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Heemskerk D, Caws M, Marais B, et al. Tuberculosis in Adults and Children. London: Springer; 2015.

Chapter 4 Diagnosis

At the turn of the century, it was widely recognized that an accurate point-of care test for TB was required to make significant reductions in the pandemic. At this time, many novel tests had been developed by research groups or small biotech companies, but had never been standardized or evaluated for scale-up and application in low-resource, high-burden settings where the need is greatest. This motivated a major drive to systematically evaluate existing tests such as commercial liquid culture and nucleic acid amplification tests (NAAT), and to develop new approaches, principally led by the Foundation for Innovative New Diagnostics (FIND www.finddiagnostics.org) in collaboration with industry, government and clinical partners. The evidence generated by this renewed focus on novel TB diagnostic tests, processes and algorithms has led to a substantial number of policy revisions and new WHO recommendations (Table 4.1, see also www.tbevidence.org).

4.1. Smear Microscopy

The confirmation of TB disease still rests upon identification or isolation of *M. tuberculosis* bacilli from a clinical sample. This can be achieved by smear microscopy for acid-fast bacilli (AFB), mycobacterial culture or nucleic acid amplification (NAAT) tests. The appropriate sample will depend upon the suspected site of disease. The quality of the sample may greatly affect the chances of a positive result therefore care should be taken to instruct the patient in producing a sputum sample. Children are often unable to produce sputum and in young children gastric aspirate is usually necessary.

Diagnosis for the majority of patients worldwide suspected of TB is still made by sputum smear microscopy for acid-fast bacilli. The test, which was developed 100 years ago by Franz Ziehl and Frederick Neelsen, is inexpensive, simple, rapid and specific but is only positive in around half of patients with active TB. The Ziehl-Neelsen smear exploits the acid-fast property of mycobacteria by staining bacilli with carbol-fuschin, using gentle heat to facilitate penetration of the dye, and then using a decolorising acid solution, which fails to penetrate the mycobacteria, leaving them stained red while other bacilli are decolorised. The slide is usually then counterstained with methylene blue to improve visualization of the mycobacteria (World Health Organisation 1998). The Kinyoun stain is an alternative cold-stain method. The sensitivity of the test is substantially lower in children and patients with HIV. In addition the test is not specific for *M. tuberculosis,* but detects all acid-fast bacilli including NTMs. Sensitivity may be increased by concentration of samples prior to microscopy, usually by centrifugation or filtration (Van Deun et al. 2000) but direct (unconcentrated) ZN stain is the most widely applied methodology due to resource limitations.

Traditional TB control focused on the identification and treatment of sputum smear-positive TB patients, considered to be most infectious cases, in the mistaken belief that systematic identification and treatment of smear-positive cases would be sufficient to reach eventual TB elimination. Recent efforts to improve the sensitivity of basic smear microscopy have developed improved fluorescent microscopes to decrease the reading time and increase the sensitivity of smear microscopy without significantly affecting specificity if training and quality control are maintained. WHO now recommends the replacement of conventional microscopy with fluorescent microscopy wherever possible, using rugged and energy efficient LED fluorescent microscopes that can be battery operated. In a further policy change, WHO recommended in 2010 that two sputum samples are sufficient, rather than the standard three samples (spot-morning-spot) which had been recommended for several decades (World Health Organisation 2010). This is due to the low diagnostic yield of a third sputum sample and the resource limitations of TB programmes. If clinical suspicion is high repeated testing may still be warranted. A single positive smear is now also considered sufficient for a TB diagnosis (Bonnet et al. 2007; Mase et al. 2007).

4.2. Mycobacterial Culture

Culture of *M. tuberculosis* is a more sensitive technique for diagnosis but due to the slow growth of the organism (replication time of 24–30 h) sputum cultures take 4–6 weeks to become positive on solid media and 10–21 days in liquid media. Solid culture is usually performed on Lowenstein Jensen (LJ), Ogawa or Middlebrook 7H10/11 agar media. Liquid culture of *M. tuberculosis* is more sensitive and rapid than solid culture but can be prone to contamination in some laboratories. Early commercial automated liquid culture systems for mycobacteria used radiometric assay but have now been replaced with fluorescence based quenching systems which has improved safety.

The most widely used system is the Bactec Mycobacterial Growth Indicator Tube (MGIT) (Becton Dickinson, Sparks, Massachusetts) system which can also be used for susceptibility testing to first line drugs using a commercially available kit. A culture is necessary to confirm drug susceptibility, particularly for second-line drugs in cases of multi-drug resistance (MDR TB). *M. tuberculosis* culture and phenotypic DST requires significant training, infrastructure, strict infection control and on-going quality assurance, which is only available in regional reference laboratories in most countries

4.3. Nucleic Acid Amplification Tests

Various commercial and in-house nucleic acid amplification tests (NAAT) have been available since the 1990s. Detection of *M. tuberculosis* in clinical samples is generally less sensitive than NAAT for other pathogens due to the relatively low numbers of bacilli present and the difficulty of efficiently extracting DNA from the tough mycobacteria. The development of Line Probe assays (LPA) allowed the simultaneous detection of *M. tuberculosis* and determination of resistance to rifampicin and later isoniazid. However these tests are only endorsed for use on smear positive sputum and therefore do not aid greatly in the diagnosis of TB itself. The MTBDR-Plus assay (HainLifesciences, Nehren, Germany) has recently been adapted to enhance detection for use on smear negative sputum samples but large-scale evaluation data is not yet available. The use of LPA for detection of drug resistance is discussed in more detail in the section on diagnosis of drug resistance below.

The most significant advance in the diagnosis of TB in the last decade has been the advent of the GeneXpert MTB/RIF test (Cepheid, California, USA). This test system was originally developed for testing for the presence of anthrax spores in the United States bioterrorism-scares. A specific cartridge was later developed to detect *M. tuberculosis* and simultaneously determine resistance to rifampicin. In 2010 results of a multi-country demonstration study sponsored by FIND demonstrated that the Xpert MTB/RIF test detected TB and rifampicin resistance with high sensitivity and specificity compared to liquid culture; confirmed by a Cochrane review in 2013. The test was officially endorsed by WHO, followed by an unprecedented rapid scale up of the new technology [http://who.int/tb/laboratory/mtbrifrollout/en/]. A key factor in wide-scale implementation was a negotiated price reduction facilitated by a large guaranteed buy-down from UNITAID, USAID, PEPFAR and the Bill and Melinda Gates Foundation which reduced the price per cartridge from more than 40 USD to less than 10 USD for public health facilities in 141 low and middle income countries. A major advantage of the Xpert MTB/RIF test is the ability to detect smear negative TB in HIV-infected individuals (World Health Organization 2013).

In 2013 WHO issued updated policy guidance on the use of Xpert MTB/RIF additionally endorsing its application for extrapulmonary and pediatric samples. This policy update expanded the recommended application of Xpert MTB/RIF to include pediatric and extrapulmonary samples, including gastric aspirate, lymph aspirate, pleural fluid and cerebrospinal fluid. There was insufficient evidence to estimate sensitivity with urine, pericardial fluid and ascitic fluid, although specificity is generally high with these sample types. Optimal sample processing for blood and stool samples has not been determined and therefore the Xpert MTB/RIF test is not recommended pending further research. Full recommendations can be found at http://tbevidence.org/wp-content/uploads/2013/11/WHOstat.pdf.

By facilitating early detection of TB, prior to smear positivity, the application of Xpert MTB/RIF should have a significant impact on transmission chains and push back the epidemic. However, many of the patients diagnosed by Xpert MTB/RIF would have been initiated on treatment due to chest X-ray findings or clinical findings consistent with TB and the extent to which the use of Xpert MTB/RIF will increase case finding is not yet clear. Theoretical modelling studies suggest that the application of the test will improve targeting of treatment, with less patients who do not have TB incorrectly started on treatment and a greater number of smear negative ‘true TB’ cases detected. South Africa has implemented the Xpert MTB/RIF test nationwide and data on the cost-effectiveness and impact on the epidemic are eagerly awaited. Although relatively simple to perform and rapid, the Xpert MTB/RIF is not a true point-of-care test and many challenges have been encountered during scale-up. The need for a reliable electricity supply is a major barrier in some settings, problems with module calibration and maintenance, the need for the bulky cartridges to be stored below 30 °C, determining optimal testing algorithms and logistics of kit supply have been some of the challenges encountered (Abdurrahman et al. 2014) (Fig. 4.1).

4.4. Diagnosing Drug-Resistant Tuberculosis

A major impact of the scale-up of Xpert MTB/RIF is increased detection of RIF resistance, which is a surrogate marker for MDR TB. Classical diagnosis of drug resistance in *M. tuberculosis* involves culture of the bacilli on solid or liquid media and comparison of growth between drug-free and drug containing media. Even with the advent of

direct liquid culture methodology, detection of drug resistance takes over two weeks, and with indirect methods, two months or more. Standardisation of drug susceptibility testing for the antituberculous drugs is difficult and should be performed in a biosafety level 3 laboratory by trained personnel participating in an external quality assurance scheme. Isoniazid, rifampicin and streptomycin are the most reliable drug susceptibility tests.

Molecular detection of drug resistance mutations provides a rapid alternative, but the accuracy of these tests varies according to the drug. Rifampicin resistance detection is the most accurate, as 95 % of phenotypically rifampicin resistant strains carry a mutation in the 81 base pair rifampicin—resistance-determining-region (RRDR) of the rpoB gene. For isoniazid, molecular methods can detect approximately 75 % of phenotypically resistant strains by detecting mutations in the katG gene or InhA promoter region. Development of commercial NAAT for the other antituberculous drugs has been hampered by incomplete understanding of the molecular mechanisms of resistance. The principal commercial NAAT for drug resistance are the Xpert MTB/RIF test and the line probe assays. The most recent pooled estimates for *M. tuberculosis* detection by Xpert MTB/RIF were sensitivity of 88 % (95 %CI; 83–92%) and specificity of 98 % (95 %CI; 97–99 %); for rifampicin resistance sensitivity 94 % (95 %CI; 87–97 %) and specificity 98 % (95 %CI; 97–99 %) (Steingart et al. 2014). Line Probe assays detect both rifampicin and isoniazid resistance simultaneously and the MTBDR-sl assay detects resistance to fluoroquinolones, ethambutol and aminoglycosides. The MTBDR-sl test has high sensitivity for fluoroquinolones, but low sensitivity for aminoglycoside and ethambutol resistance (Feng et al. 2013). However, specificity is high for all drugs and therefore the test can be used to detect resistance but should not be used to rule-out resistance. Unfortunately, the need remains to confirm susceptibility by laborious phenotypic DST. Rapid sequencing techniques provide more comprehensive drug susceptibility data but are not yet widely available beyond research settings.

Several non-commercial phenotypic DST approaches have been developed including microscopic observation drug susceptibility testing (MODS), nitrate reductase assay (NRA) and colorimetric redox indicator (CRI) tests. A MODS test kit is now available to improve standardization (Hardy diagnostics). In 2010 WHO issued a recommendation that MODS could be used as an ‘interim’ approach for increased DST in high-burden countries but concluded that there was insufficient data to recommend NRA or CRI. Reservations particularly around biosafety and quality control have limited scale-up of the techniques (Image 4.1).

4.5. Other Diagnostic Methods

In 2011 WHO issued an unprecedented negative advisory on the use of serodiagnostic tests for TB (Steingart et al. 2011). These tests are appealing because of they are simple, rapid, inexpensive and non-invasive and are marketed with claims of high sensitivity and specificity. However, systematic evaluation of 19 commercially available tests using a well-characterised serum bank, and systematic review of all published studies concluded that none was accurate for use in clinical practice (Steingart et al. 2011). The search for accurate biomarkers for use in serodiagnostic tests continue, but has so far yielded little promise. Tests under evaluation by the Foundation for Innovative New Diagnostics (FIND; www.finddiagnostics.org) include the loop-mediated isothermal amplification test (LAMP) assay, mobile NAAT devices, volatile organic compound (VOC) or ‘electronic nose’ tests, an adapted interferon-gamma release assay, and enzymatic detection systems. It is unlikely that a true point-of-care test for TB will be available in the next 2–3 years given the current pipeline of diagnostic tests under evaluation (Thwaites et al. 2003; UNITAID 2014) (Fig. 4.2).

4.6. Diagnosing Latent Tuberculosis Infection

The interferon gamma release assays (IGRA) were developed as an alternative to the tuberculin skin test which is confounded by BCG vaccination. Two commercial IGRAs are currently FDA approved for the diagnosis of *M. tuberculosis* infection: The QuantiFERON-TB Gold In-Tube test (QFT-GIT) (Cellestis Limited, Carnegie, Victoria, Australia, approved 2007); T-Spot test (Oxford Immunotec Limited, Abingdon, United Kingdom, approved 2008). Whole blood or Peripheral blood mononuclear cells (PBMC) are stimulated with antigens from *M. tuberculosis* and the interferon gamma release stimulated is measured. IGRA which use ESAT-6 and CFP-10 antigens for stimulation are not confounded by prior BCG vaccination because these antigens are found in a region (RD1) of the *M. tuberculosis* genome which is deleted from BCG and thought to be partially responsible for the loss of virulence.

In the United States, IGRA are widely used for the diagnosis of latent TB infection and are recommended by CDC guidelines (available at: http://www.cdc.gov/mmwr/pdf/rr/rr5905.pdf). WHO do not recommend the use of IGRA in endemic settings or for the diagnosis of active TB. It should be noted that 95 % of IGRA positive individuals do not go on to develop active TB and therefore the predictive value of a positive IGRA is extremely low; no statistically

significant difference in the incidence of active TB between IGRA positive and IGRA negative individuals has been demonstrated in the small number of studies which have addressed this question.

Figures

Fig. 4.1 Current development in TB diagnostics. Only the Xpert® MTB/RIF has received WHO endorsement. *From* UNITAID, Diagnostic technology and market landscape, 3rd edition, 2014. Reprinted with permission

Image 4.1 Mycobacterial cording in MODS. *Image* courtesy of Dr. Dang Thi Minh Ha

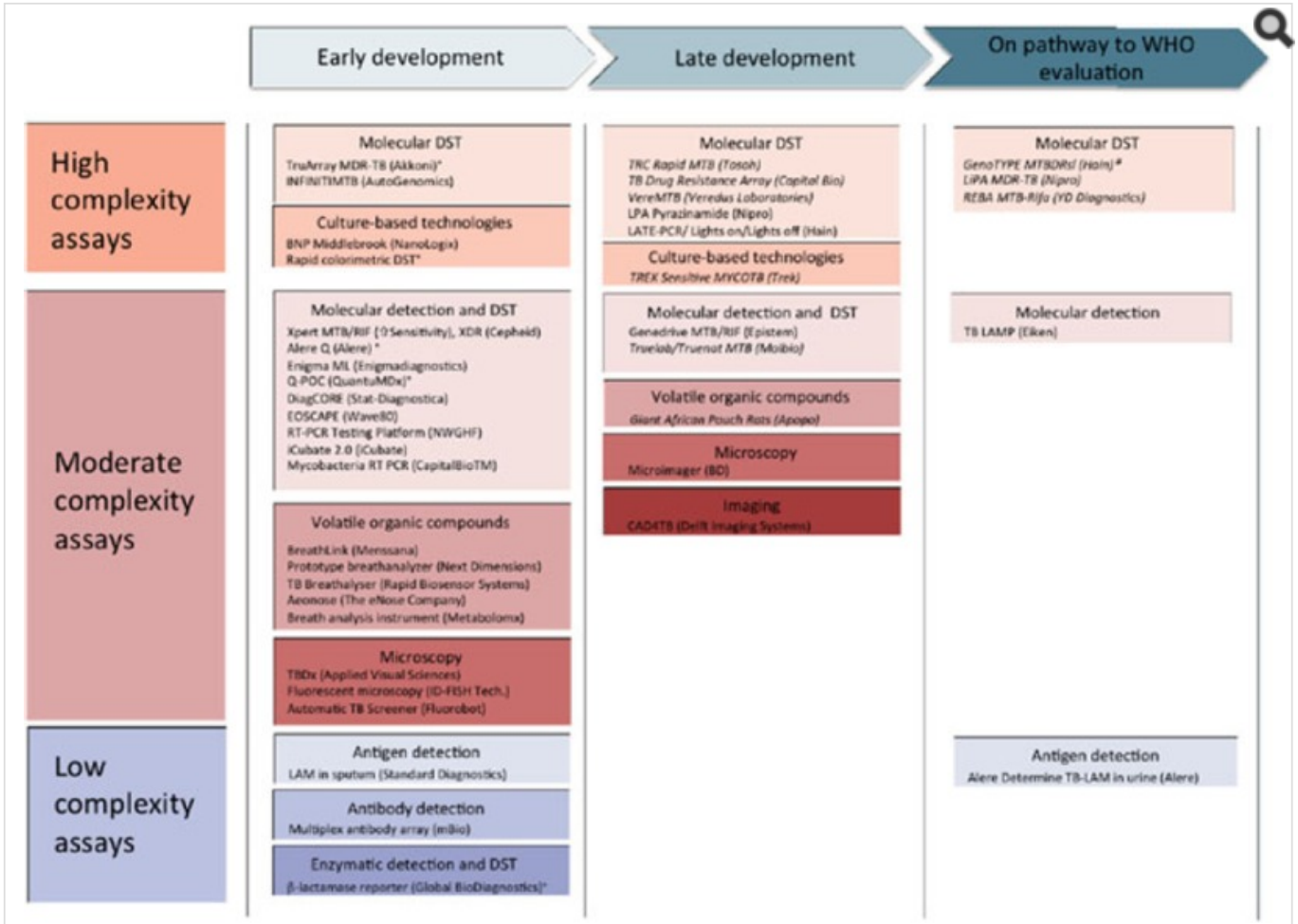

Fig. 4.2 Current FIND TB diagnostics pipeline listing the development phases and the types of technologies in development or evaluation, from UNITAID diagnostic technology and market landscape, 2014, reprinted with permission

Tables

Table 4.1 Laboratory tests for diagnosis of active tuberculosis and drug resistance

Diagnostic tests for active TB					
Test type	**Principal commercial tests**	**WHO policy recommendation**	**Advantages**		**Limitations**
Smear microscopy	Non-commercial	Recommended	Inexpensive, simple, rapid, specific		Cannot differentiate NTM[a] and *M. tuberculosis*
LED microscopy		Recommended	Inexpensive, simple, rapid		Cannot differentiate NTM[a] and *M. tuberculosis*
Automated real-time nucleic acid amplification	GeneXpert MTB/RIF	Recommended	Rapid (2 h to result). Detects smear-negative TB. Also detects RIF resistance		Higher cost than smear
Loop-mediated isothermal amplification test kit for TB	LAMP assay	Not recommended. Under further development	Rapid, simple		Subjective interpretation and poor specificity
Rapid speciation strip technology		Recommended	For rapid differentiation of NTM[a] and *M. tuberculosis*		Expensive
Serodiagnostic tests	Over 20 commercial variants	Not recommended			Poor sensitivity and specificity
Interferon-Gamma release assays	QuantiFERON-TB Gold In-Tube test, T-Spot test	Not recommended for diagnosis of active TB			Complex to perform and indeterminate results relatively common
Drug susceptibility tests					
Test type	Principal Commercial tests	WHO policy recommendation	Drugs tested	Advantages	Limitations
Phenotypic DST on solid or liquid media	Non-commercial	**Recommended for USE**	All drugs[b]	Gold-standard	Extremely long time to result (6–12 weeks)
Commercial liquid culture and DST systems	Bactec MGIT	**Recommended for USE**	STR, INH, RIF, EMB, PZA	Faster than solid culture media. Ten days if direct testing	Expensive
Line probe assay first-line	MTBDR-Plus; INNO LiPA-RIF TB	**Recommended for USE on smear-positive samples**	RIF, INH	Result in 2 days	Expensive

Diagnostic tests for active TB					
Test type	**Principal commercial tests**	**WHO policy recommendation**	**Advantages**		**Limitations**
Line probe assay second-line	MTBDRsl	**Not yet recommended due to insufficient evidence**	Fluoroquinolones, aminoglycosides and EMB	Result in 2 days	Low sensitivity for ethambutol
Automated real-time nucleic acid amplification	GeneXpert MTBRIF	**Recommended for USE**	RIF	Result in 2 h	Cartridge price reductions only available in low middle income countries
Microscopic observation drug susceptibility (MODS)	Non-commercial	**Recommended for USE**	RIF, INH	Low-tech. 10–14 days for result	Subjective interpretation. Laborious manual plate reading[c]
Colometric redox indicator (CRI)	Non-commercial	**Not yet recommended due to insufficient evidence**	RIF, INH	Low-tech. 10–14 days for result	Subjective interpretation
Nitrate reductase assays (NRA)	Non-commercial	**Not yet recommended due to insufficient evidence**	RIF, INH	Low-tech 10–14 days for result	Subjective interpretation
Phage assays	FASTplaque, lucerferase reporter phage assay	Not recommended	RIF, INH	N/a	Poor specificity
Sequencing	Non-commercial	No policy	Depends on gene regions sequenced	Can provide information on multiple drugs simultaneously	Requires specialist interpretation. Not generally available outside research centres

Details of policy guidance at: http://www.who.int/tb/laboratory/en/

a NTM: non-tuberculous mycobacteria

b Reliable for first-line drugs (except pyrazinamide), fluoroquinoloes and aminoglycosides. Second-line DST should be interpreted in context of treatment history and local prevalence of resistance (if known)

c Indicator well must be incorporated to differentiate NTM from *M. tuberculosis*

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