Future Trend in Laboratory Diagnosis of Tuberculosis

Angkana Chaiprasert, B.Sc., M.Sc., Dr.rer.nat.

Department of Microbiology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok 10700, Thailand.

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Tuberculosis (TB) is still a major public health challenge worldwide. One third of the world population is infected with *M. tuberculosis*. The World Health Organization (WHO) reported 9.2 million new cases of TB and 1.7 million patients died in 2006.[1] WHO recommends, for the stepwise success of TB, to control by an international achievement of 70% detection rate and 85% cure rate of TB patients. Early diagnosis, adequate therapy and prevention measures against transmission of TB are essential for TB control. Laboratory diagnosis of TB patients traditionally relies on direct smear microscopy of sputum and isolation of *M. tuberculosis* by culture. Active engagement of agencies such as the Stop TB Working Group on New Diagnostics, the Foundation of Innovative New Diagnostics and the TB Diagnostics Initiative of the Special Programme for Research and Training in Tropical Diseases (TDR) WHO, have led to renewed interest in the development of new tools for TB diagnosis.[2] In this special issue on TB, we describe some recent advances and emerging technologies in the diagnosis of TB both for latent TB and active TB, including serological tests, direct detection of *M. tuberculosis* complex in clinical samples, identification of mycobacterial species from cultures and detection of multi-drug resistant TB..

For the diagnosis of latent TB, a tuberculin skin test (TST) has been used by clinicians for decades. Recently, interferon-gamma release assays (IGRAs) such as T-SPOT.*TB* and the QuantiFERON-TB GOLD have emerged as attractive alternatives.[3,4] Diagnostic tests for latent tuberculosis are based on the region of difference (RD)1-specific antigens, early secretory antigen target (ESAT-6) and culture filtrate protein (CFP-10), which correlate better with intensity of exposure, and therefore are more likely than TST/purified protein derivative (PPD)-based assay to detect latent TB infection accurately.[4,5]

For diagnosis of active TB, many new tools have been introduced to the TB scientific community. Serological tests have been used for decades. Two meta-analyses have convincingly shown that existing commercial antibody-based tests have poor accuracy and limited clinical utility in the diagnosis of pulmonary TB. Attempts to search for new antigens with immunodiagnostic potential need to be intensified.[2,4,6] Lipoarabinomannan (LAM) is one of the most frequently used target antigens. Its monoclonal and polyclonal antibodies have been used to detect antigens in sputum and urine samples. LAM antigen has been detected in 74-93% of urine samples from TB patients and in 4-13% of those from healthy controls. Since urine samples are easy and non-invasive to obtain, the antigen detection by immunochromatographic assay in urine is promising for the rapid and simple diagnosis of TB especially in high burden TB countries with limited resources. However, urine antigen detection methods need to be assessed further before they will be implemented in TB control programs.[7]

Nucleic acid amplification tests (NAATs) are a major breakthrough in TB diagnosis. A series of meta-analyses have shown that NAATs have high specificity and positive predictive value, but variable sensitivity, especially in smear-negative and extra-pulmonary TB. New molecular methods have been introduced, including modified versions of the polymerase chain reaction (PCR) such as PCR-Restriction Fragment Length Polymorphism (PCR/RFLP), real-time PCR, Loop-mediated isothermal amplification (LAMP), DNA sequencing, and DNA strip assays, as mycobacterial diagnostic tools.[2,5,8-12] Manufacturers have developed NAATs for *Mycobacterium tuberculosis* complex that enable direct detection of the organism in sputum samples. Two commercially available NAATs approved by the U.S. Food and Drug Administration (FDA), the Amplified *Mycobacterium tuberculosis* Direct test (MTD test; Gen-Probe, San Diego, CA, USA) and the Cobas Amplicor *M. tuberculosis* assay (Roche Diagnostic, Mannheim, Germany), had excellent performance when used for testing smear-positive specimens (sensitivity>95%, specificity 100%). Cobas Amplicor *M. tuberculosis* assay is based on PCR amplification of the 584-bp segment of the 16SrRNA gene followed by hybridization of the biotin-labeled amplified products of a *M. tuberculosis* complex-specific oligonucleotide probe, coated onto the microtiter plates. The assay includes an internal control and has a

turnaround time of 6.5 hours. The MTD test is based on amplification of the released ribosomal RNA sequences (amplicons) from the *M. tuberculosis* complex and is detected by hybridization with an acridinium ester-labeled DNA probe. The test takes approximately 3.5 hours.[12] The FDA has recommended the use of NAATs only for smear positive respiratory specimens from patients who had not received antituberculous drugs for 7 or more days or within the last twelve months. Gen-Probe has enhanced the performance of the MTD test which was later approved by the FDA for testing respiratory specimens, regardless of the smear result.[2,10] In addition to the commercial NAATs, a number of in-house tests have been developed over the years. Each laboratory uses its own protocol for pretreatment, DNA extraction and detection of amplification products. The insertion element IS*6110* and the *16SrDNA* are the most common targets used. In general, the accuracy of in-house PCR tests has been more heterogeneous than commercial kits. The use of IS*6110* as an amplification target and the use of nested PCR were associated with higher accuracy.[2,10,12] Our own developed one-tube nested PCR based on *16SrDNA* revealed 93.2% sensitivity and 85.0% specificity for direct detection of *M. tuberculosis* complex DNA in sputum samples, (including smear-positive and smear-negative). These are comparable to the positive isolation of *M. tuberculosis* on Loewenstein Jensen egg-based medium.[13]

The *BDProbe Tec ET* (energy-transfer) *M. tuberculosis* Direct Detection Assay *(DTB)*, (*BDProbe Tec*: Becton Dickinson Bioscience, Sparks, Md.) is based on homogeneous Strand Displacement Amplification (SDA) and fluorescent energy transfer detection on an instrumental system. It targets isothermal co-amplification of sequences of a 95-bp region in IS*6110*, specific to *M. tuberculosis* complex and the *16SrRNA* gene, common to most mycobacteria. The process is based on the nicking of a modified recognition sequence in double-stranded DNA, by the restriction endonuclease *BsoB1*, and the extension and repair of that site by the DNA polymerase *Bst*, which synthesizes a new strand of DNA while displacing the existing strand. The displaced strand can then serve as a template for further amplification. All steps occur at 52.2°C. The assay is recommended by the manufacturer for use with respiratory specimens and is completed within 4 hours with comparable sensitivity and specificity with MTD and Cobas Amplicor.[12]

The *Genotype mycobacteria direct assay* can also be used for detection of *M. tuberculosis* complex and four non-tuberculous mycobacteria (NTM), (Hain Lifescience, Nehren, Germany). This is a novel assay, based on the nucleic acid sequence-based amplification (NASBA) applied to DNA strip technology. According to the manufacturer, the assay has three steps. The first step consists of isolation of 23S rRNA, the second step includes amplification of RNA by a NASBA method, and the third step involves the reverse hybridization of the amplified products on membrane strips using an automated system. The assay has the ability for rapid simultaneous detection of *M. avium, M. intracellulare, M. kansasii, M. malmoense* and *M. tuberculosis* complex with reliable sensitivity and specificity of 92% and 100% respectively.[12]

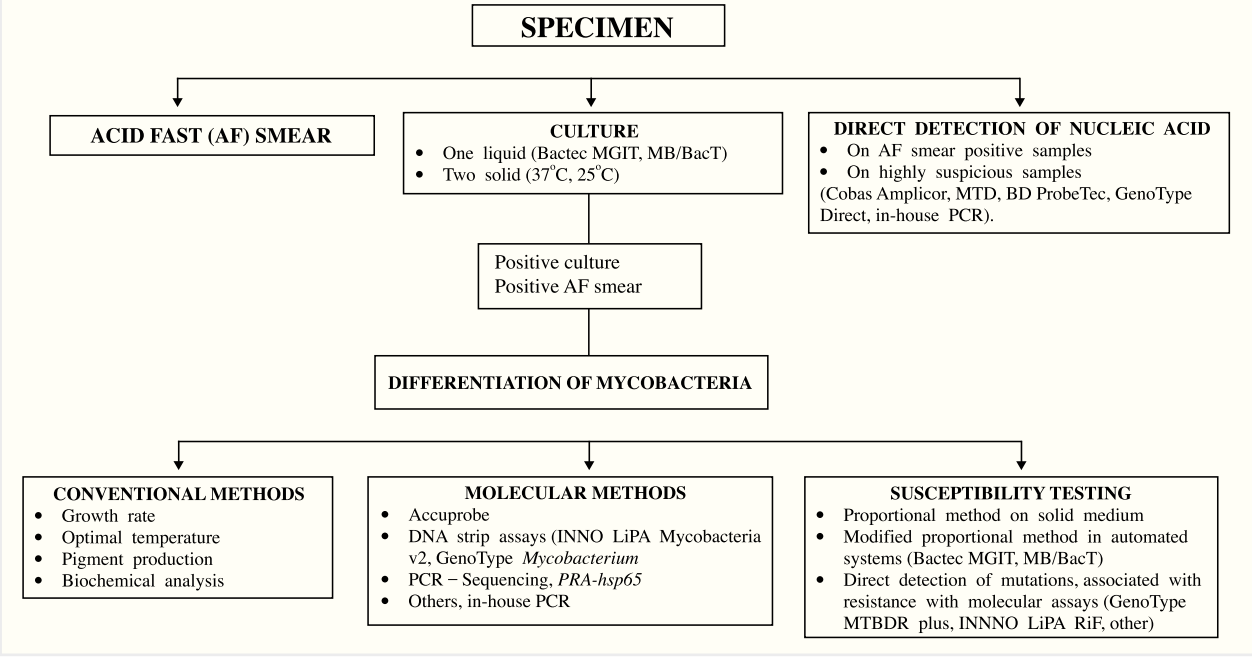
The identification of mycobacterial species has been conventionally performed on the basis of biochemical reactions and phenotypic characteristics for many decades. The methods are labor-intensive and time-consuming and often give ambiguous results. The molecular methods, such as PCR-based sequencing, DNA probe technology, hybridization in strips, and PCR/RFLP, now provide more rapid and accurate mycobacterial identification.[8,12,14] PCR-based sequencing has recently been considered the gold standard for identification of mycobacteria. Several target genes have been used for the procedure, but the most common is the *16SrRNA* gene. This gene is widely sequenced because it contains both highly conserved and variable regions. It consists of more than 1,500 bp but usually the first 500 bp are adequate for identification of a common *Mycobacterium* species. There are also other target genes, such as 65 kDa heat shock protein and the 32 kDa protein encoding genes, the *16S-23S rRNA* internal transcribed spacer (ITS) and the recA gene, used for identification purposes. The PCR/RFLP-*hsp65* or PRA-*hsp65* is an alternative cost effective, rapid and highly reliable method to serve as the routine technique for speciation of mycobacteria.[14-17] In identification of 434 NTM isolates, PRA-*hsp65* was significantly more accurate than by phenotype methods 90.3% vs. 77.9% respectively.[14] Genotype *Mycobacterium* (Hain Lifescience, Nehren, Germany) is based on a multiplex PCR and is identified by line probe hybridization technology. It consists of three kits: i) the GenoType MTBC for distinguishing members of the *M. tuberculosis* complex, and ii) the GenoType *Mycobacterium* CM (Common Mycobacteria) and iii) GenoType *Mycobacterium* AS (Additional Species) for NTM.[8,12] The GenoType MTBC is based on the *gyrB* gene polymorphism. The AS and CM assays use *23S rDNA* as their target, thus the amplicon generated in the CM assay can be used for the AS assay without the need to perform a second PCR. The combined use of CM and AS can distinguish almost 30 different NTM. The sensitivity and the specificity of the GenoType *Mycobacterium* compared with *16S rRNA* gene sequencing, were 97.9% and 92.4% for CM and 99.3% and 99.4% for AS, respectively.[12]

Over the last few years, increasing resistance rates of *M. tuberculosis* have been observed in many parts of the world. Problems with inadequate treatment and compliance are the usual causes of drug resistance development. The rapid determination of drug resistance in clinical isolates of *M. tuberculosis* is the prerequisite for the initiation of effective chemotherapy ensuring successful treatment of the patient and preventing further spread of drug resistant isolates.[9] Resistance to anti-tuberculous drugs is primarily due to mutations in a series of genes, (see Therdsak Prammananan in this issue). Molecular assays have the ability to detect these mutations and reveal the underlying resistance mechanism within hours. They are PCR-DNA sequencing, hybridization-based techniques such as line probe technology; *INNO-LiPA RifTB* (Innogenetics, Ghent, Belgium), and *GenoType MTBDR* plus (Hain Lifescience, Nehren, Germany), DNA chips, PCR-single strand conformation polymorphisms, real-time PCR and mycobacteriophage D29-based assay.[2,4,9-12] Line-probe assays are a family of novel DNA strip-based tests that use PCR and reverse hybridization methods for the rapid detection of mutations associated with drug resistance. Commercially available line-probe assays include

the INNO-LiPA RifTB kit and the GenoType MTBDR plus. INNO-LiPA *RifTB* contains 10 oligonucleotide probes: one specific for *M. tuberculosis* complex, five wide type probes (S1-S5), and four probes (R) for the detection of the most frequent mutations that cause resistance to rifampicin (RMP). More than 95% of the RMP-resistant strains have mutations within an 81-bp hot spot region (codon 507-533) of the *rpoB* gene. The R probes used are: R2: Asp516Val, R4: His526Tyr, R4b: His526Asp, R5: Ser531Leu. All the probes are immobilized on a nitrocellulose strip. A *M. tuberculosis* isolate is considered susceptible to RMP, if all the wide type probes give a positive signal and all the probes for resistance are negative. The absence of hybridization of one or more of the five S probes is indicative of a mutation that may be identified by one of the R probes.[12] GenoType MTBDR plus offers the simultaneous identification of *M. tuberculosis* complex and detection of the most common resistant mutations in *rpoB* (RMP resistance), *katG* and *inhA* gene (INH resistance). A recent meta-analysis demonstrated that line-probe assay has high sensitivity and specificity when culture isolates are used. The majority of studies had a sensitivity of 95% or greater, and nearly all were 100% specific.[9,11,12] However, the results are less accurate when the test is directly applied on sputum samples. Since the tests rely on the occurrence of only certain mutations related to INH resistance, there are some drawbacks when compared with the standard BACTEC MGIT960 system and DNA sequencing.[2,12]

In conclusion, there has been continual progress in the discovery and evaluation of new techniques in the diagnosis of tuberculosis in the last decade. Due to their rapidity and accuracy, molecular techniques are of great significance for detection, identification and susceptibility testing of mycobacteria. Although these techniques cannot yet fully replace conventional methods, they should be integrated in the workflow of a routine laboratory. The scale of such integration depends primarily on the available economic resources, on the burden of TB in the region and the intention as far as the level of diagnostic capability of each laboratory concerned. At an initial level, all microbiology laboratories worldwide should have the ability to perform and evaluate an acid-fast staining. At a second level, laboratories of general or regional hospitals should perform cultivation of the samples using solid or/and liquid media, along with susceptibility testing. At this level in developed countries, the use of NAATs is advocated for all smear-positive specimens and furthermore for smear-negative, when clinical suspicion of TB is high. A positive NAAT would indicate disease with a very good degree of accuracy, whereas a negative result is less helpful. A line-probe assay that can genetically detect the resistance of *M. tuberculosis* to RMP or/and INH should also be performed as soon as possible. At a third level of diagnosis, any reference laboratory should additionally include characterization of mycobacteria with sequencing and, furthermore, should perform molecular epidemiology techniques (Figure 1). In developing countries, new low-cost TB diagnostic assays are needed. In high burden, low-income countries, rapid alternative tests like the phage system may be useful, due to its simplicity and no sophisticated equipment is required. In TB-endemic countries, the molecular methods like in-house PCR and RFLP may improve the diagnostic and epidemiologic research. The design and evaluation of new rapid diagnostic tools for *M. tuberculosis* infection for use in low-income countries is a challenge for all researchers.[12]

Fig 1. Algorithm for laboratory diagnosis of tuberculosis (modified from ref.12)



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