Laboratory Techniques for the Diagnosis of TB – what’s available?

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Outline of Topics to be Covered

Laboratory processing of sputum samples
Culture techniques
Molecular diagnostics
New developments
Current standards

• National SOP (BSOP 40, July 2006)
  • How to do the tests; reporting standards

• NICE guidance (*Clinical guideline 117*, March 2011)
  • How to manage the patient (including laboratory diagnosis)
  • Updates the CG 33 guidelines from 2006 to include new guidance on the use of IGRAs for the diagnosis of latent TB

• Laboratory guidelines for the diagnosis of *Mycobacterium tuberculosis* infection (DOH TB monitoring & laboratory services working group, August 2006)

• WHO laboratory strengthening taskforce (October 2005)
  • Outlines basic standards for diagnostic laboratories
Summary of laboratory processes for mycobacterial culture and sensitivity testing

MICROSCOPY (ZN or fluorescence)

POSITIVE

PCR for MTbc and rif resistance

NEGATIVE

CULTURE – liquid-based recommended in Standards

PCR on direct sample

NEGATIVE at 6 weeks

POSITIVE

In-house identification of MTbc eg MPB64 assay

(1) Identification of culture
(2) 1st-line susceptibility testing if MTbc using liquid-based or molecular methods

MDR

Not MDR, but resistant to 1 or >1 1st line drugs

Fully susceptible

Second line susceptibility testing

XDR

Not XDR, but resistant to some 2nd line drugs

Susceptible

Routine laboratory

Reference laboratory
To diagnose active pulmonary TB (NICE, 2006):

- PA CXR

- At least 3 sputum samples (including 1 early morning) -> lab for TB microscopy & culture

- Spontaneously produced sputum if possible; otherwise induced sputum or sample from BAL (NB Inf Control!)

- In children unable to expectorate sputum, induction of sputum should be considered (if can be done safely), with gastric washings considered as 3rd line

- Ensure specimens are sent in a sterile LEAKPROOF container in a sealed plastic bag!
Sputum microscopy

• Specimen(s) should be decontaminated and **concentrated by centrifugation**. A thin smear of the deposit is placed on a slide and heat-fixed, after which it is stained using auramine-phenol

• Auramine-phenol staining is more sensitive than Ziehl-Neelson, but less specific. Aur+ve specimens are therefore re-examined using the Z-N method

NB – an urgent (out-of-hours) smear may not be concentrated, and should always be formally repeated the next working day
The evidence…

(from Peterson et al, J Clin Micr 1999; 37(11): 3564-8)

US study compared cumulative proportion of +ve smears upon microscopy for direct vs concentrated sputum (all patients were subsequently \textit{M. tuberculosis} culture +ve); 3 or more specimens were received:

<table>
<thead>
<tr>
<th>Sample</th>
<th>Direct (%)</th>
<th>Concentrated (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>57</td>
<td>74</td>
</tr>
<tr>
<td>1,2</td>
<td>76</td>
<td>83</td>
</tr>
<tr>
<td>1,2,3</td>
<td>81</td>
<td>91</td>
</tr>
</tbody>
</table>
An auramine-stained smear
A Z-N positive smear
Limitations of Microscopy

• Cannot distinguish between dead and living bacilli
• High bacterial load (> 3000 – 5000 AFB/mL) is required for detection
• Cannot distinguish between species
• No indication of drug susceptibility
Decontamination of specimens from non-sterile sites

- Essential to kill unwanted normal flora present
- Mycobacteria able to withstand short periods during the process

1. 4% NaOH added to specimen and shaken for 15 mins
2. Centrifuge at 3,000rpm for 15 mins
3. Decant and add 15mls phosphate buffered saline
4. Centrifuge at 3,000rpm for 15 mins
5. Decant, culture and stain with auramine phenol
Why is decontamination necessary?

- In 18 hours, 1 Mtb bacillus will become 2 bacilli.

- In the same time, a single Staphylococcus will become 63,000 million…

» (M. Yates)
Culture methods

Löwenstein-Jenson media and Kirschner's broth:
For culture of tissues, swabs and bloody samples
  • Traditional culture method
  • 12 week incubation period, checked weekly for growth

At Imperial, these have largely been superseded by the liquid culture method
and L-J slopes are reserved for the storage of positive cultures
BACTEC 960 using the Mycobacterial Growth Index Tube (MGIT) system

- Used for the majority of specimens received in the TB lab
- A rapid liquid culture method (positive cultures usually within 10 – 12 days)
- Utilises fluorescence technology (O₂ reduction)
- Continuous monitoring (every 60 minutes)
- 6 week protocol
- Automated reading of cultures
- Capable of holding up to 960 patient samples
How does the MGIT work?

- A fluorescent oxygen sensor is embedded in the base of the tube
- Detects any decrease in $O_2$ dissolved in broth
- Oxygen sensor will emit light when exposed to UV
- Actively respiring organisms consume $O_2$
- Reduction in $O_2$ is detected by machine
- Machine flags tube as positive
Positive tubes flagged by machine
Remove tube, centrifuge for 15 mins
Stain using a Ziehl-Neelsen (ZN) stain
Note appearance i.e cording or non-cording

Typical cording morphology of M.tb
“clumping” morphology of other mycobacteria
Identification of culture in the routine laboratory

• BD MGIT™ TBc Identification Test
  A rapid “card” test that can differentiate M. Tb complex from other mycobacterial species based upon the detection of MPT64, a Mycobacterial protein secreted by MTbc cells during culture

• Genprobe
  • DNA hybridisation technique for the identification of M.tb complex or MAI from positive culture
  - Fast and accurate results
  - Results allow sensitivity testing to take place
  - Quicker results enable clinicians to treat appropriately
Sensitivities of Sputum Smear and Culture for Mycobacteria (ARRD 1966; 95:998)

• Smear +ve threshold: minimum 5,000 – 10,000 bacilli/mL

• Culture +ve threshold: minimum 10 bacilli per mL (liquid media) – 100 bacilli per mL (solid media)

• NB: negative sputum AFB smears never exclude pulmonary TB!
Culture sites for suspected extra-pulmonary tuberculosis (from NICE guidelines)

<table>
<thead>
<tr>
<th>Site</th>
<th>Imaging</th>
<th>Biopsy</th>
<th>Culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymph node</td>
<td>Node or aspirate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bone/joint</td>
<td>Plain X-ray and CT, MRI</td>
<td>Site of disease</td>
<td>Biopsy or para-spinal abscesses, Site or joint fluid</td>
</tr>
<tr>
<td>Gastrointestinal</td>
<td>Ultrasound, CT abdomen</td>
<td>Omentum, Bowel</td>
<td>Biopsy, Ascites</td>
</tr>
<tr>
<td>Genitourinary</td>
<td>Intravenous urography, Ultrasound</td>
<td>Site of disease</td>
<td>Early morning urine, Site of disease, Endometrial curettings</td>
</tr>
<tr>
<td>Disseminated</td>
<td>High resolution CT thorax, Ultrasound abdomen</td>
<td>Lung, Liver, Bone marrow</td>
<td>Bronchial wash, Liver, Bone marrow, Blood</td>
</tr>
<tr>
<td>Central nervous system</td>
<td>CT brain, MRI</td>
<td>Tuberculoma</td>
<td>Cerebrospinal fluid (CSF)</td>
</tr>
<tr>
<td>Skin</td>
<td>Site of disease</td>
<td>Site of disease</td>
<td></td>
</tr>
<tr>
<td>Pericardium</td>
<td>Echocardiogram, Pericardium</td>
<td>Pericardial fluid</td>
<td></td>
</tr>
<tr>
<td>Cold/liver abscess</td>
<td>Ultrasound</td>
<td>Site of disease</td>
<td></td>
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</tbody>
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Molecular Diagnostics for Mycobacterial Identification

From Mycobacterial culture

• Nucleic acid hybridisation (gen-probe)
• Line probe assays (eg “Hain strips”)
• PCR +/- sequencing

Directly from clinical specimens

• MTD (Gen-probe) or Amplicor (Roche) for MTb
• PCR
Identification of Mycobacteria from culture using nucleic acid hybridisation probes

This system uses a bioluminescent probe that binds to a complementary region of the mycobacterial 16SrRNA; useful for early identification of MTbc from positive culture in the diagnostic laboratory. Accuprobes® also available for the identification of M. avium complex, M. gordonae and M. kansasii
Example of a “DNA Strip” (reverse hybridisation analysis) for identification of a cultured Mycobacterial isolate

- Amplified DNA is applied to the DNA strip matrix where specific probes are located
- Hybridisation occurs
- Hybridisation reaction causes an enzymatic colour change (visible band formation)
Example of Mycobacterial identification from DNA strips (from HPA presentation by Tim Brown)
Possible future role in laboratory identification of positive cultures: MALDI-ToF

- Matrix Assisted Laser Desorption Ionisation Time of Flight MS
- Rapid analytical technique
- Applications in clinical monitoring, quality control in food processing, research and clinical Microbiology

MALDI-TOF Biotyper
TB...

- Application of MALDI-TOF
  - Rapid ID (<1 hour) to Genus or Species
    - TBC vs nTBC
  - Positive Impact? Patient management?
- Impact upon the bench?
- Database...
- Methodology (previously done via LJ slopes, what about MGIT tubes?)
Several methods available – all target and amplify specific regions of mycobacterial DNA -> rapid result. Usually available in Reference laboratories.

However, all such tests can result in false +ve or false -ve findings.

For example, the Amplicor is a DNA-based test that amplifies the 16S rRNA gene using genus-specific primers with detection in a colorimetric reaction.

Technologies are addressing the issue of low DNA volumes and real-time PCR methods have the advantage of speed (results may be available 1.5 – 2 hrs after DNA extraction) and lower risks of contamination.
The study, carried out in Peru, Azerbaijan, South Africa & India, using untreated sputum samples, demonstrated that among culture positive patients, a single MTB/RIF test identified 98% of patients with smear positive TB and 72.5% of patients with smear negative TB. The test showed a specificity of 99%. As compared with phenotypic DST, the MTB/RIF test correctly identified almost 98% of patients with rifampicin-R bacteria and 98% of those with rifampicin-S bacteria.

The assay was carried out using the MTB/RIF test platform of the GeneXpert, provided by Cepheid.
TB PCR using the GeneXpert system is used at Imperial for the diagnosis of TB using small tissue aspirates (e.g., from lymph nodes) or where there is a high suspicion of rifampicin resistance.
Percentage sensitivity and specificity of nucleic-acid amplification tests in different clinical samples (from Curr Opin Pulmonary Med 2006, 12: 172-178)

<table>
<thead>
<tr>
<th></th>
<th>Smear-positive pulmonary</th>
<th>Smear-negative pulmonary</th>
<th>Extrapulmonary</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sensitivity</td>
<td>Specificity</td>
<td>Sensitivity</td>
</tr>
<tr>
<td>Amplicor</td>
<td>97</td>
<td>&gt; 95</td>
<td>40–73</td>
</tr>
<tr>
<td>AMTD</td>
<td>92–100</td>
<td>&gt; 95</td>
<td>40–93</td>
</tr>
<tr>
<td>BD Probe Tec</td>
<td>90–100</td>
<td>92</td>
<td>33–100</td>
</tr>
<tr>
<td>Real-time PCR</td>
<td>78</td>
<td>100</td>
<td>78</td>
</tr>
</tbody>
</table>

NB – as 20-30% of pulmonary cases (and a higher proportion of non-pulmonary cases) are not culture proven, the actual performance of molecular tests is difficult to assess!
Genotypic Methods for Detection of Drug Resistance (cf DST, which is the traditional phenotypic methodology generally in use)

Each drug has a target protein. Changes or distortions of the active site due to gene mutations -> reduced activity.

Rifampicin resistance results from mutations in the \( \beta \)-subunit of RNA polymerase, usually the result of mutations in \( \alpha \alpha \) 512 – 534 in the \( rpoB \) gene.

The molecular basis of resistance to isoniazid is more complex: several genes have been defined, eg \( katG, inhA, kasA, ahpC \), etc.
Example of line probe assays for detection of drug resistance

Mutations in the rpoB gene and location of the probes

INNO-LiPA Rif.TB simultaneously detects the *Mycobacterium tuberculosis* complex and the presence of mutations in the rpoB gene associated with resistance to rifampicin (RMP), which is considered a marker for multidrug resistant strains.

The strip contains 5 probes for detection of sensitive genotypes (S1-S5) and 4 probes for detection of resistant genotypes (R2, R4a, R4b, R5). RMP resistance is indicated by the absence of one or more "sensitive" probes, possibly accompanied by the appearance of one or more "mutant" probes.
Where are we going?

• Expanding role for diagnostic tests that are already available
  • IGRAs
  • NAATs
  • MALDI-ToF MS

• Other developments over the next 5 years
  • Rapid colorimetric DST
  • Molecular markers to facilitate diagnosis of XDR-Tb
  • Point of care diagnostics
Tools in an early phase of development

Researchers continue to develop and evaluate diagnostic tools beyond those described in this document. There is a wide array of products in the pipeline with different expected dates of availability—and a new tool may be superceded quickly by a newer tool, where appropriate evidence exists to support it. It is important that programmes continue to monitor the progress of research and carefully consider the potential of future tools when planning to invest in existing tools. Below is a short summary of several promising new diagnostic tools that still require further development and/or evaluation, but evidence for some could be ready for review by WHO in the next few years.

Breathalyser screening test
A test for respiratory forms of the disease that could be used in the community to screen high risk groups. Patients cough into a disposable device which is placed in the instrument for detection of volatile organic compounds. The instrument is fully portable and runs off rechargeable AA batteries. The test is performed and readout obtained in under 10 minutes. Limited performance data is available and further evaluation studies are required. Developers: Rapid Biosensor Systems Ltd (www.rapidbiosensor.com). Level of health system: community or point-of-care.

First-generation loop-mediated isothermal amplification (LAMP) assay
Molecular amplification methods are proven technologies for the detection of TB but have not been widely used in remote settings because of the cost and complexity. LAMP is a simple DNA amplification method that does not require a thermostable or detection system and reportedly allows visual detection of amplification, possibly allowing it to be used at lower levels of the health system. Developers: Fiken Chemical Co Ltd (www.alexam.com) and FMB. Level of health system: Peribificial laboratory.

Lipoarabinomannan (LAM) detection in urine
At tuberculosis LAM has been shown to be excreted in the urine of TB patients. Urine is an easier specimen to obtain than sputum, and may be less variable in quantity and easier to handle. There are several versions of this assay in development, including ELISA and direct methods. Urinary antigen detection may be of particular value in diagnosing TB in HIV-infected patients. It may prove valuable for rapid and simple diagnosis of TB in particular in developing countries at peripheral levels. Developers: Inverness Medical Innovations, Inc. (www.invernessmedical.com). Level of health system: Peribificial laboratory.

Phage based tests
A method for detecting rifampicin resistance directly from sputum smear positive samples or indirectly from culture. The test has a manual format and results are read by eye. Results are reportedly available within 9 days and utilizes basic microbiological equipment and skills available in most laboratories. The performance of the FastPlaque Response has been evaluated in a TDR sponsored Phase III clinical trial in Peru. Phase IV demonstration evaluations are necessary to assess use of the diagnostic in the public sector of low income countries. Developers: FastPlaque Response Test by BTI Technologies, Ltd. (www.btite.com), academic laboratories. Level of health system: Reference laboratory.

Sodium hypochlorite (bleach) microscopy
The digestion of sputum with household bleach prior to sputum smear preparation and microscopy has been reported to be an effective, simple method to improve the yield of smear microscopy even in high HIV prevalence settings. Progress on the development of a bleach microscopy method has been complicated by the wide heterogeneity, and lack of standardization, in methods described. A standardized bleach method, the Mbilane Sodium Hypochlorite (MaSH) method, has recently been developed and evaluated in MOP-suggested studies in Malawi, Namibia. The addition of a standard sodium hypochlorite solution to smears followed by overnight sedimentation resulted in a 15% increase in TB cases detected. The MaSH Method is now being evaluated by TDR under operational conditions in large multi-country studies. Developers: TDR Level of health system: Peribificial laboratory.

Sputum filtration
In this method, sputum is liquefied and passed through a filter, which is then stained or cultured by standard techniques. Filtration considerably concentrates mycobacteria, increasing sensitivity. Another advantage of using concentrated sputum is the reduction in time spent on sputum examination. Some studies have been published on this method, but further validation studies are required. Developers: academic laboratories. Level of health system: Peribificial laboratory.

TB Patch Test
The TB patch test is a point-of-care diagnostic used to diagnose active TB. The Patch Davies matrix, a protein specific to the organisms that cause TB in those with active, infectious TB, is a localized immune response consisting of erythema and/or vesiculation appears 3-4 days after application to the skin. The patch reportedly avoids responding to BCG vaccination, to other mycobacteria or to previous TB infection that has been cured with drugs. Enrollment in a Peru clinical trial complete, data analysis ongoing. Additional trials in the Philippines began in 2007. Developers: Sequella (www.sequella.com). Level of health system: Health post.

Vital fluorescent staining of sputum smears
Unlike most fluorescent stains, fluorescent dye only stains living, culturable organisms. While a positive finding provides a basis for initiating antibiotic treatment, the sensitivity of the direct smear is highly variable. Note that use of this dye requires a fluorescent microscope. This technique might be most appropriate for use with patients not responding to therapy. Developers: Academic laboratories. Level of health system: Peribificial laboratory.

From: WHO/StopTB partnership retooling task force, 2008
Figure 1. The tuberculosis diagnostics pipeline
Technologies in boxes have been endorsed by WHO. DST = drug susceptibility test. MOOS = microscopic observation drug susceptibility. NNA = nitrate reductase assay. RIF = rifampicin (redox indicator assay). LPA = line-probe assay. NAAT = nucleic acid amplification test. LED = light-emitting diodes. PO C = point of care. LTBI = latent tuberculosis infection. *Manual NAAT: technology for Mycobacterium tuberculosis drug-susceptibility testing. †Manual NAAT: technology for M tuberculosis detection at the peripheral laboratory. ‡Manual NAAT: technology for M tuberculosis detection at the community health care level. Source: adapted from Stop TB Partnership, Global Plan to Stop TB, 2006-2015, and reinvigorated with permission from author and publisher.

From the Lancet 210;375:1920 – 37, Wallis, Pai, et al