Tuberculosis in the developing world: recent advances in diagnosis with special consideration of extensively drug-resistant tuberculosis (XDR-TB)

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Abstract

Purpose of Review—Globally tuberculosis is mainly diagnosed by sputum smear microscopy, which fails to detect half of all cases and fails to identify drug resistance. Inadequate global TB control through the DOTS strategy alone plus the growing threat of MDR and XDRTB has driven recent development of new commercial and non-commercial tests, which are most desperately needed in resource-limited, high-burden settings. This review outlines the range of options currently available, highlighting particularly those recent developments with greatest potential for addressing the growing MDR and XDR disaster as it affects those communities least technically and financially capable of controlling it.

Recent Findings—Simplification of molecular diagnostic techniques, rapid liquid culture and the use of colorimetric indicators have improved the sensitivity, speed and reliability of TB and MDRTB detection, while decreasing cost and bringing diagnosis closer towards (though still some way from) the point-of-care.

Summary—Global TB control in 2008 demands the use of new tools for more sensitive and rapid detection of active disease and of drug resistance. Improved technologies are available for reference laboratories but for settings where resources and technical capacity are limited there is little ready for field implementation. The pipeline is promising, but in the interim wider use of liquid culture and manual or colorimetric DST should be promoted.

Keywords

tuberculosis; diagnosis; drug resistance

Introduction

For over 100 years sputum smear microscopy has been the cornerstone of tuberculosis diagnosis. Recently the growing acknowledgement – particularly with the HIV pandemic and global spread of MDRTB - that this will be insufficient to control TB, has driven development of a new generation of tests, described here. How this toolbox is utilised will depend upon technical, financial and logistical factors as much as upon test performance.

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Though beyond the scope of this review, the reader should recognise that in resource-limited, high-burden settings sustainable human resources, sample transport, biosafety, information systems, and laboratory maintenance are as important as having a good test.

Diagnostic test requirements

There are three core diagnostic needs in TB control – detection of latent tuberculosis infection (not the subject of this article but recently reviewed extensively (1, 2)), detection of active tuberculosis, and identification of drug resistance. Sputum culture and (if positive) DST are the industrialised world standard of care, yet most TB patients worldwide are diagnosed by sputum smear microscopy (3). Minor incremental improvements in microscopy performance (4, 5) and new LED microscopes (6) will facilitate wider use of fluorescence microscopy (7), but microscopy remains limited by low sensitivity (on average 50% of culture-positive samples are smear-positive (8, 9)) particularly in HIV co-infection (10), and lack of DST capability.

Access to DST is increasingly important in this era of emerging MDR and XDRTB (table 1), (11) which threatens to push tuberculosis control into a 'post-antibiotic era' with no available effective therapies (12). Citing limited resources to justify failure to implement measures to identify patients with MDR and XDRTB is both iniquitous and a false economy. Patients failing therapy continue to transmit their difficult-to-treat infection to other patients and healthcare workers, propagating the spread of resistant strains (13-15).

Considerable energy has been directed in recent years towards development of improved TB and MDRTB diagnostics suitable for where resources are limited; market potential (16) has encouraged commercial interest and non-proprietary methodologies have also emerged. Technologies in development worthy of attention but beyond the remit of this article include VOC detection devices (17), microarrays (18), proteomic signatures (19) and a lateral flow test for speciation of *M tuberculosis* from cultures (20). Serological tests are widely marketed in many developing countries despite a very weak evidence base – none of the currently available tests is sufficiently accurate to be recommended for use (21).

The front end - sample preparation and biological hazard

TB diagnostic tests can be categorised as phenotypic, for which the substrate is usually a decontaminated sputum sample, or genotypic for which chromosomal DNA is required. Sample preparation is an important factor that can limit test implementation. Prior to culture and DST, sputum decontamination and concentration usually requires the use of a biosafe centrifuge and appropriate infection control measures (22), which may be financially and logistically challenging in resource-limited settings. Biosafety is not a concern for genotypic tests (MTB is heat-killed at the outset), rather the issue is DNA cross-contamination during concentration, DNA extraction and amplification, thus separate clean rooms dedicated purely to DNA extraction and amplification, and special attention to procedural care and detail, are needed.

Indirect DST entails manipulation of suspensions of highly concentrated MTB from primary cultures for secondary inoculation – this important biohazard requires BSL3 containment (23) beyond the reach of laboratories in most resource-limited settings. In contrast direct DST, though regarded as heresy by the conservative world of mycobacteriology, by obviating the need for strain manipulation amounts to no more than culture inoculation and can thus be performed in the far more attainable setting of a BSL2 laboratory. Elegant occupational exposure and TB infection risk data from Korea confirm the low risk of simple culture inoculation compared to performance of indirect DST (24).

Microscopy to predict drug resistance (vital staining)

First developed for *Mycobacterium leprae* in 1982 (25), vital staining with FDA and fluorescence microscopy can determine the viability of MTB. FDA is hydrolysed intracellularly and fluorescein which rapidly accumulates is detected under ultraviolet illumination. Serial sputum examination (26) can be used to follow the response of a patient to treatment; persistent MTB viability may predict treatment failure due to drug resistance (27). Though FDA staining is not specific to *M. tuberculosis* this straightforward technique may become increasingly useful as low-cost LED fluorescence microscopy becomes more widespread.

Conventional indirect DST by the proportions method, minimum inhibitory concentration (MIC) and resistance ratio method

In the 1960's a WHO-convened expert committee attempted to establish a standardised method for MTB DST (28). The Pasteur Institute proposed the proportions method and the UK MRC the minimum inhibitory concentration and resistance ratio methods.

The proportions method (using LJ media) despite its limitations (29) is widely considered the DST standard. MTB is inoculated in equal amounts on drug free and drug containing media and colonies are counted in both. If the ratio of drug containing to drug free colonies is greater than 0.01 (1%, in the case of isoniazid and rifampicin), the strain is regarded as resistant.

To determine the MIC, MTB is inoculated in equal amount on culture media prepared with drug dilutions. The MIC is the lowest drug concentration that achieves growth inhibition. The resistance ratio is an adaptation which compares the test MIC to that of a known susceptible wild type strain. Conceptually, this "normalization" of the MIC by a strain with known MIC, deals with inherent lot to lot media variability. If the test strain/control strain ratio is >8 the strain is resistant; <2 is sensitive; 2-8 is intermediate. All three methods are regarded as DST reference standards (28, 30).

E-Test indirect DST

The E-test is an MIC method in which a strip containing an exponential gradient of antimicrobial is placed on an agar surface onto which MTB has been inoculated. Use with *M. tuberculosis* was first described in 1994 (31). Subsequent evaluations have yielded mixed results (32-34). Modest performance, high cost and the need for BSL 3 laboratories limit utility (35).

TK colorimetric solid media for detection and direct DST

Early reported but unpublished promise has yet to be realised for Dio-TK colorimetric solid media. The only published study to mid-2008 confirmed rapidity of detection (15 days vs. 26 for LJ) but lower detection sensitivity than LJ (36); further development and refinement is understood to be underway.

Thin Layer Agar (TLA) for TB detection

Detection of MTB microcolonies on clear agar was first described in 1970 (37) and evaluated on thin layer 7H11 agar in 1993 (38). Sensitivity of TLA compares favourably with LJ (38-40) and time to detection is significantly shorter at 7-11 days (40). At USD\$3 per plate TLA is one of the cheapest diagnostic tests available, and use of a conventional microscope facilitates implementation in low-resourced settings. Drawbacks include the

training required to recognise colony formation and the lack (as of mid-2008) of performance data supporting TLA use for direct DST.

Automated liquid culture platforms

The principle of a radiometric liquid culture system for TB was first described in 1975 (41). Radioactive palmitic acid in 7H12 media is taken up by growing mycobacteria; $^{14}CO_2$ is released as a metabolic by-product and measured. Middlebrook demonstrated that the technique could be fully automated in 1977 and subsequently the Bactec460 automated liquid culture system became widely used and regarded as the first and second-line DST reference standard (42-45).

Non-radiometric platforms have superseded Bactec460 over the past decade, including Bactec MGIT960 (a.k.a. MGIT) (46); MB Redox (47); BacT/Alert3D (a.k.a. MB/BacT (48)) and the VersaTrek (a.k.a. ESP Culture II, (49)), both of which have similar performance characteristics to the MGIT 960 and can reliably determine first-line drug susceptibility (50, 51)); BacT/Alert3D has also been shown to reliable for second-line DST (52). Automated MGIT detects MTB growth using a non-radiometric ruthenium salt colorimetric method in which the unquenched salt fluoresces under ultraviolet light as oxygen in the tube is consumed by tuberculosis growth. MGIT is faster and more sensitive than solid media; performance is equivalent to other liquid culture techniques (46, 50, 53, 54), with reliable performance for first and second line indirect DST (55-60).

Data from MGIT implementation projects led the STAG TB board of WHO to recommend in 2007 wider adoption of liquid culture and DST (61) in global TB control. Technical and economic restraints will likely limit the developing world feasibility of automated MGIT implementation to reference laboratories.

Microscopic observation drug susceptibility assay (MODS)

MODS is a non-proprietary methodology developed in Peru which depends upon observation of characteristic cord formation of MTB in liquid media (62). In head-to-head evaluation with MBBacT and LJ culture comparative TB detection sensitivities using MODS, MBBacT and LJ were 98%, 89% and 84% respectively (63); median times to culture positivity were 7 days, 13 days and 26 days respectively; and to MDR detection 7, 22 and 68 days. DST agreement between MODS and reference testing was 100% for rifampin, 97% for isoniazid, 99% for rifampin and isoniazid, 95% for ethambutol and 92% for streptomycin. The cost of MODS is USD$2 per test (based on 2006 prices), one of the cheapest diagnostics available (www.modsperu.org).

Recent publications provide evidence that rapidity and high performance are maintained when MODS is used in other settings (Brazil/Honduras (64) and Ethiopia (65, 66)) and for other indications such as diagnosis of pleural TB (67) and TB meningitis (68). Sample preparation without a centrifuge could facilitate MODS usage at a more peripheral level of the laboratory network and this has been evaluated (69).

Principal limitations are the need for an inverted microscope ($600 USD in India) and training to recognise cord formation. Evaluation in regions where *M. bovis* and non-tuberculous mycobacteria are prevalent would more thoroughly test specificity. Cost-benefit analyses and investigation of MODS for second-line DST are underway.

Manual MGIT

The fluorescence of a MGIT tube can also be detected manually by inspecting it under an ultraviolet lamp, obviating the need for an expensive automated platform and opening the way to more widespread uptake. Performance is comparable to Bactec460 and MGIT960 providing reliable and sensitive DST (albeit indirect) for rifampicin and isoniazid (70). Shortcomings in streptomycin and ethambutol DST are the same as for the automated system (42, 55).

Colorimetric DST methods (used in conjunction with culture)

Colorimetric methods are a low-tech, low cost approach to MTB growth detection, recently reviewed comprehensively (71). These methods either make use of an oxidation-reduction indicator that changes colour in response to the metabolic products associated with MTB growth, or nitrate reduction which is revealed by an added indicator. When isolates are cultured in a range of concentrations of anti-TB drugs, an MIC may be determined by noting at what concentration colour change is inhibited.

The indicators Alamar Blue (known in microplate format as MABA) and resazurin change from blue (oxidised state) to pink in the presence of bacterial growth. Both deliver reliable first and second line DST by MICs (72-74). The tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) also performs well in microplate format for both first and second line DST (75), changing colour from yellow to violet .

The nitrate reductase assay (NRA) can be used for direct DST (76) (a.k.a. Griess assay) – KNO_3 incorporated into LJ media is reduced to nitrite by MTB growth, a process detected by addition of a colorimetric reagent after 28 days. Use is limited to smear-positive sputa, for which rifampicin and isoniazid resistance are detected with 100% and 93% sensitivity respectively (77, 78).

For indirect DST a recent systematic review established sensitivity for resistance detection of >94% for rifampicin and >92% for isoniazid; direct DST data are too limited for firm conclusions (79). Use of conventional LJ media is a significant advantage – laboratories already doing LJ should be able to move to the NRA relatively easily. No published data yet indicate performance of NRA for second-line DST.

Phage-based testing

The principle underlying phage assays is that mycobacteriophages (viruses that infect mycobacteria) added to sputum replicate within viable MTB and thus can only be propagated when a sample contains MTB. Detection of this propagation relies upon subsequent infection and lysis of indicator cells (*M smegmatis*) plated in a lawn on agar – lysis leaves plaques in this lawn, which by inference proves the sputum sample contained MTB (80). Incorporation of anti-TB drugs into the system permits direct DST (81-84).

Though an attractive methodology for many reasons (rapid, no specific equipment requirements, no amplification of MTB thus safe) sensitivity is little better than smear microscopy (85, 86), thus the strength would be in rapid MDR detection. Unfortunately, demonstration projects of a commercial phage-based diagnostic have been hampered by excessively high contamination rates so further refinement is needed.

Genotypic testing

TB PCR is less sensitive than culture for detection of TB from clinical samples, thus in general the role of molecular tests in TB diagnosis is limited to rapid identification of mutations associated with resistance to isoniazid and rifampicin. The validity of genotypic testing hinges on the observation that 90-95% of isolates phenotypically resistant to isoniazid or rifampicin demonstrate common resistance mutations (87) (88) (89). Theoretically it is possible for laboratories to detect such resistance in over 90% of isolates within 2 days (90).

The Loop Mediated Isothermal Amplification Assay (LAMP) is a novel nucleic acid amplification technique that does not require a thermal cycler, potentially enhancing operational feasibility (91). LAMP shows promise for TB detection though sensitivity still lags behind culture (92) (93). Adaptation to perform DST directly upon sputum samples would represent an important advance.

The Hain GenoType MTBDRplus (94) a solid phase hybridisation assay detects the common resistance mutations in the rpoB (rifampicin) and katG (isoniazid) genes (95). In a demonstration project in a South African Public Health Laboratory, with MGIT960 as the reference standard (96), sensitivity and specificity for MDR detection in smear-positive samples were 98.8% and 100% respectively. The INNO-LiPA RifTB line probe assay has a sensitivity of 70% when used directly on unselected clinical specimens and a specificity of 98% (97). However when used only with smear positive sputum samples, sensitivity increased to 92% and the technique and diagnosed rifampicin resistance (a good predictor of MDRTB (98)) with 90% sensitivity.

Mycolic acid high performance liquid chromatography (HPLC)

The use of HPLC to distinguish mycobacterial species by their mycolic acid profile is long established (99-101). Only with recent standardization has the impressive potential of HPLC in identifying drug resistance of MTB strains inoculated into BACTEC bottles been demonstrated (99% agreement with Bactec460 for all first line drugs (102, 103). Evaluation against MGIT960 and for use in second line DST is underway.

Conclusion

Before a test can be recommended for use in resource-poor, high-burden settings it must be proven to be rapid, simple, reliable, cost effective and easy to establish and maintain without compromising bio-safety. Ideally diagnostic tests should be performed as close to the point of care as possible, thus minimizing delays in transport of samples and results to and from reference laboratories.

In industrialised countries all culture isolates undergo first-line drug DST at the regional reference laboratory. In the UK, rapid molecular testing for MDRTB is performed on sputa from all smear positive patients with MDR risk factors (104, 105). In contrast, fewer than half of the 22 highest-TB-burden countries have >3 DST-capable laboratories (16). Streamlining DST requirements could facilitate wider uptake and access. Given the redundancy of ethambutol and streptomycin DST for non-MDR patients, a more rational and resource-conserving approach would entail initial DST focussed on MDR detection, followed by a secondary panel if MDR is identified, which includes streptomycin, ethambutol, pyrazinamide as well as second line agents.

Few diagnostic tests have had their performance evaluated with second-line drugs (table 2). Thus XDRTB will continue to be diagnosed at the level of the national/regional reference

laboratory rather than the district hospital. Whilst it appears impractical to apply in resource-poor settings the model used in industrialised countries, it is possible to strategically and quickly diagnose multidrug resistant tuberculosis and streamline samples from these patients for rapid second-line drug susceptibility testing. Formal evaluation of the cost effectiveness of new and existing tests in resource poor settings will enable health programmes to make informed decisions about implementation and long term feasibility.

Acknowledgments

Abbreviations List

BSL3	Biosafety Level 3
DNA	Deoxyribonucleic Acid
DST	Drug Susceptibility Test
FDA	Fluorescein Diacetate
HIV	Human Immunodeficiency Virus
HPLC	High Performance Liquid Chromatography
LED	Light Emitting Diode
LAMP	Loop Mediated Isothermal Amplification Assay
LJ	Löwenstein Jensen
MABA	Microplate Alamar Blue Assay
MDR	Multidrug-resistant
MGIT	Mycobacteria Growth Indicator Tube
MIC	Minimum Inhibitory Concentration
MODS	Microscopic Observation Drug Susceptibility
MRC	Medical Research Council
MTB	*Mycobacterium tuberculosis*
NRA	Nitrate Reductase Assay
PCR	Polymerase Chain Reaction
STAG TB	Strategic and Technical Advisory Group on Tuberculosis
TB	Tuberculosis
TLA	Thin Layer Agar
UK	United Kingdom
USD	United States Dollar
VOC	Volatile Organic Compound
WHO	World Health Organisation
XDR	Extensively Drug Resistant

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Table 1

Definitions

Drug panel	Anti-Tuberculous Drugs	Resistance definition	
		MDR	XDR
1st Line Drugs	Ethambutol Pyrazinamide Streptomycin Rifampicin Isoniazid		
2nd line drugs (conventional)	Kanamycin Amikacin Capreomycin Any Fluoroquinolone (ofloxacin/ciprofloxacin/moxifloxacin etc.) Cycloserine Ethionamide Prothionamide PAS		

Table 2

Test[a]	Detection	Direct DST[b]		Indirect DST[b]	
		1st Line Drugs	2nd Line Drugs	1st Line Drugs	2nd Line Drugs
Bactec MGIT960	×	×			
MODS			×	×	×
Vital Stains		/×	×	×	×
Thin Layer Agar		/×	×	×	×
Proportions		×	×	×	
Mycolic Acid HPLC		/×	×	×	×
Hain MTBDR Assay			×	×	×

[a] Many of the diagnostic tests listed are undergoing evaluation for diagnosis of second-line DST

[b] Compared to Indirect DST, Direct DST takes less time to diagnose drug susceptibility and minimises handling of concentrated clinical samples.