (coding for the β -subunit of the RNA polymerase). For testing the high-level isoniazid resistance, the *katG* gene (coding for the catalase peroxidase) is examined and for testing the low level isoniazid resistance, the promoter region of the *inhA* gene (coding for the NADH enoyl ACP reductase) is analyzed. Recently the GenoType MTBDRsl test has been released designed to test for resistance to second-line anti-TB drugs (fluoroquinolones, ethambutol, aminoglycosides and cyclic peptides). This test can be used in combination with the MTBDRplus test to identify XDR-TB

The WHO has issued a recommendation for the use of molecular LiPA for the rapid diagnosis of MDR-TB in high TB-burden, low-income settings. Evaluation and demonstration studies indicate that the Line Probe Assays are highly accurate in detecting MDR-TB in a variety of geographical settings, and cost-effective when compared with TB culture followed by DST (31-33).

CONCLUSIONS AND DISCUSSION

In the past decades, several molecular methods have been developed for direct detection, species identification, and rapid drug susceptibility testing of mycobacteria. These methods reduce the diagnostic time of TB from weeks to days. Some techniques are simple, but others are technically demanding and increase the costs of the diagnosis considerably. Several of these novel methods have been endorsed by the WHO and have shown their potential to significantly improve case detection and management of patients, including drug-resistant TB

cases (34, 35). However, for most resource-poor countries, with high rates of TB, where the technology will be most needed, the new technology is just too expensive and requires a complex technical infrastructure. According to reports of the WHO, eighty percent of all cases worldwide, occur in 22 high-burden, mainly resource-poor settings. In most of these countries, the TB diagnosis is done with smear microscopy only and in addition, laboratories are marginalized by their TB programs, understaffed and with untrained personnel and inadequate or poorly maintained equipment. Quality assurance programs for these laboratories including quality control and external quality assessments (EQAs) are often lacking (36). Priority for these countries is the improvement of the national laboratory system providing good-quality microscopy and the access to conventional culture and drug susceptibility testing (DST).

Another important point is that the new technology cannot replace the standard diagnostic methods; culture and conventional DST. Culture remains necessary for the diagnosis of TB in smear negative patients and conventional DST is required to confirm the molecular detection of resistance. Molecular detection of resistance depends on the detection of the resistance-conferring mutation. However, alternate mechanisms of resistance may develop or mutations may appear for which the test was not designed to detect. Thus, to provide reliable and fast results for TB diagnostics and patient care, a combination of tests that include smear staining using a fluorescence microscopy, liquid and solid medium culture methods, and a molecular assay for TB identification and drug resistance detection are necessary. Of course, the implementation of all of these tools in routine laboratory practice requires the implementation of appropriate quality assurance systems.

Some main problems in TB diagnosis has not been resolved with the introduction of the new

techniques. There is still the need to increase the sensitivity of TB detection among patients with extrapulmonary TB or paucibacillary disease, among immunocompromised (HIV) patients and among children. There is also a great need for a simple, low-cost, point-of-care assay for use in primary health clinics, which see the majority of TB patients but cannot provide laboratory-confirmed diagnosis of TB (37). To have an impact on the TB problem in these resource-limited settings, the ideal TB diagnostic would be a sensitive, specific, inexpensive real-time test. Progress toward a robust point-of-care test has been limited, but perhaps in a near future, novel biomarkers that can be measured by point-of-care tests, will enable the diagnosis of active TB with a simple real-time test. Several new point-of-care tests for TB including improved serologic assays, hand-held molecular devices, breath-based assays for the detection of volatile organic compounds in the diseased patient, microchip technologies and proteomics-based and metabolomics-based tests are in investigation (34, 37). As the technology advances, new point-of-care tests for TB and drug-resistant TB tests will become available on the market and, hopefully, prices for such tests will become affordable for low-income settings where the budget to health care is less from the ideal.

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