Pathology (April 2015) 47(3), pp. 250–256

MOLECULAR DIAGNOSTICS IN MICROBIOLOGY

Molecular diagnostics for tuberculosis

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Summary

The phenotypic methods of smear microscopy, culture and indirect drug susceptibility testing (DST) remain the 'gold standard' diagnostics for tuberculosis (TB) in 2015. However, this review demonstrates that genotypic methods are in the ascendancy. Current-generation nucleic acid amplification tests (NAATs) are important supplementary tests for the rapid direct detection of (multidrug-resistant) TB in specific clinical settings. Genotypic detection is already the preferred method of detecting rifampicin and pyrazinamide resistance. Next-generation NAATs able to detect about 10 colony forming units/mL of sputum could replace culture as the initial test for detecting TB. Whole genome sequencing could also plausibly replace phenotypic DST but much work is required in method standardisation, database development and elucidation of all resistance gene determinants. The challenge then will be to rollout these increasingly complex and expensive diagnostics in the low-income countries where TB is prevalent.

Key words: DNA, diagnosis, molecular diagnostic techniques, sequence analysis, tuberculosis.

Received 24 November, revised 2 December 2014, accepted 7 January 2015

INTRODUCTION

An estimated 9.0 million new cases of active tuberculosis (TB) disease occurred worldwide in 2013.[1] Early detection and treatment is crucial in reducing the resulting 1.5 million annual deaths.[1] The incident cases in 2013 included an estimated 480,000 patients with multidrug-resistant (MDR) TB, of whom about 9.0% had extensively drug-resistant (XDR) TB.[1] MDRTB is defined as resistance to at least isoniazid and rifampicin, the two key drugs in 'short course chemotherapy' for TB. XDRTB is defined as MDRTB with resistance to a fluoroquinolone and one of the injectable second-line agents (amikacin, kanamycin or capreomycin), fluoroquinolones and an injectable agent being the key drugs in an MDRTB treatment regimen.

Molecular diagnosis of TB has advanced rapidly over the past two decades in an attempt to aid early detection of TB and MDRTB. Point of care tests (POCT) and assays with the ability to detect antibiotic resistance could have major impacts in low- and middle-income countries with high burdens of (MDR)TB. This review will focus on molecular assays that detect *Mycobacterium tuberculosis* directly in patient specimens, genotypic tests for drug resistance in *M. tuberculosis*, and the burgeoning applications of whole genome sequencing (WGS) in the TB field.

DIRECT DETECTION TESTS FOR *M. TUBERCULOSIS*

The introduction of liquid culture for *M. tuberculosis* has improved tremendously the turnaround time for the laboratory diagnosis from weeks to only 10–14 days. To further reduce this turnaround time, several molecular detection methods were introduced to detect *M. tuberculosis* directly in the clinical samples. Currently, there are two methods which are endorsed by the World Health Organization (WHO) to be used on patient samples: the Xpert MTB/RIF assay (Cepheid, USA) and line probe assays (LPAs). Another method, TB loop-mediated isothermal amplification (LAMP), is awaiting WHO approval. These methods plus several other polymerase chain reaction (PCR) assays are reviewed below. The sensitivities and specificities of each method are outlined in Table 1 and a summary of all methods is provided in Table 2.

XpertMTB/RIF assay for direct detection

The assay uses real-time (rt) PCR technology to detect TB and rifampicin resistance concurrently using unprocessed clinical specimens, regardless of their smear status. The target DNA sequence is the 81 bp core region of the bacterial RNA polymerase (*rpoB*) gene which encodes the active site of the enzyme.[2] More than 95% of all rifampicin-resistant strains contain mutations localised within this region. In addition, the *rpoB* core region is flanked by *M. tuberculosis*-specific DNA sequences. Therefore, it is possible to test for *M. tuberculosis* and rifampicin resistance simultaneously.[2]

The assay utilises molecular beacon technology to detect DNA sequences amplified by a hemi-nested rt-PCR assay.[2] Extraction, amplification and detection processes occur in an automated closed cartridge system. The assay may be used as a POCT since the system is self-contained, fully-integrated, automated, requires minimal expertise, and the result is available in 2 hours.

Previous nucleic acid amplification tests (NAATs) have usually been expensive, technically demanding, restricted to centralised laboratories, at risk of cross contamination, and not generally recommended in smear-negative clinical specimens.[3] Xpert is able to address many of these issues except for the high cost.

Line probe assays

In 2008, LPAs became the first molecular method endorsed by WHO for detection of *M. tuberculosis* and drug resistance from smear-positive patients at risk of MDRTB. Line probe assays may be used for the diagnosis of TB, speciation of non-tuberculous mycobacteria (NTM) and drug resistance

Print ISSN 0031-3025/Online ISSN 1465-3931 © 2015 Royal College of Pathologists of Australasia
DOI: 10.1097/PAT.0000000000000232

Table 1 Sensitivity and specificity of nucleic acid tests in clinical specimens

	Smear positive pulmonary		Smear negative pulmonary		Extrapulmonary	
	Sensitivity	Specificity	Sensitivity	Specificity	Sensitivity	Specificity
Xpert[2]	98–100	>98	57–83	>99	53–95	98–99.6
LPA[45]	93.4	85.6	NA	NA	NA	NA
In-house NAAT[6]	Sensitivity: 84–100 Specificity: 83–100					
Amplicor[6]	97	>95	40–93	>95	27–28	>95
AMTD[6]	92–100	>95	40–93	>95	93	>95
LAMP[9]	92.1	98.3	53.8	98.3	NA	NA

AMTD, amplified mycobacterium tuberculosis direct test; LAMP, loop-mediated isothermal amplification; LPA, line probe assay; NA, not applicable; NAAT, nucleic acid amplification test.

detection, and are based on the reverse hybridisation principle.[4,5] Specific oligonucleotides are immobilised at known locations on a membrane strip and are hybridised under strictly controlled conditions with the biotin-labelled PCR product. The hybrids formed are detected colorimetrically.

Commercially-available LPAs include the INNO-LiPA Mycobacteria (Inno-genetics, Belgium) and the GenoType MTBC (Hain Lifesciences, Germany) for mycobacterial species identification and differentiation within the *M. tuberculosis* complex, respectively.[6] The MDRTB*plus* assay (Hain Lifesciences) allows direct detection of *M. tuberculosis*, isoniazid and rifampicin resistance from smear-positive pulmonary specimens.

LPAs are designed for use in reference and intermediate-tier laboratories and can be manual or semi-automated. The manual version is quite laborious with an advertised turnaround time of 6 hours but is usually 1–2 days in most ‘real world’ settings. Like all open-system PCR assays, there is a risk of cross contamination. Separated laboratories for DNA extraction, amplification and analysis are required, as is exemplary technique.[6]

Other nucleic acid amplification-based techniques

In-house NAATs use different targets, either DNA or RNA, genus or species specific, followed by a detection step performed by various formats. One of the most commonly used targets for identification of *M. tuberculosis* is the insertion sequence IS*6110*.[6] There have been reports of false-negative results due to absence or very low numbers of IS*6110* in certain strains.[7,8] A meta-analysis and meta-regression of multiple published studies found the use of IS*6110* as the amplification target together with nested-PCR techniques were associated with a higher diagnostic accuracy.[6] Considerable expertise is needed to run most in-house NAATs and there is a risk of contamination, limiting their use to the reference laboratory only.

NAATs are also commercially available such as the COBAS Taqman MTB test (Roche Diagnostics, Switzerland) and the m2000 RealTime MTB assay (Abbott Molecular, USA), which cater for high-throughput testing in the reference laboratory. Both systems involve automated extraction, reaction preparation and manual transfer to their amplification platforms. The tests are meant for decontaminated and concentrated smear-positive respiratory samples.

Loop-mediated isothermal amplification technique

This assay is based on autocycling strand displacement DNA synthesis using the large fragment of DNA polymerase. The test targets the *gyrB* gene and the IS*6110* insertion sequence. The main characteristic of LAMP is the ability to synthesise large amounts of DNA.[6] The whole procedure is carried out in a single tube with the isothermal reaction held at 63°C for an incubation time of 1 hour with a visual detection. With the exception of a water bath or heating block, no other laboratory equipment is necessary,[6] therefore the platform is suitable for resource-poor settings.[5] The LoopAMP MTBC detection kit (Eiken Chemical Company, Japan) was launched in Japan and was assessed by a WHO expert group in 2013 but was not endorsed due to insufficient evidence.[8] Presently, a larger evaluation is in progress in 14 countries.

Indications for performing direct molecular detection tests for TB

WHO has strongly recommended the Xpert MTB/RIF assay as the initial diagnostic test in adults and children suspected of having MDRTB or HIV-associated TB.[10] The assay is also

Table 2 Summary of direct molecular detection tests

Test	Location	Throughput	Function	Complexity	Hardware cost	Cost/test
Line probe assay	Reference/intermediate lab	Moderate	MTBC/NTM diagnosis DST	Moderate	Moderate	Moderate
Modular NAAT (Xpert)	Reference/intermediate lab; point-of-care	Low/Moderate	Diagnosis and DST MTBC	Low	High	Moderate
In house NAAT	Reference/intermediate lab	High/Moderate	MTBC/NTM diagnosis	High	High	Moderate
Automated batched PCR	Reference lab	High/Moderate	MTBC diagnosis	High	High	Low
LAMP	Reference/intermediate lab	Moderate	MTBC diagnosis	Moderate	Moderate	Low

Adapted from UNITAID, World Health Organization.[3]
DST, drug susceptibility testing; LAMP, loop-mediated isothermal amplification; MTBC, *Mycobacterium tuberculosis* complex; NAAT, nucleic acid amplification test; NTM, non-tuberculous mycobacterium; PCR, polymerase chain reaction.