TB Diagnostics and Laboratory Services
Information Note

Introduction

Care of patients with tuberculosis (TB) starts with a quality assured diagnosis. Successful DOTS expansion, as well as programmatic management of drug-resistant and HIV-associated TB therefore require - at its core - a robust network of TB laboratories with adequate biosafety, modern methods for diagnosis, standard operating procedures and appropriate quality assurance.

Arguably the weakest component of health systems, laboratory services have historically been grossly neglected, under-staffed and underfunded. Diagnostic capacity is therefore a major bottleneck for scaling up management and control of drug-resistant and HIV-associated TB, largely as a result of:

- Slow policy change and technology transfer, especially in low-and middle-income countries;
- Insufficient and underfunded laboratory strengthening plans;
- Inadequate laboratory infrastructure and biosafety;
- Vastly inadequate numbers of skilled staff;
- Insufficient technical assistance.

Strengthening TB laboratory services offers one of the best avenues for overall laboratory improvement as an essential health systems activity. Fundamental to this activity is collaboration between TB control programmes and public health laboratory services at country level, as adequate laboratory capacity consists of several essential elements which need to be addressed simultaneously, within comprehensive strategies and national laboratory strengthening plans.

An unprecedented effort to improve and expand TB laboratory capacity is currently under-way, spearheaded by the WHO and Stop TB Partnership Global Laboratory Initiative (GLI) and its network of international collaborators ([http://www.stoptb.org/wg/gli](http://www.stoptb.org/wg/gli)). At the same time research on new TB diagnostic tools has been accelerated and the diagnostic pipeline is now rapidly growing.¹

Genotypic (molecular) methods have considerable advantages for scaling up programmatic management and surveillance of drug-resistant TB, offering speed of diagnosis, standardized testing, potential for high throughput, and fewer requirements for laboratory biosafety. The development of the Xpert MTB/RIF assay for the GeneXpert platform was completed in 2009 and is considered an important breakthrough in the fight against TB. For the first time, a molecular test is simple and robust enough to be introduced outside conventional laboratory settings. The assay provides results directly from sputum in less than 2 hours.

The WHO evidence synthesis process confirmed a solid evidence base to support widespread use of Xpert MTB-RIF for detection of TB and rifampicin resistance. It is therefore recommended that 1) Xpert MTB/RIF should be used as the initial diagnostic test rather than conventional microscopy, culture and DST in individuals suspected of MDR-TB or HIV-associated TB (strong recommendation); and that 2) Xpert MTB/RIF may be used as a follow-on test to microscopy in settings where MDR and/or HIV is of lesser concern, especially in smear-negative specimens (conditional recommendation, recognising major resource implications). Xpert MTB/RIF technology does, however, not eliminate the need for conventional microscopy culture and DST, which are required to monitor treatment progress and to detect resistance to drugs other than rifampicin.

Robust, point-of-care diagnostic tests for TB are not expected before 2015; therefore, uptake of existing WHO-recommended technologies must be accelerated, which requires adequate laboratory infrastructure and clear policies at country level on their use in TB screening and diagnostic algorithms. Because of the complexity of laboratory strengthening, the involvement of an expert laboratory consultant is recommended to guide the implementation process at country level.

Technologies must be used in appropriate laboratory services

Establishing, equipping and maintaining laboratory networks are challenging, complex and expensive. Introducing new technologies is bound to fail if all core elements of laboratory services are not addressed at the same time. These include:

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• Laboratory infrastructure, appropriate biosafety measures and maintenance;
• Equipment validation and maintenance;
• Specimen transport and referral mechanisms;
• Management of laboratory commodities and supplies;
• Laboratory information and data management systems;
• Laboratory quality management systems;
• Appropriate, adequate strategies and funding for laboratory human resource development.

The GLI has developed a Roadmap for TB laboratory strengthening aimed at ensuring quality TB diagnostics in appropriately laboratory services within the context of national laboratory strategic plans, available at http://www.who.int/tb/dots/laboratory/policy/en.

Laboratory biosafety

*M. tuberculosis* is classified as a Risk Group 3 pathogen but handling of specimens poses different risks based on the methods employed. Using a risk-based assessment of different technical procedures performed in a TB laboratory permits the development of a set of minimum requirements for laboratory facilities. The risk assessment approach considers the bacillary load of materials (specimens, cultures), the viability of bacilli, whether the material handled is prone to generate aerosols, the number of manoeuvres generating infectious aerosols with each technique, the workload of the laboratory, the epidemiological characteristics of patients, and the medical fitness of the laboratory workers. A summary of relative risks follows below:

Preparing direct smears for AFB microscopy and processing samples for Xpert MTB/RIF

Minimum requirements

- Adequate ventilation*;
- Laboratory separated from other areas;
- Access to the laboratory restricted to authorized persons;
- The bench for smear-preparation separated from other work benches in the laboratory.

*Adequate ventilation can be ensured by opening windows if local climatic conditions allow. An exhaust fan can be used to ensure adequate room air changes. When climatic conditions prevent window opening, consideration should be given to mechanical ventilation systems that provide an inward flow of air without recirculation in the room.*

Processing sputum specimens for primary culture inoculation, direct nitrate reductase assays (NRA), direct MODS or direct line-probe assays (LPA)

Minimum requirements

- Laboratory separated from other areas;
- Access to the laboratory restricted to authorized persons;
- Floors, walls, ceilings, benches and furniture have impervious surfaces;
- Windows permanently closed. Air supply either passive or mechanical without recirculation;
- Centrifuge with aerosol tight buckets;
- Handling of specimens in appropriate biological safety cabinets (BSC), class I (EN12469/NSF49) or Class IIA2 (NSF49) or Class II (EN12469) equipped with HEPA filters H14;
- BSCs designed by certified manufacturers, properly installed, regularly maintained and re-certified at least annually on site;
- Controlled ventilation system that maintains a directional airflow into the laboratory from functionally clean to dirty areas, with a minimum of 6 up to 12 air changes per hour*.

*Installation of a controlled ventilation system should be planned with engineering specialists.*

Manipulating cultures for identification and drug-susceptibility testing (DST) with phenotypic methods and/or line probe assays

Minimum requirements:

- Meeting ALL requirements for abovementioned tests, and in addition:
- Containment laboratory with double door entry;
- Autoclave available on site and in close vicinity of the laboratory, for safe waste disposal.

**Technologies are suitable for different laboratory service levels**

The specialised nature of technical procedures, laboratory management and administration, and ensuring laboratory quality require different levels of laboratory testing, with clear specimen referral mechanisms.

Conventional tiered laboratory services for TB diagnosis are described in many resource documents. Three main laboratory service levels are common to the majority of countries:

- **Peripheral (typically district) level**: Performing sputum smear microscopy; TB and rifampicin resistance testing using Xpert MTB/RIF; referring specimens or patients in need of further tests to higher level laboratories.
- **Intermediate (typically regional) level**: Performing smear microscopy; TB and rifampicin resistance testing using Xpert MTB/RIF; and conventional culture, with or without species identification and first-line drug susceptibility testing (DST); referring cultures in need of further tests (eg. second-line DST) to higher level laboratories.
- **Central (typically national or reference) level**: Performing sputum smear microscopy, TB and rifampicin resistance testing using Xpert MTB/RIF, conventional and rapid culture and phenotypic DST, and molecular tests; referring isolates in need of further tests (eg. second-line DST or molecular sequencing) to Supranational Reference Laboratories in other countries or regions.

**WHO-recommended technologies**

**MICROSCOPY**

Mycobacteria are distinguished from other micro-organisms by thick lipid-containing cell-walls that retain biochemical stains despite decolourisation by acid-containing reagents (so-called ‘acid-fastness’).

**Advantages**: Microscopy of sputum smears is simple and inexpensive, quickly detecting infectious cases of pulmonary TB; Sputum specimens from patients with pulmonary TB - especially those with cavitary disease - often contain sufficiently large numbers of acid-fast bacilli to be readily detected by microscopy.

**Disadvantages**: Direct smear microscopy is relatively insensitive as at least 5,000 bacilli per millilitre of sputum are required for direct microscopy to be positive. Smear sensitivity is further reduced in patients with extra-pulmonary TB, those with HIV-co-infection, and those with disease due to nontuberculous mycobacteria (NTM).

**Limitations**: Microscopy for acid-fast bacilli (AFB) cannot distinguish *Mycobacterium tuberculosis* from NTM, nor viable from non-viable organisms, or drug-susceptible from drug-resistant strains.

**Conventional light microscopy**

Ziehl-Neelsen (ZN) light microscopy performed directly on sputum specimens is suitable for all laboratory service levels, including peripheral laboratories at primary health care centres or districts hospitals.

There is not sufficient evidence that processed (eg. concentrated or chemically treated) sputum specimens provide superior results to direct smear microscopy. Implementation of such methods in programmatic settings is therefore not recommended.

The number of ZN smears examined per microscopist per day should not exceed 20 as visual fatigue leads to a deterioration of reading quality; on the other hand, proficiency in reading ZN smears can only be maintained by examining at least 10-15 ZN smears per week.

In general, one ZN microscopy centre per 100,000 population is sufficient; however, expansion of ZN microscopy services should also take into account the location and utilisation of existing services, urban/rural population distribution, and specimen transport mechanisms.

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Conventional fluorescent microscopy

Conventional fluorescence microscopy typically uses quartz-halogen or high-pressure mercury vapour lamps as light sources. A lower magnification objective is used to scan smears, allowing a much larger area of the smear to be seen and therefore taking less time than ZN microscopy.

Conventional fluorescence microscopy is on average 10% more sensitive than ZN microscopy, but requires considerable technical expertise. Capital and running costs are also considerably higher. Conventional fluorescent microscopy has therefore been recommended by WHO at intermediate laboratory level where more than 100 smears are examined per day.5

Light-emitting diode (LED) fluorescent microscopy

LED technology allows the use of fluorescent microscopy with a much less expensive light source. LED microscopes or -attachments require less power, are able to run on batteries, the bulbs have a very long half-life and do not release potentially toxic products if broken.

Recent WHO evaluation confirmed the diagnostic accuracy of LED microscopy compared to conventional fluorescent microscopy, and superior efficiency of LED over conventional ZN microscopy. It is therefore recommended that conventional fluorescence microscopy be replaced by LED microscopy and that LED microscopy be phased in as an alternative for conventional ZN light microscopy in both high- and low-volume laboratories.6

CULTURE AND SPECIES IDENTIFICATION

Advantages: Mycobacterial culture and identification of \textit{M. tuberculosis} provide a definitive diagnosis of TB, significantly increases the number of cases found (often by 30-50%), and can detect cases earlier (often before they become infectious). Culture also provides the necessary isolates for conventional DST.

Disadvantages: Culture is much more complex and expensive than microscopy to perform, requiring facilities for media preparation, specimen processing, and growth of organisms, specific laboratory equipment, skilled laboratory technicians, and appropriate biosafety conditions.

Limitations: Specimens have to be decontaminated prior to being cultured to prevent overgrowth by other micro-organisms. All decontamination methods are to some extent also harmful to mycobacteria, and culture is therefore not 100% sensitive. Good laboratory practices maintain a delicate balance between yield of mycobacteria and contamination by other micro-organisms.

Solid and liquid culture methods are suitable for central/national reference laboratories (or regional laboratories in large countries). Usually, one culture laboratory is adequate to cover 500,000 - 1 million population. Solid culture methods are less expensive than liquid culture systems, but results are invariably delayed due to the slow growth of mycobacteria. Liquid culture increases the case yield by 10% over solid media, and automated systems reduce the diagnostic delay to days rather than weeks. Liquid systems are, however, more prone to contamination and the manipulation of large volumes of infectious material mandates appropriate and adequate biosafety measures.

Positive cultures have to be identified to differentiate \textit{M. tuberculosis} from NTM. NTM are more common in HIV-infected patients and the prevalence varies from country to country. Treatment of NTM is entirely different from treatment of TB and drug-resistant TB. As a minimum, laboratories performing DST must differentiate \textit{M. tuberculosis} from other NTM (further speciation is not recommended at programmatic level).

Confirmation is usually done by a combination of biological characteristics of the culture growth and selected molecular or biochemical tests (which invariably delay the final result).7 Rapid immunochromatographic assays (so-called strip speciation tests) for species identification on culture isolates provide a definitive identification of \textit{M. tuberculosis} in 15 minutes and are recommended.8 Molecular tests, biochemical methods and strip speciation assays are suitable for laboratories where culture and DST are performed.

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DRUG SUSCEPTIBILITY TESTING

Advantages: DST provides a definitive diagnosis of drug-resistant TB. A number of different DST techniques are available:

- Phenotypic methods involve culturing of \textit{M. tuberculosis} in the presence of anti-TB drugs to detect growth (indicating drug resistance) or inhibition of growth (indicating drug susceptibility).
- Genotypic methods target specific molecular mutations associated with resistance against individual drugs.

Phenotypic DST methods are performed as direct or indirect tests on solid or liquid media. In direct testing, a set of drug-containing and drug-free media is inoculated directly with a concentrated specimen. Indirect testing involves inoculation of drug-containing media with a pure culture grown from the original specimen.

Indirect phenotypic tests have been extensively validated and are currently regarded as the gold standard. Three methods are commonly used: proportion, absolute concentration, and resistance ratio. DST results do not differ significantly between the three methods for first-line anti-TB drugs.

Disadvantages: DST methods are suitable for use at central/national reference laboratory level only, given the need for appropriate laboratory infrastructure (particularly biosafety) and the technical complexity of available technologies/methods.

Limitations: The accuracy of DST varies with the drug tested (see below).

For both first- and second-line DST, formal links with one of the laboratories in the Supranational Reference Laboratory (SRL) network is recommended to ensure adequate expert input on laboratory design, specimen and process flow, biosafety, standard operating procedures, maintenance of equipment and external quality assurance.

First-line DST

DST is most accurate for rifampicin and isoniazid and less reliable and reproducible for streptomycin, ethambutol and pyrazinamide.

As a minimum, national TB control programmes treating MDR-TB patients should establish laboratory capacity to detect MDR-TB. Rifampicin resistance is a valid and reliable indicator/proxy of MDR-TB in high burden settings.

Rapid DST is essential for identifying patients at risk of MDR-TB, as the first priority. Automated liquid systems and molecular line probe assays (see later) for first-line DST are recommended as the current gold standard. Xpert MTB/RIF is recommended as a stand-alone diagnostic test in individuals at risk of MDR-TB (see below).

Once MDR-TB has been confirmed, additional first- and second-line drug susceptibility results should be obtained following current WHO recommendations.\(^9\)

Second-line DST

Second-line DST is complex and expensive. Commercial liquid methods and the proportion method on solid medium have been studied; methods for the absolute concentration or resistance ratio on solid medium have not been validated. Automated liquid systems for second-line DST are recommended as the current gold standard.

Routine second-line DST is not recommended unless the required laboratory infrastructure and capacity has been established, rigorous quality assurance is in place, and sustainable proficiency has been demonstrated. In order to retain proficiency and expertise, it is recommended that second-line DST only be performed if at least 200 specimens from high-risk patients are expected per year.

Aminoglycosides, polypeptides, and fluoroquinolones have been shown to have relatively good reliability and reproducibility, allowing a quality-assured diagnosis of XDR-TB.

Routine DST for other second-line drugs (ethionamide, prothionamide, cycloserine, terizidone, \textit{P}-aminosalicylic acid, clofazimine, amoxicillin-clavulanate, clarithromycin, linezolid) is not recommended as reliability and reproducibility of laboratory testing cannot be guaranteed.

**DST using non-commercial methods**

Non-commercial culture and DST methods are less expensive than commercial systems; however, non-commercial methods are prone to errors due to a lack of standardization and local variations in methodology. Performance of these methods is highly operator-dependent and good laboratory practice, good microbiological technique, and adequate quality assurance, supported by adequate training, are therefore imperative. As for commercial systems, stringent laboratory protocols, standard operating procedures, and internal quality control mechanisms must be implemented and enforced.

The evidence base for selected non-commercial culture and DST methods has been reviewed by WHO and the performance of these methods found to be acceptable under stringent laboratory protocols in reference/national laboratories in selected settings. These methods include microscopic observation of drug susceptibility (MODS), colorimetric redox indicator (CRI) methods, and the nitrate reductase assay (NRA). Recommendations for their respective use are:

- **MODS**: A microcolony method in liquid culture, based on inoculation of specimens to drug-free and drug-containing media followed by microscopic examination of early growth;
  - Recommended as direct or indirect tests, for rapid screening of patients suspected of having MDR-TB;
- **CRI methods**: Indirect testing methods based on the reduction of a coloured indicator added to liquid culture medium in a microtitre plate after in vitro exposure of *M. tuberculosis* strains to anti-TB drugs;
  - Recommended as indirect tests on *M. tuberculosis* isolates from patients suspected of having MDR-TB, and acknowledging that time to detection of MDR-TB is not faster (but less expensive) than conventional DST methods using commercial liquid culture or molecular line probe assays (see below);
- **NRA**: A direct and/or indirect method on solid culture based on the ability of *M. tuberculosis* to reduce nitrate, which is detected by a coloured reaction;
  - Recommended as direct or indirect tests, for screening of patients suspected of having MDR-TB, and acknowledging that time to detection of MDR-TB in indirect application is not faster than conventional DST methods using solid culture (see below).

Both commercial and non-commercial culture and DST systems/methods are suitable for implementation at central/national reference laboratory level only.

**MOLECULAR TESTING**

The ultimate aim should be to implement molecular assays (such as the line-probe assay, Xpert MTB/RIF or other WHO-endorsed molecular platforms in the future) for rapid first-step identification of MDR-TB or HIV-associated TB. Molecular line probe assays (LPAs) focused on rapid detection of rifampicin resistance (alone or in combination with isoniazid) have been endorsed by WHO in 2008 with detailed policy guidance on its introduction at country level. Xpert MTB/RIF assay was endorsed by WHO in December 2010 and supported by a document describing practical considerations for rapid implementation and operational how-to.

**Advantages**:
- Genotypic methods have considerable advantages for scaling-up programmatic management of drug-resistant and HIV-associated TB, in particular with regard to speed, standardised testing, potential for high throughput, and reduced biosafety needs.
- **Xpert MTB/RIF** detects both TB and rifampicin resistance in a single test. Rifampicin resistance is a good and reliable proxy for MDR-TB in high burden settings. Xpert MTB/RIF is suitable for all levels of laboratories but capacity of one device is limited to 20 specimens per day. Higher-volume settings may require more than one device. Xpert MTB/RIF can be used as a stand-alone diagnostic test in individuals at risk of MDR-TB.

**Disadvantages**: LPAs do not eliminate the need for conventional culture and DST capability. Currently available LPAs are registered for use only on smear-positive sputum specimens *M. tuberculosis* isolates grown from smear-negative specimens by conventional culture methods.

**Limitations**:
- LPAs are suitable for implementation at central/national reference laboratory level, with potential for decentralisation to regional level if appropriate infrastructure can be ensured. Conventional culture (solid or liquid) is required to monitor treatment response (culture conversion) of DR-TB patients.

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**Xpert MTB/RIF** requires uninterrupted and stable electrical power supply and yearly calibration of the cartridge modules. The positive predictive value of Xpert MTB/RIF is low in settings where rifampicin resistance is rare and results need to be confirmed by phenotypic DST or LPA.

**Selecting appropriate algorithms and technologies/methods**

1. Currently available technologies are not mutually exclusive. Molecular line probe assays and selected non-commercial culture and DST methods are suitable for direct application on smear-positive specimens only. Conventional culture capacity is still required for smear-negative specimens while conventional phenotypic DST capacity is needed to detect XDR-TB;
2. Liquid culture and molecular line probe assays are regarded as international gold standards, to be phased in without loss of existing solid culture and DST capacity;
3. Xpert MTB/RIF is appropriate for district and intermediate level use but can be used at all levels of laboratory service. Interim algorithms are available to guide Xpert MTB/RIF use\(^\text{14}\). Conventional culture (solid or liquid) is required to monitor treatment response (e.g. culture conversion and cure) of DR-TB patients.
4. Rapid phenotypic DST methods present an interim solution, especially in resource-constrained settings, while capacity for genotypic testing is being developed;
5. Implementation of new technologies/methods for TB should be decided by Ministries of Health within the context of national strategic plans for laboratory strengthening and with input from laboratory experts;
6. TB diagnostic capacity should be linked to drug access and programmatic capacity to ensure treatment of patients under appropriate standards of care.

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