Chapter 7

Laboratory diagnosis

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Laboratory diagnosis of TB needs to be accurate, rapid and able to provide sufficient data to start patients on proper treatment. While culture and phenotypic DST remain pillars in the diagnostic process, molecular methods have the advantage of providing key data in a very short time. Worldwide roll-out of automated platforms able to detect TB and rifampicin resistance have substantially contributed to the increase of detection and treatment of MDR-TB cases. New, more comprehensive platforms are under development or have just started commercialisation, where the main advantage will be the capacity to detect resistance to isoniazid in addition to rifampicin. Some of the platforms are designed to be placed at a more centralised and high workload level. Whole-genome sequencing (WGS) and WGS-based technologies are the promise for the future. These tests will provide a very comprehensive range of information, from extensive resistance prediction to data on transmission in the population.

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The availability of more sensitive, accurate and affordable diagnostics for TB and DR-TB, including new point-of care tests, is one of the key pre-requisites to reach the 2035 targets of the End TB Strategy http://ow.ly/cfVQ30lqCfE

Rapid and accurate diagnosis of TB is key to avert death and to prevent further transmission of the disease. However, of the 10.4 million estimated new TB cases that occurred in 2016, 40% remained undiagnosed or underreported, including 450 000 cases (>75%) of RR-TB and MDR-TB [1]. Finding these 4 million missing cases will require 1) the development of improved diagnostics and to ensure their accessibility in low- and middle-income countries, and 2) the correct implementation and most efficient use of the existing tools.

The past few years have seen an increased interest in new technologies and progress in the field of TB diagnostics. The list of the TB diagnostic tools currently under development and validation is shown in table 1 [2]. The WHO has recently endorsed and issued policy

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Table 1. TB diagnostic landscape

	Development	Validation	Regulatory	WHO evaluation	Country transition
Rapid biomarker-based non-sputum-based test for detecting TB		Sensitive lipoarabinomannan assay (Fujifilm, Tokyo, Japan)			Determine TB LAM Ag (Alere, Waltham, MA, USA)
Rapid sputum-based test for detecting TB at the microscopy centre level of the healthcare system		ID-FISH assay (ID-FISH Technology, Palo Alto, CA, USA)	Truenat MTB (Molbio Diagnostics, Goa, India)		Loopamp MTBC assay (Eiken Chemical, Tokyo, Japan)
Next-generation DST at microscopy centres	TruArray MDR-TB and XDR-TB (Akonni Biosystems, Frederick, MD, USA) PoC (Bioneer, Daejon, South Korea) Xpert XDR (Cepheid, Sunnyvale, CA, USA) Omni (Cepheid) Q-POC TB/MDR-TB (QuantuMDx, Newcastle upon Tyne, UK) TB MultiTest (SelfDiagnostic, Leipzig, Germany) Stool testing with molecular tools (various)		Truenat MTB RIF Dx (Molbio Diagnostics)		Xpert MTB/RIF Ultra (Cepheid)
Centralised DST	FluoroType MTBXDR version 1.0 (Hain Lifescience, Nehren, Germany) LabChip-based rapid POCT (MicoBiomed,	INFINITI MDR-TB (AutoGenomics, Carlsbad, CA, USA) AccuPower XDR-TB real-time PCR (Bioneer)	TB resistance module series (Autoimmun Diagnostika, Strassberg, Germany) AccuPower TB&MDR real-time PCR (Bioneer)	RealTime MTB RIF/INH (Abbott Laboratories, Des Plaines, IL, USA)	

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Table 1. Continued

	Development	Validation	Regulatory	WHO evaluation	Country transition
	Geumcheon-gu, Seoul Korea)			BD MAX MDR-TB (BD, Franklin Lakes, NJ, USA)	
	UltraFast LabChip real-time PCR MDR-TB kit (MicoBiomed)	INNO-LiPA Rif.TB (Fujirebio Europe, Gent, Belgium)	TB drug resistance detection array kit (CapitalBio Technology, Beijing, China)	MGIT bedaquiline and delamanid (Becton Dickinson)	
		AdvanSure MDR-TB GenoBlot assay (LG Life Sciences, Seoul, South Korea)	MTB drug-resistant mutation test kits (QuanDx, San Jose, CA, USA)	FluoroType MTBDR version 1.0 (Hain Lifescience)	
		PrimeSuite TB (Longhorn Vaccines and Diagnostics, San Antonio, TX, USA)	Mycolor TK platform (Salubris, Shenzhen, China)	Cobas MTB-RIF/INH (Roche, Basel, Switzerland)	
		QMAC DST (QuantaMatrix, Seoul, South Korea)	Anyplex assays for MDR/XDR (Seegene, Seoul, South Korea)		
		VereMTB detection kit (Veredus Laboratories, Singapore)	VersaTREK (Thermo Fisher, Waltham, MA, USA)		
			Sensititre MYCOTB MIC plate (Thermo Fisher)		
			MolecuTech REBA MDR/XDR assays (YD Diagnostics, Gyeonggi-do, South Korea)		
			MeltPro MTB (MDR-TB, XDR-TB) kits (Zeesan Biotech, Fujian, China)		

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guidelines on multiple tests, including Xpert MTB/RIF and Xpert MTB/RIF Ultra (Xpert Ultra) assays [3, 4], line probe assays (LPAs) for the detection of resistance to isoniazid and rifampicin and to second-line anti-TB drugs [5, 6], loop-mediated isothermal amplification (TB-LAMP) [7], the lateral flow urine lipoarabinomannan (LF-LAM) assay [8], and a policy framework to guide countries in the implementation of TB diagnostics [9]. Nonetheless, many challenges for active TB diagnosis remain, including the lack of a point-of-care test deployable at the most decentralised level of care and having minimal infrastructure and training requirements, the unavailability of sensitive tools for the diagnosis of EPTB and TB in children, and the lack of molecular tools for accurate DST to more anti-TB drugs and implementable in decentralised settings.

In this chapter we provide an overview of the current diagnostics for active TB endorsed by the WHO and of tools that are currently undergoing multicentre evaluation. In addition, we describe the current applications of whole-genome sequencing (WGS) as next-generation DST and the use of targeted next-generation sequencing (NGS) for drug susceptibility and resistance prediction starting from clinical specimens. Finally, we provide some considerations on the future perspectives of the TB diagnostic landscape.

Current methodologies for tuberculosis detection

Direct microscopy

Microscopy still plays a major role in TB diagnosis and follow-up. The high lipid content of the mycobacterial cell wall makes the uptake of dyes problematic; however, once the primary colour has penetrated, it forms stable complexes resistant to decolourisation with acid-alcohol solutions (acid-fast bacilli (AFB) microscopy). Classical methods, *i.e.* Ziehl–Neelsen and Kinyoun staining, use different carbol fuchsin concentrations requiring, or not requiring, heating for cell wall penetration [10–12]. A valid alternative is represented by the fluorescence-based methods using auramine O as the primary stain. Mycobacteria stained with fuchsin appear red; those stained with auramine are yellow-orange fluorescent. The need for an expensive fluorescence microscope has long hampered the spread of florescence microscopy, despite its higher sensitivity in comparison with classical staining; in recent years the availability of cheaper and more durable light-emitting diode microscopes has made the latter approach preferred worldwide [13]. The reading of fluorescent AFB microscopy requires lower magnification with a significant reduction of the reading time in comparison with fuchsin.

Although smear microscopy on concentrated sputum samples has an increased sensitivity compared with the direct method, the WHO does not recommend it because of the increased biosafety risk of centrifugation methods at the peripheral microscopy laboratory level and the feasibility and costs of implementing such methods on a large scale [14].

AFB microscopy provides important information of a patient's infectiousness and allows the response to therapy to be evaluated. Its major limit is the poor sensitivity, in particular with extrapulmonary specimens and in HIV-positive patients (2000–10 000 bacilli·mL^{-1} are needed for a positive result).

The use of fluorescein diacetate, a viability stain that fluoresces only when hydrolysed by esterases of viable bacteria, has been proposed since the 1980s for mycobacteria [15].

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The method revealed excellent correlation with quantitative culture and potentially represents an inexpensive tool for treatment monitoring [16].

Culture methods

Culture is at least 100 times more sensitive than microscopy in detecting mycobacteria. Due to the slow growth rate of *Mycobacterium tuberculosis*, the risk of overgrowth by the contaminating flora present in specimens from nonsterile sites needs to be minimised. Of the various methods developed for this purpose, the one using *N*-acetylcysteine-NaOH is recommended; *N*-acetylcysteine is mucolytic and 2% NaOH eliminates most of the contaminant flora. The objective is to keep the contamination rate of cultures between 3% and 5%. A contamination rate <3% may indicate a too harsh procedure detrimental for mycobacteria, while a rate >5% is at risk of missing positive cultures.

Different solid media are available for culture of mycobacteria, either egg- or agar-based. Liquid media were rarely used because of problems with frequent contamination; it was only following the introduction of supplements consisting of antimicrobial blends active against common bacteria and yeasts that they have become more widely used. Culture in liquid media is both more sensitive and rapid in comparison with solid media. At present, to get maximum yield, the international guidelines recommend culture to be performed in parallel on both solid and liquid media, with Löwenstein–Jensen medium and the Mycobacteria Growth Indicator Tube (MGIT) being the most used, respectively [10, 11, 17]. The MGIT is a commercial liquid medium consisting of Middlebrook 7H9 broth supplemented with an antibiotic mixture (PANTA) (BD, Franklin Lakes, NJ, USA). An indicator, embedded in a silicon film at the bottom of the tube, becomes fluorescent when the oxygen concentration decreases in the medium, consumed by mycobacterial metabolism. Despite the high costs, the BACTEC MGIT 960 System, consisting of a fully automated machine for incubation and detection of positivity in MGIT tubes, is nowadays used in many laboratories worldwide, including in low-income countries [18, 19].

Rapid molecular tools for active tuberculosis detection

Xpert MTB/RIF

Xpert MTB/RIF (Cepheid, Sunnyvale, CA, USA) is a cartridge-based real-time PCR assay for the simultaneous detection of MTBC and rifampicin resistance from clinical specimens [20]. The test is fully automated, and takes ∼2 h for sample processing, DNA extraction, PCR amplification and data analysis. The WHO issued policy recommendations on using Xpert MTB/RIF in early 2011 [21] and a policy update in 2013 [3], stating that Xpert MTB/RIF may be used as the initial diagnostic test for all adults and children with signs and symptoms of TB, rather than microscopy and culture (conditional recommendation acknowledging resource implications). Xpert MTB/RIF can be performed directly on sputum, processed sputum sediment and selected extrapulmonary specimens from adults and children [3]. The Xpert MTB/RIF pooled estimates of sensitivity and specificity for PTB detection are 88% and 99%, respectively [22]. However, the test sensitivity remains suboptimal compared with culture in smear-negative TB patients, in patients with HIV infection and in children [22, 23]. For extrapulmonary samples, the sensitivity varies depending on the sample type, being higher for lymph node tissues and aspirates, cerebrospinal fluid [24], and gastric aspirates, but low for pleural fluid [25].

Xpert MTB/RIF Ultra

The Xpert Ultra assay (Cepheid) was launched in 2017 as a next-generation more sensitive assay for TB and rifampicin resistance detection [26]. Xpert Ultra uses the same GeneXpert platform and sample processing procedures as the Xpert MTB/RIF assay, but targets additional genetic regions, *i.e.* the multicopy genes IS6110 and IS1081, to enhance its sensitivity for MTBC detection. In addition, Xpert Ultra is characterised by a larger DNA reaction chamber, more rapid thermal cycling, and improved fluidics and enzymes [4]. Xpert Ultra uses the same semiquantitative categories used in the Xpert MTB/RIF assay with an additional category "trace" to identify the paucibacillary samples positive to IS6110/IS1081 targets but negative to *rpoB* [26].

In January 2017, a WHO expert consultation found the Xpert Ultra cartridge noninferior to the original assay for TB and rifampicin resistance detection based on data from a multicentre study coordinated by the Foundation for Innovative New Diagnostics (FIND) [4]. The study showed that Xpert Ultra's overall sensitivity was 5% higher than that of Xpert MTB/RIF with a higher incremental sensitivity among paucibacillary forms of TB: 17% higher for smear-negative, culture-positive patients and 13% higher for HIV-positive patients [27]. In a study assessing the performance of Xpert Ultra for the diagnosis of tuberculous meningitis against uniform clinical case definition, the assay sensitivity was 70% compared with 43% for Xpert MTB/RIF and liquid culture [28]. In a recent study on the accuracy of Xpert Ultra for the diagnosis of PTB in children, the assay detected 73.7% of microbiologically confirmed TB cases compared with 63.2% for Xpert MTB/RIF and 82.9% for culture [29].

However, Xpert Ultra's increased sensitivity came at a trade-off of decreased specificity, with an overall reduction of 2.7% [27]. In patients with a history of TB, Xpert Ultra specificity was 93% compared with 98% for Xpert MTB/RIF, possibly due to the presence of *M. tuberculosis* DNA or intact *M. tuberculosis* bacilli in the participant's respiratory system [27]. Based on these results, the WHO Technical Expert Group agreed that an Xpert Ultra "trace" result is sufficient to start therapy in individuals with known or suspected HIV infection and in children, and for the diagnosis of EPTB. To mitigate the risk of overtreatment in HIV-negative adults, Xpert Ultra should be repeated on a fresh specimen, with a second "trace" positive result being sufficient to make a diagnosis of PTB, unless there is a recent history of TB [4].

Loop-mediated isothermal amplification

A TB-LAMP assay for the detection of MTBC has been developed by Eiken Chemical Company (Tokyo, Japan). It is based on a rapid nucleic acid amplification method that occurs at a constant temperature of ~65°C. The assay leads to results in <1 h, does not require special reagents or sophisticated equipment and has the potential to be applied in peripheral facilities. Following a review of the latest evidence, in 2016 the WHO issued a policy guideline recommending the use of TB-LAMP as a replacement for smear microscopy in adults and children with signs or symptoms consistent with TB, but not among people with HIV due to the lack of data [7]. TB-LAMP has limited application in settings with high rates of HIV and drug resistance.

Lateral flow urine lipoarabinomannan assay

The commercially available LF-LAM assay Determine TB LAM (Alere, Waltham, MA, USA) allows the presence of LAM antigen released from metabolically active or degenerating mycobacterial cells to be detected in the urine of people with active TB

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disease. This is a technically simple, instrument-free, low-cost, rapid and point-of-care assay easily implemented in remote health facilities in low-resource settings. Results from a meta-analysis showed a pooled sensitivity and specificity of LF-LAM for TB diagnosis of 56% and 90%, respectively, in participants with CD4 $\leqslant$100 cells·μL^{-1} *versus* 26% and 92%, respectively, in those with CD4 >100 cells·μL^{-1} [30]. The WHO released policy guidance in 2015 stating that the test may be used to assist the diagnosis of HIV-positive TB presumptive patients with very low CD4 counts (<100 cells·mm^{-3}) or who are seriously ill [8]. In 2016, a multicentre RCT conducted in Africa showed that LF-LAM-guided, prompt anti-TB treatment initiation could reduce all-cause 8-week mortality in HIV-positive hospital inpatients [31]. Despite the positive results of the trial and the WHO recommendations, no country has yet committed to using LAM testing on a large scale.

Abbott RealTime MTB assay

Abbott Molecular (Des Plaines, IL, USA) has recently launched an automated RealTi*m*e MTB assay for the detection of MTBC in respiratory specimens through the amplification of both the IS6110 sequence and the gene for protein antigen B. The company offers a fully automated system from DNA extraction, to amplification and detection, with a flexible throughput to up to 94 samples per run and ~7 h to complete the entire process. Published studies have reported good analytical performances with sensitivity ranging from 71.7% to 96.7% in smear-negative, culture-positive samples and specificity ranging from 97% to 100% [32–36].

Current methodologies for drug resistance detection

Phenotypic-based drug-susceptibility testing

Drug resistance is defined as a significant reduction in sensitivity to a specific drug leading to affected strains being unlikely to demonstrate clinical responsiveness to the drug. Resistance to anti-TB drugs, especially MDR- and XDR-TB, is an emerging problem worldwide, and effective management of DR-TB largely relies on rapid and accurate identification of resistant *M. tuberculosis* strains.

The principal objectives for DST include: 1) determination of the resistance/sensitivity pattern of individual *M. tuberculosis* strains to ensure adequate treatment and management of a TB case, 2) assessment of the need for institutional isolation of patients, and 3) assisting in drug resistance surveillance at various levels and/or determination of the scope of institutional and community outbreak investigations/interventions required [37, 38]. The implementation of the WHO End TB Strategy requires provision of access to DST for all patients with signs and symptoms of TB [39].

Culture-based phenotypic DST methods remain the gold standard for the detection of drug resistance despite being time consuming, labour intensive and requiring sophisticated laboratory infrastructure [38]. The currently employed indirect phenotypic methods are based on inoculation of mycobacteria grown in bacterial culture onto drug-containing solid media or liquid media followed by a direct observation of growth, or an indirect monitoring of growth through oxygen consumption. Prior identification of species to ensure purity of MTBC culture is very important as many NTM are intrinsically resistant to many anti-TB drugs, thus increasing the risk of false-resistance results.

The principal phenotypic DST methods endorsed by the WHO include liquid media and egg- or agar-based solid media assays [37, 40–43]. Three solid culture methods (*i.e.* the proportion method, the resistance ratio method and the absolute concentration method) are relatively inexpensive and remain in use worldwide, but have been standardised predominantly for testing of first-line drugs only (rifampicin, isoniazid, ethambutol and pyrazinamide) and require up to 8 weeks to produce DST results. Turnaround times for liquid culture methods are significantly shorter and DST results are usually obtainable within 2 weeks.

Automated liquid culture-based systems monitor mycobacteria growth through oxygen consumption, thus enabling accurate reading and interpretation of DST results. This method has been extensively validated and is proposed as the reference method for performing DST for the majority of anti-TB drugs, including second-line and novel drugs (bedaquiline and delamanid) [38]. Recently developed microtitre plate-based liquid culture methods (*e.g.* Sensititre MYCOTB; Thermo Fisher, Waltham, MA, USA) allow MICs for multiple drugs to be determined in one plate, thus achieving an unprecedented level of precision for phenotypic DST [44–46]. Details of both liquid and solid media-based methodologies are described elsewhere [11].

Modern phenotypic DST methods traditionally use critical concentrations of anti-TB agents to determine if an isolate is resistant or sensitive to a given drug, where the critical concentration is defined as the lowest concentration of a drug *in vitro* that inhibits the growth of a pre-defined (usually 99%) proportion of a wild-type MTBC strain [38, 47, 48]. Definitions and utility of critical concentrations as well as other cut-off values (clinical breakpoints, MICs and epidemiological cut-off values) in phenotypic DST have considerably evolved over the last decade and are considered in detail elsewhere [49–51]. The updated list of critical concentrations and clinical breakpoints for drugs recommended for treatment of DR-TB was published in 2018 [38].

Several noncommercial phenotypic assays, including the nitrate reductase (NRA), microscopic observation drug susceptibility (MODS) and colorimetric redox indictor (CRI) assays, could be considered as inexpensive and rapid alternatives to complex and technically demanding commercial assays, especially in resource-constrained settings. The WHO has endorsed the use of the NRA and MODS assays for direct DST, and the NRA, MODS and CRI assays for indirect DST, in reference laboratories under clearly defined operational conditions following strict laboratory protocols [52]. Performance characteristics and the current status of phenotypic DST assays are summarised in table 2.

Rapid molecular tools for detection of drug resistance

Line probe assays

LPAs are a family of DNA strip-based tests that allow the drug resistance profile to be determined through the binding of amplicons to probes targeting the most common mutations to first- and second-line drugs and to wild-type probes. They can be used for the direct testing of smear-positive sputum specimens as well as indirect testing on culture isolates. Given the moderate complexity of the assay, the necessity of multiple pieces of equipment and the laboratory infrastructure requirements, LPAs are usually implemented at upper- and middle-tier health facilities. Compared with phenotypic DST, the use of LPAs allows the drug susceptibility profile to be obtained within 24–48 h, and presents a

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Table 2. Principal characteristics of phenotypic-based DST for *Mycobacterium tuberculosis*

Assay	Direct or indirect	Turnaround time	WHO status or recommendations	Cost	Labour intensity	Quantitative or qualitative
Solid media	Indirect	⩽8 weeks	First-line drugs only	++	+++	Qualitative
Liquid media: automated	Indirect	1–2 weeks	First, second, reserve drugs	+++	+++	Qualitative
Liquid media: plate based	Indirect	1–2 weeks	Not endorsed/ research in progress	+++	++	Quantitative
NRA	Direct/ indirect	Days	Conditional endorsement	++	++	Qualitative
MODS	Direct/ indirect	Days	Conditional endorsement	++	++	Qualitative
CRI	Indirect	Days	Conditional endorsement	++	++	Quantitative

NRA: nitrate reductase; MODS: microscopic observation drug susceptibility; CRI: colorimetric redox indictor.

lower biosafety risk and a higher throughput. In 2016, the WHO approved the use of several commercially available LPAs for the detection of MTBC and resistance to rifampicin and isoniazid, *i.e.* GenoType MTBDR*plus* versions 1 and 2 (Hain Lifescience, Nehren, Germany), and Nipro NTM+MDRTB detection kit 2 (Nipro, Osaka, Japan) [5], and a third LPA for the detection of resistance to SLIDs and fluoroquinolones, *i.e.* GenoType MTBDR*sl* versions 1 and 2 (Hain Lifescience) [6].

Line-probe assays for detection of resistance to first-line anti-tuberculosis drugs

The GenoType MTBDR*plus* and Nipro NTM+MDRTB detection kit 2 assays target mutations in the *inhA* promoter (from −15 to −8 nucleotides upstream) and *katG* regions (codon 315) to identify isoniazid resistance, and in the rifampicin resistance-determining region (RRDR) of the *rpoB* gene (from codon 505 to 533) for rifampicin resistance. The Nipro assay also differentiates *Mycobacterium avium*, *Mycobacterium intracellulare* and *Mycobacterium kansasii* from other NTM. A noninferiority study conducted by FIND showed equivalence among the three commercially available LPAs for detection of rifampicin and isoniazid resistance in smear-positive samples [53]. The overall pooled estimates of the sensitivity and specificity of the three LPAs for rifampicin resistance were 96.8% and 98.1%, respectively. For isoniazid, the pooled estimates of the sensitivity and specificity were 90.2% and 99.2%, respectively [5]. The WHO recommends the use of LPAs for persons with a sputum smear-positive specimen or a cultured isolate of MTBC, as the initial test, instead of phenotypic DST, to detect resistance to rifampicin and isoniazid [5]. However, given the different accuracy of detecting resistance to these two drugs, phenotypic DST for isoniazid may still be used when the LPA does not detect isoniazid resistance [5].

Line-probe assays for detection of resistance to second-line anti-tuberculosis drugs

The second version of GenoType MTBDR*sl* includes the *gyrA* and *gyrB* genes for detection of resistance to fluoroquinolones, and *rrs* and the *eis* promoter region for detection of resistance to SLIDs. In addition to culture isolates, the assay may be used on both smear-positive and smear-negative specimens. For fluoroquinolones, the sensitivity and specificity were 97% and 98% on smear-positive specimens and 80% and 100% on

smear-negative specimens, respectively [54]. For SLIDs, the sensitivity and specificity were 89% and 90% on smear-positive specimens and 80% and 100% on smear-negative specimens, respectively [55]. The WHO recommends the use of second-line LPAs for patients with confirmed RR-TB or MDR-TB, as the initial test, instead of phenotypic DST, to detect resistance to fluoroquinolones and to SLIDs. However, given the suboptimal sensitivity of the assay, culture and phenotypic DST is still required to completely exclude resistance to these classes of drugs as well as to other second-line drugs [6].

Line-probe assays for detection of resistance to pyrazinamide

The Genoscholar PZA-TB II assay (Nipro) is the first commercial molecular test available for the rapid detection of resistance to pyrazinamide starting from direct sample or culture isolates. The assay targets a 700-bp fragment covering the entire *pncA* coding region and 18 nucleotides upstream. The Genoscholar PZA-TB II assay comprises a total of 48 probes which recognise the wild-type sequence of the gene, while no specific mutant probes are included. Resistance is identified by the absence of probe binding. Two studies assessed the performance of the test, and reported a sensitivity ranging from 93.2% to 94.3% and a specificity ranging from 91.2% to 94.9% [55, 56]. The accuracy of the assay may be affected by the detection of mutations not causing resistance and this may vary depending on the regional context. In addition, the test has poor capacity in detecting heteroresistance and insertions. Despite these limitations, the Genoscholar PZA-TB II assay offers a valid alternative for rapid susceptibility testing for pyrazinamide.

Xpert MTB/RIF and Xpert Ultra

The Xpert MTB/RIF assay uses a molecular beacon technology to detect rifampicin resistance. A failure in, or delayed binding of one or more of the five probes to, the RRDR of the *rpoB* gene indicates potential rifampicin resistance. The pooled estimates for rifampicin resistance detection on pulmonary samples were 95% and 98% for sensitivity and specificity, respectively [22]. Despite the good analytical performance, the assay has some limitations including a poor sensitivity of specific probes in detecting certain mutations [57] or in the case of heteroresistance [58, 59] and may lead to false-positive results in the presence of some synonymous mutations [60] or in the case of paucibacillary samples or extrapulmonary samples [61].

The new version of the test, *i.e.* Xpert Ultra, provides more reliable detection of rifampicin resistance. Four sloppy molecular beacon probes targeting the RRDR of the *rpoB* gene have been designed to detect mutations by measurable shifts in melting temperature peaks. The assay allows a vast range of mutations to be identified, has improved capacity to detect resistant mutations at codon 533, correctly identifies mutations in mixed and paucibacillary samples, and differentiates synonymous mutations from other RRDR mutations [26]. Results from the multicentre diagnostic accuracy study showed comparable diagnostic performance of Xpert Ultra and Xpert MTB/RIF for detection of rifampicin resistance [27].

FluoroType MTBDR

The FluoroType MTBDR test (Hain Lifescience) is a fluorescence-based single-tube multiplex PCR for the rapid detection of MTBC and resistance to rifampicin and isoniazid directly from clinical samples. The assay combines LATE (linear-after-the-exponential)-PCR [62] together with specific probes using a lights-on/lights-off detection technology, where the presence of specific mutations is detected by shifts in the melting curves of the probes [63]. The test is semiautomated, with amplification and detection occurring in a closed system, and allows the simultaneous processing of up to 96 samples including an internal control. Results are generated within 3 h and are interpreted by software. Recent studies evaluated the

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performance of the test for rifampicin and isoniazid resistance detection on culture isolates and sputum specimens, showing very good results in terms of sensitivity and specificity for both drugs, and high accuracy for the identification of mutations in *rpoB*, *katG* and the *inhA* promoter [64, 65]. In theory, new mutations and the corresponding melting curve properties could be entered in the analytical software, potentially allowing the performance of the test to be improved.

BD MAX MDR-TB assay

BD has recently developed a multiplex real-time PCR assay (BD MAX MDR-TB assay) for the simultaneous detection of MTBC and mutations conferring resistance to rifampicin and isoniazid from sputum specimens [66]. The assay runs on a fully integrated platform, the BD MAX System, including automated DNA extraction and five-colour detection real-time PCR. Each run allows the simultaneous testing of up to 24 sputum specimens within 4 h, making the platform suitable for use in central laboratories. The assay has recently undergone evaluation for rapid detection of *M. tuberculosis* DNA and rifampicin and isoniazid resistance in a multicountry study, showing promising performance [67].

Next-generation sequencing

Whole-genome sequencing

WGS utilising high-throughput NGS technologies interrogates the entire bacterial genome, and can be used for a reliable prediction of drug-resistant or -sensitive phenotype through identification of single nucleotide polymorphisms and small insertions/deletions (indels) within regions associated with resistance to anti-TB drugs, thus offering completely new opportunities in TB diagnostics and clinical management [68–73].

Regardless of the platform and/or chemistry employed, WGS workflow can be broadly subdivided into four major steps: 1) DNA extraction, 2) library preparation (comprising fragmentation and adapter linkage), 3) automated NGS generating millions of reads and 4) data analysis. Existing chemistries (*e.g.* Illumina (San Diego, CA, USA), Ion Torrent (Thermo Fisher), Pacific Biosciences (Menlo Park, CA, USA) and MinION (Oxford Nanopore, Oxford, UK)) vary, sometimes considerably, in terms of read length, error rate, cost and other parameters [74]. The minimum concentration of genomic *M. tuberculosis* DNA required for good quality WGS is generally 5–10 ng·μL^{-1} but lower (>0.5 ng·μL^{-1}) concentrations could be acceptable, enabling successful WGS of mycobacteria from 1–2 mL of early MGIT cultures for the purposes of speciation, drug resistance prediction and relatedness [72]. DNA could be isolated using a variety of commercial and in-house methods. Details of wet laboratory procedures, including DNA extraction, quantification, library preparation and WGS, are described elsewhere [72, 75, 76].

WGS data analysis for drug resistance prediction predominantly involves identification of polymorphisms in genes associated with drug resistance using analytical pipelines [69, 70, 77]. This could be done using a variety of free online tools (*e.g.* TB Profiler, PhyReSE, Mykrobe, *etc.*) as well as commercial/proprietary software [78–81]. Online tools have the advantage of being free, but may need to be extensively validated in the laboratory to enable their use in routine diagnostic management.

Over 100 genes have been shown to be associated with resistance to anti-TB drugs, and the availability of high-quality curated up-to-date databases is of utmost importance for the

correct interpretation and reporting of WGS DST results [68, 82]. While much is known about polymorphisms associated with resistance to principal first-line drugs, including rifampicin and isoniazid, molecular mechanisms of resistance to selected reserve and new drugs are yet to be fully understood, which may have an impact on the performance of WGS for some of these drugs [68, 78]. Importantly, the quality of phenotypic DST data plays a major role in establishing the associations between polymorphisms and phenotypic resistance [82]. Ideally, specific mutations should be linked to specific MICs, and WGS should be able to predict both resistant and sensitive phenotypes, thus enabling clinicians to adjust treatment regimens, including dosage, depending on whether specific mutations are associated with low or high levels of resistance [69, 70].

Implementation of WGS-based systems for routine diagnosis of TB in selected high-income settings in Europe and North America has already provided evidence about WGS methodology to inform clinical management of DR-TB [83, 84].

Targeted sequencing

One of the main goals for the near future is to move towards culture-free NGS for rapid DST. This requires the capacity to extract high-molecular-weight genomic DNA from an extremely low number of *M. tuberculosis* bacilli (100–10^5 cells·mL^{-1}) present in a complex matrix which contains large amounts (<99%) of both human and other bacterial DNA. A process of enrichment is thus required to increase the proportion of mycobacterial DNA to enhance the sequencing efficiency. Although in principle direct NGS from smear-positive clinical sputum samples has proven to be feasible [85, 86], the protocols are rather cumbersome or very expensive. A recent study presented a new method for extracting *M. tuberculosis* DNA directly from smear-positive respiratory samples, allowing sufficient data for antibiotic susceptibility prediction to be retrieved from >60% of the samples tested [87]. However, the bioinformatic analysis was computationally intensive as human DNA reads had to be removed *a priori*.

An alternative approach for the rapid sequencing of primary specimens is to perform targeted NGS (amplicon NGS), which allows the ultra-deep sequencing of specific genomic regions of interest, such as those associated to drug resistance and species identification. Although this method requires pre-existing knowledge of the targets, in principle it is easily customisable and scalable with the addition of new targets of interest. Only a few studies have evaluated the performance of amplicon sequencing of *M. tuberculosis* DNA from sputum specimens to provide rapid DST, showing a high level of concordance (97%) with phenotypic testing [88, 89]. Importantly, amplicon NGS allows the identification of resistant subpopulations with high accuracy thanks to the high coverage depth of the targets [89]. GenoScreen (Lille, France) has recently released the Deeplex-MycTB assay for research purposes, which is a 24-plexed amplicon mix allowing the simultaneous prediction of resistance to 13 anti-TB drugs, genotyping and mycobacterial identification, directly on primary specimens. The kit includes access to a secure, cloud-based application for the rapid analysis and interpretation of the sequencing data. Recently, the assay has been used for rapid DST of sputum specimens collected during the anti-TB drug resistance survey of Djibouti [90].

Future perspectives

Despite a promising pipeline in new diagnostics, major gaps remain in the diagnosis of TB and DR-TB, including 1) a highly sensitive low-cost triaging test perhaps with an acceptable

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lower specificity that could be used as a point-of-care test to exclude TB and 2) a biomarker for cure. A next generation of LAM assays working on sputum is under field trials and preliminary unpublished data seem really promising. Other biomarker assays are under development. At present none of them have been fully validated as able to be used as a marker of cure [66].

A "closer to point of need", fully portable and wireless device (Omni; Cepheid) compatible with Xpert Ultra cartridges is also close to commercialisation. If expectations are confirmed by performance data, the detection of TB and MDR-TB cases will be further decentralised. Other devices and portable platforms are in different stages of development. The ability to rapidly detect resistance to isoniazid has important implications, especially in light of the new WHO recommendations on the treatment of patients with confirmed rifampicin-susceptible, isoniazid-resistant TB [91]. Roll-out of molecular assays able to predict isoniazid monoresistance is needed to allow implementation of the WHO guidelines in high-burden countries: several platforms are in the pipeline or close to commercialisation in the European Union or global market, although the majority are designed for centralised work.

The Xpert XDR cartridge has been developed to complement the Xpert MTB/RIF assay, providing additional information on isoniazid, fluoroquinolone and SLID resistance of TB-positive samples [92, 93].

In the next few years we expect an increasing role of WGS/NGS-based technologies. These technologies have the incomparable advantage of providing "complete" sets of data, allowing us not only to predict drug resistance with higher sensitivity (interrogating larger numbers, full genes and relevant noncoding sequences), but also providing an indication of the transmission of the disease. Data on transmission of TB and MDR-TB should inform TB programme strategies in low- and medium-burden countries engaged in eliminating TB.

Molecular data coupled with the knowledge obtained by MIC data (now more easily obtainable using microtitre plates) will allow a truly personalised treatment to be designed. Phenotypic DST will not disappear soon; it may be restricted to a few molecules for which genomic data are insufficient for clear-cut interpretation.

Conclusion

The TB diagnostic pipeline has flourished in the past 5 years as never before with many companies becoming interested and new tests/platforms entering in the process. How many of the proposed tests will pass the "reality check" (*i.e.* high-level performance in high-burden settings at a competitive cost) and become reality is unknown. Phenotypic DST will be gradually replaced by molecular interpretation of mutations for some drugs, but not for all. With more information on drug MIC distribution becoming available, the use of a single critical concentration to categorise drugs as susceptible or resistant is insufficient to properly guide treatment. MIC values or "clinical concentration" tests should be provided for key drugs when alternative or less toxic regimens are not possible.

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