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TUBERCULOSIS
Diagnostic Technology Landscape

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Terms and Acronyms

AFB	Acid fast bacteria
amplicon	A piece of DNA that is formed as a product of natural or artificial amplification
AMTD	Amplified Mycobacterium tuberculosis Direct
CD4	Cluster of differentiation 4. A glycoprotein found on the surface of T helper cells, monocytes, macrophages, and dendritic cells
CDC	Centers for Disease Control and Prevention (USA)
CDRC	Tuberculosis Clinical Diagnostics Research Consortium at NIAID (USA). https://www.tbcdrc.org/home.aspx
CE / CE-marking	A mark placed on products in the European Economic Area that indicates the product conforms with requirements of EU directives. CE stands for *Conformité Européenne* (European Conformity)
CPA	Cross priming amplification
CRI	Colorimetric redox indicator
CSF	Cerebrospinal fluid
DNA	Deoxyribonucleic acid
DOTS	Direct observed therapy, short courses. An internationally recommended strategy for TB control
DST	Drug susceptibility testing
ECDC	European Centre for Disease Prevention and Control (EU)
ELISA	Enzyme-linked immunosorbent assay
EQA	External quality assurance
FDA	Food and Drug Administration (USA)
FIND	Foundation for Innovative New Diagnostics
FM	Fluorescence microscopy
GLI	Global Laboratory Initiative. A partnership with Stop TB comprised of "a network of international partners dedicated to accelerating and expanding access to quality assured laboratory services" for diagnosing tuberculosis, particularly HIV-associated and drug-resistant tuberculosis. http://www.stoptb.org/wg/gli
HBC	High-burden country
HDA	Helicase dependent amplification
HIV	Human immunodeficiency virus
IFN-γ	Interferon-gamma
IGRA	Interferon gamma release assay
L J	Lowenstein-Jensen media. A solid culture media used for tuberculosis diagnosis
LAM	Lipoarabinomannan, a lipopolysaccharide located on the cell wall of MTBC
LAMP	Loop mediated amplification
LBC	Low-burden country
LED	Light emitting diode
LIC	Low-income country
LPA	Line probe assay
LRP	Luciferase reporter phage assay
LTBI	Latent TB infection
MBA	Mycobacterial phage assay

MIC	Middle-income country
Middlebrook 7H9	Liquid medium used for the culture of Mycobacteria
Middlebrook 7H10 or 7H11	Agar-based media used for the culture of Mycobacteria
MDR-TB	Multidrug resistant tuberculosis
MODS	Microscopic-observation drug-susceptibility
MTB	*Mycobacterium tuberculosis*
MTBC	*Mycobacterium tuberculosis* complex. A genetically related group of *Mycobacterium* that cause tuberculosis
MTB/RIF	*Mycobacterium tuberculosis* resistant to rifampicin
NAT	Nucleic acid-based test
NAAT	Nucleic acid amplification test
NEAR	Nicking enzyme amplification reaction
NIAID	National Institute of Allergy and Infectious Diseases (USA)
NIH	National Institutes of Health (USA)
NRA	Nitrate reductase assay
NTM	Non-tuberculosis mycobacteria
PATH	Program for Appropriate Technology in Health
PCR	Polymerase chain reaction
PhaB	Phage amplified biologically assay
PLHIV	People living with human immunodeficiency virus
PM	Proportion method
PNB	*para*-Nitrobenzoic Acid
PPD	Purified protein derivative
POC	Point-of-care
RDT	Rapid diagnostic test
RIF	Rifampicin
RNA	Ribonucleic acid
RPA	Recombinase polymerase amplification
SDA	Strand displacement amplification
STAG-TB	Strategic Technical Advisory Group of TB experts (WHO)
TB	Tuberculosis. Also referred to as MTB. A common, potentially lethal infectious respiratory disease
TDR	Special Programme for Research and Training in Tropical Diseases (WHO)
TLA	Thin layer agar
TMA	Transcription mediated amplification
TST	Tuberculin skin test
VOC	Volatile organic compounds
WHO	World Health Organization
XDR-TB	Extensively drug-resistant tuberculosis. A form of TB caused by bacteria resistant to all of the most effective drugs
ZN	Ziehl-Neelsen staining

TUBERCULOSIS DIAGNOSTIC TECHNOLOGY LANDSCAPE

Foreword

The inadequate ability to rapidly and accurately diagnose active tuberculosis (TB) in developing countries remains a major obstacle in global control of the disease.(1) When appropriately diagnosed and treated, TB is largely curable. Yet, in 2010, an estimated 8.8 million people became ill with TB, of which 3.1 million with active disease were not diagnosed and notified to national TB control programs.(2) To enable widespread use in resource-constrained settings, new diagnostic tools for TB are needed urgently.

Currently available diagnostic technologies—including smear microscopy and culture—have notable shortcomings. While technological advances have brought about largely incremental improvements, more profound change may be seen with new or expected diagnostics, particularly nucleic acid amplification technologies (NAAT). In 2010, WHO endorsed GeneXpert® MTB/RIF, an automated, bench-top device that tests for TB and rifampicin resistance. It returns results within hours, is relatively easy to use, and can be used at decentralized health levels.

Despite its advantages, however, the diffusion of GeneXpert® MTB/RIF is not without challenges: it is still relatively expensive, is not a point-of-care (POC) test, does not eliminate the need for drug sensitivity testing, and will require some evaluation to determine its most effective use in resource-constrained environments. The pipeline promises new technologies, including a POC manual NAAT kit using loop-mediated isothermal amplification from Eiken / FIND, and a handheld NAAT device from Epistem / Xcelris. However, these products require further development and validation, and are not expected to be widely commercially available until the end of 2012 or later.

In summary, despite existing technologies and incremental improvements in these, there is a persisting unmet need for new TB diagnostic tools and approaches that:

- Have high specificity and sensitivity, and are appropriate for use in patient groups that are currently underserved (e.g., those with suspected extra-pulmonary infections, children, and people living with HIV);
- Are amenable to decentralized use—ideally available at the point of patient care;
- Require limited infrastructure (e.g., power supply, biosafety equipment, human resource time and training);
- Provide rapid results at low cost (particularly for detection of smear negative cases and drug susceptibility testing); and
- Use specimens other than sputum.

Market-based interventions will be key in facilitating access to new TB diagnostic tools. Specifically, interventions that foster development and dissemination of pipeline technologies that address the needs above could

facilitate access to game-changing innovations. In parallel, interventions that support infrastructure developments are needed: in the absence of appropriate POC diagnostic tests, laboratory capacity building is key for improving the effectiveness of screening and diagnostic algorithms. Indeed, although peri-urban or rural communities are often underserved due to inadequate infrastructure, 60% of suspected TB cases use the lowest tier test centres: namely, the microscopy centres and health clinics.(7) Appropriate solutions therefore must be tailored to specific needs of individual countries, and settings within countries.

This report reviews current technologies to identify critical unmet needs, and incremental improvements and pipeline technologies to highlight areas of potential promise for improved TB diagnosis. UNITAID will consider contents of this report in conjunction with complementary TB diagnostics market intelligence.

This report is part of a broader effort by UNITAID to identify opportunities to create new markets, catalyse markets for underutilized products, and address market inefficiencies towards increased access to medicines. UNITAID's landscape analyses for medicines and diagnostics markets for HIV, tuberculosis and malaria guide priority setting for UNITAID and many other organizations.

Executive Summary

Rapid, accurate diagnosis of tuberculosis (TB) is critical for timely initiation of treatment, and ultimately, control of the disease. Currently available diagnostics are time- and labour-intensive, and the lack of appropriate diagnostic tools remains a major obstacle for TB control in low-income countries. There is an urgent need for new TB diagnostics that can be used in settings with resource constraints.

The purpose of this report is to:

- describe existing TB diagnostics and the pipeline of expected future methods and tools;
- characterize unmet needs and the extent to which the pipeline may address these; and
- highlight areas of persisting market shortcomings and potential opportunities for market-based interventions.

Approaches to TB diagnosis and control recognize the importance of early, accurate detection of TB. In the absence of effective and rapid diagnostic technologies that can be used at the point of care (POC) for patients, laboratory capacity and other considerations are essential, particularly in resource-limited settings. For example, the most common **specimen type** for the diagnosis of active pulmonary TB is sputum. However, the use of sputum is limited by infection risk; lack of effectiveness in diagnosing extra-pulmonary TB; and challenges in manipulating a viscous, non-uniform sample. In addition, the difficulty of collecting an adequate sputum specimen is a significant challenge in people living with HIV (PLHIV), who may have a low bacterial load in their lungs, and in paediatric patients, who may be unable to produce an adequate volume of sputum (or indeed, any sputum at all) for testing. To address these challenges, researchers are investigating sputum induction methods and alternative specimen types, but many of these are not appropriate for use in resource-limited settings.

TB diagnostics currently in use include culture, phage-based TB diagnostics, smear microscopy, and nucleic acid amplification technologies (NAAT). **Culture**—considered the current diagnostic gold standard—is essential for detection of smear negative cases, and is the first step for drug susceptibility testing (DST). However, culture is relatively slow and expensive, requiring specialized laboratories and highly skilled labour. Recent innovations have improved techniques for culture speciation (confirmation of TB bacteria in samples), including lateral flow strips that can detect TB bacteria in 15 minutes. Step-wise advancements also have been made by alternative, non-commercial, culture-based drug susceptibility methods: colorimetric redox methods; nitrate reductase assays; and microscopic-observation drug-susceptibility (MODS) assays. These techniques offer improved speed, cost, and sensitivity, but must be run under strict conditions in reference laboratories. As such, they have been endorsed by the World Health Organization (WHO) as an interim measure while capacity for genotypic and/or automated liquid culture and DST are developed.

Phage-based TB diagnostics use mycobacteriophage-based approaches to detect TB cells in sputum or from culture, and include luciferase reporter phage assay (LRP) and phage-amplified biological assay (PhaB) or mycobacteriophage-based assay (MBA). Phage-based TB diagnostics offer faster diagnosis of *Mycobacterium*

tuberculosis complex (MTBC) disease and DST than culture and the potentially greater sensitivity than microscopy, described below. However, low specificity and persistent contamination problems associated with phage-based TB diagnostics can lead to unacceptable numbers of false positive test results.

Smear microscopy, a low-cost method and the frontline tool for TB diagnosis across the developing world, is effective in detecting the most infectious cases and can be performed in basic laboratories. The introduction of fluorescence microscopy has increased the sensitivity of the test, and procedural improvements have increased throughput. Despite these gains, detection is poor in specimens without a relatively high bacterial load; many cases remain undiagnosed. Extra-pulmonary TB diagnosis and DST cannot be performed with microscopy. In addition, microscopy is labour-intensive and reliant on highly trained staff.

Nucleic acid amplification technologies hold promise for significant gains in speed and performance. GeneXpert® MTB/RIF, for example, returns automated results on TB detection and rifampicin resistance in hours and can be used at decentralized locations. However, GeneXpert® MTB/RIF is not a true POC test and does not eliminate the need for DST, performance is suboptimal in some patient populations, and its high cost has limited uptake despite WHO endorsement in 2010. Pipeline products include manual isothermal NAAT based assays that would allow POC testing with minimal equipment, but further development is needed to ensure adequate performance and stability.

Molecular **line probe assays (LPA)**, endorsed by WHO in 2008, apply principles of nucleic acid amplification to detect both TB bacteria and mutations that indicate drug resistance. They can be used with sputum samples and culture isolates, and when used with the former can deliver results within days, rather than weeks. However, they require specialized facilities, can detect only well characterized drug resistance alleles, and are best utilized on smear positive TB cases (thereby limiting their utility in PLHIV and in children).

Immune response-based diagnostic tests—including tuberculin skin test (TST) and interferon gamma release assays (IGRAs)—have a place for latent tuberculosis infection (LTBI) screening in low TB incidence settings. However, the performance of these tests in high TB burden settings is poor, limiting their role and value. **Serodiagnostic assays** offer low-cost, rapid results, but unfortunately currently available tests do not offer acceptable performance. Indeed, WHO made the unprecedented step of recommending that current TB serodiagnostic assays not be used. Further research is needed to develop immune response-based or serodiagnostic tests with appropriate performance.

Alternative antigen assays detect antigen biomarkers derived from MTBC disease, but the performance of currently available tests is poor for most patient populations. An exception is the sensitivity of lipoarabinomannan (LAM) assay in patients with CD4 counts below 200/μL. This suggests that these tests may have some utility in very high HIV-burden areas where advanced HIV-associated immunodeficiency is common, but further investigation is required.

TB diagnostics that detect **volatile organic compounds** (VOCs) as a metabolic biomarker of TB could allow rapid testing, non-invasive specimen collection, and use with PLHIV and paediatrics. However, this technology is still in the early development stage, and may not be suited to POC use.

Despite existing technologies and incremental improvements, unmet needs persist. Novel diagnostic technologies are needed to provide better performance in diagnosing TB in currently underserved patient groups (e.g., those with suspected extra-pulmonary infections, children, and PLHIV). Diagnostics amenable to use in resource-limited settings are also needed; these would provide rapid results at low cost, and would be characterized by appropriateness for decentralized use, limited infrastructure requirements, and potential to use specimens other than sputum, detect smear negative cases, and test for drug susceptibility. Pipeline technologies that address these needs have the potential to radically reshape approaches to TB diagnosis.

A dynamic understanding of existing and pipeline technologies is key for UNITAID to facilitating access to appropriate TB diagnostic tools through market-based interventions. As such, this landscape is intended to be a living document updated as the TB diagnostics market evolves, to review current technologies and critical unmet needs and to highlight areas of potential promise for improved TB diagnosis in the pipeline.

This landscape was developed from primary sources (e.g. meetings and interviews with technology developers, targeted analyses where needed) and extensive review of secondary sources (e.g. published and unpublished

reports, WHO policies and systematic reviews, corporate prospectuses, developer web sites). For further detail, refer to Methodology (below).

Introduction

This document is a landscape analysis of *Mycobacterium tuberculosis* complex (MTBC) diagnostic technologies. Technologies described include existing products and methods that are currently in use, as well as emerging diagnostic technologies with the potential to improve MTBC diagnosis. These pipeline technologies require further evaluation or consideration of how these tools can solve the inherent problems faced in providing appropriate diagnosis to aid tuberculosis (TB) treatment in developing countries.

This document is the result of an effort by UNITAID to understand market shortcomings and opportunities in diagnostic technologies as part of the core work of the Market Dynamics team. This effort is a first step in providing a larger overview for TB with regard to medicines, diagnostics, and preventatives. The diagnostics landscape for TB will be published annually and may be updated at least once each year.

The purpose of this report is threefold. First, the report describes TB diagnostics currently in use and the pipeline of expected future TB diagnostic methods and tools. The report provides context through an overview of approaches to TB control (e.g. goals of TB diagnosis and treatment, laboratory infrastructure required to support TB diagnosis, constraints and limitations specific to low-income countries). Current and pipeline TB diagnostic products are then described, including culture-based systems, phage-based TB diagnostics, smear microscopy, nucleic acid amplification technologies (NAAT), immune response-based diagnostic tests, serodiagnostic assays, alternative antigen assays, and diagnostics to test for volatile organic compounds. Second, the report characterizes unmet needs and comments on the extent to which the TB diagnostic pipeline can be expected to address these. Uncertainty in the pipeline depends largely on the stage of development: pipeline products in very early stages of development may require significant investment and breakthroughs in the basic science on which they are based (e.g., identification of appropriate biomarkers), while late-stage products can be better characterized in terms of expected commercial attributes (e.g., timing of commercial availability, cost) and performance.

Third, the report highlights areas of persisting market shortcomings. Where well matched to UNITAID's model of market-based interventions, market shortcomings can represent potential opportunities for UNITAID intervention in the TB diagnostic market.

Methodology

This report was prepared by David Boyle (PATH, Seattle) and Madhukar Pai (McGill University, Montreal) with support from UNITAID. The material in this landscape is current through February 2012.

In general, the material in this landscape was gathered from an extensive review of publicly available information, published and unpublished reports, WHO policies and systematic reviews, corporate prospectuses, and developer web sites, as well as meetings and interviews with technology developers.

In addition to this broad approach, specific targeted analyses were carried out in areas where little information was publically available, such as a survey of Chinese diagnostics developers to identify current pipeline products.

Acknowledgements and conflicts of interest

David Boyle holds an NIH grant to develop a diagnostic assay (not tuberculosis related) with TwistDx (UK) (R01 AI097038-02) and a second grant with Ustar Biotechnologies (China), also unrelated to TB (BMGF OPP 1044825). He has no other commercial/financial conflicts pertaining to information described in this document.

Madhukar Pai has no commercial/financial conflicts. He previously served as Co-Chair of the Stop TB Partnership's New Diagnostics Working Group, and as a consultant for the Foundation for Innovative New Diagnostics. He is currently serving as a consultant for the Bill & Melinda Gates Foundation (BMGF). BMGF had no involvement in the production of this report.

Approaches to TB diagnosis and control

The inadequate ability to rapidly and accurately diagnose active TB in developing countries remains a major obstacle in the global control of TB.(1) This obstacle persists despite improvements and investments in the directly observed therapy, short-courses (DOTS) program as part of the Stop TB Strategy, where the incidence rate was reduced by 1% per annum and the mortality rate by approximately one-third since 1990. From 1990 until 2009, it is estimated that the implementation of DOTS have treated 49 million people infected with TB, with 41 million of those treated successfully.(3) It is the current goal of Stop TB to further diagnose and treat 32 million people with TB using the Stop TB Strategy/DOTS approach, with 28 million successfully treated by 2015.(3)

The first of five goals stated in *The Global Plan to Stop TB* (4) is to improve access to and use of diagnostic services in an effort to increase early TB case detection and to improve the diagnosis of drug-sensitive and resistant MTBC and TB/HIV coinfection. Current diagnostic strategies must be improved by both developing existing TB diagnostic capacity and integrating effective and rapid diagnostic technologies close to or at the point of care (POC) for patients. In the absence of appropriate POC diagnostic tests, laboratory capacity building is essential for ensuring an effective TB response. To be effective, TB diagnostics need to be supported by a well-maintained and fully functional laboratory system. For the uptake of new TB diagnostic technologies, the World Health Organization (WHO) has identified that both policy reform and appropriate laboratory infrastructure are key areas if the uptake of new diagnostic technologies are to impact TB programs. Several areas were identified for improvement, including (5):

1. Laboratory infrastructure, appropriate biosafety measures, and maintenance.
2. Equipment validation and maintenance.
3. Specimen transport and referral mechanisms.
4. Management of laboratory commodities and supplies.
5. Laboratory information and data management systems.
6. Laboratory quality management systems.
7. Appropriate, adequate strategies and funding for laboratory human resource development.

Without implementation of these key recommendations, new TB diagnostic technologies will not have a sustained impact. A concerted effort to improve and expand TB laboratory capacity is underway, spearheaded by WHO in conjunction with the Stop TB Partnership Global Laboratory Initiative (GLI) and its network of international collaborators.(5)

Access to treatment and preventive TB health care is variable and limited in developing countries due to a variety of factors, including poor health-care infrastructure, inadequate financial support, and a lack of trained health-care workers and laboratory professionals. From the patient's perspective, social factors—including stigmatization, discrimination, and inadequate education in addition to economic or employment constraints—pose additional challenges.(6) Infrastructure limitations mean most clinical laboratories with the greatest test capacity and more accurate test methods are located in urban settings. As a result, peri-urban or rural communities are often underserved. The diagnostic capability of health-care systems decreases as the facility level moves from the reference laboratory to the periphery, or POC. In developing countries, most patients access health care at levels where diagnosis is most basic (see Figure 1). It is estimated that 60% of suspected TB cases use the lowest tier test centres: namely, the microscopy centres and health clinics.(7)

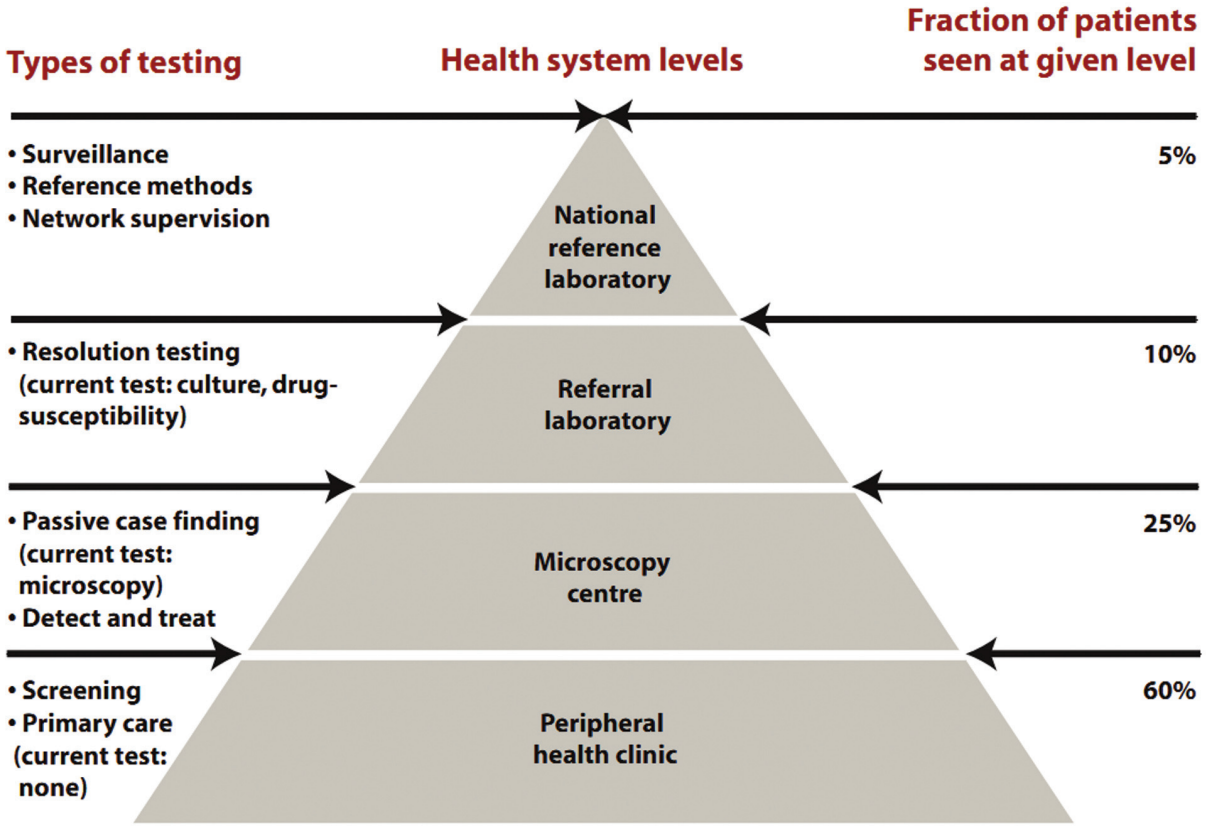
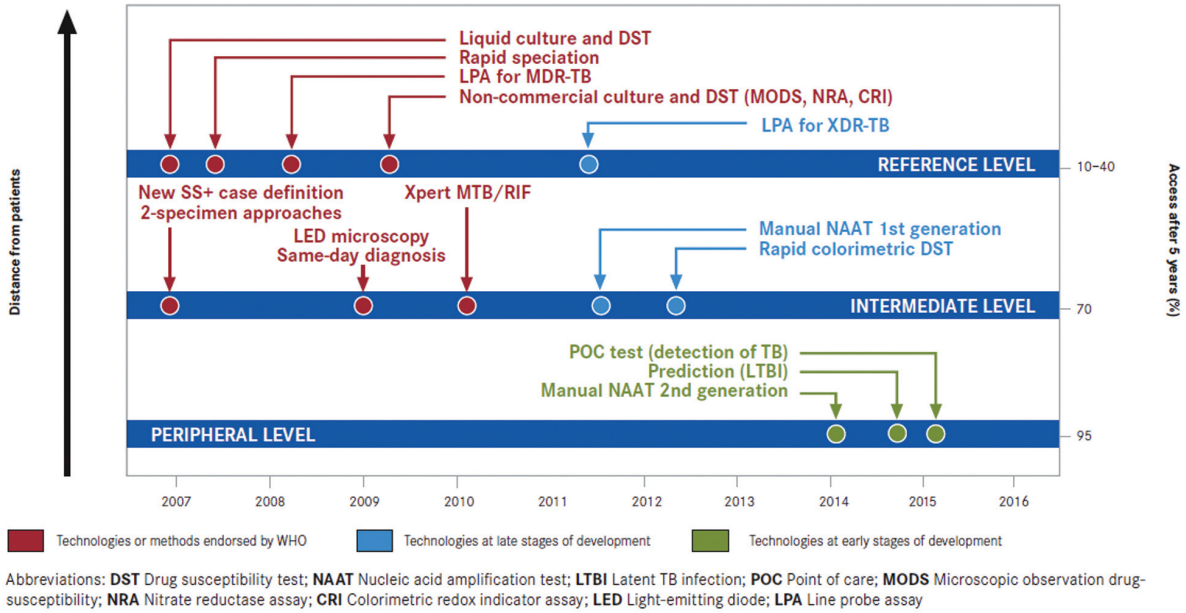


Figure 1. Tiered laboratory capacity for TB control in resource-constrained health-care settings in relation to the location and sophistication of laboratory tests employed and patient access.(8)



Abbreviations: **DST** Drug susceptibility test; **NAAT** Nucleic acid amplification test; **LTBI** Latent TB infection; **POC** Point of care; **MODS** Microscopic observation drug-susceptibility; **NRA** Nitrate reductase assay; **CRI** Colorimetric redox indicator assay; **LED** Light-emitting diode; **LPA** Line probe assay

Figure 2. The landscape of the technology pipeline and intended areas of use for TB diagnostic tests and methodologies endorsed or under consideration by the WHO STAG TB. Timelines of emerging diagnostics for TB with recommendation for use by WHO (red dots) and diagnostic tests under late stage development or evaluation (blue dots) or in early stage (green dots).(9) Also displayed are the proposed test location (e.g. distance from patients) and the availability to patient after five years.

Source: Global Tuberculosis Control 2011. Geneva, World Health Organization, 2011.

Available at http://www.who.int/tb/publications/global_report/2011/gtbr11_full.pdf. Accessed on 24 November 2011.

TB specimen types

Sputum is the most common specimen type for diagnosis of active pulmonary TB. Patients with a cough for 2 or more weeks should be screened for active TB, particularly if it is in conjunction with other clinical symptoms such as night sweats, weight loss, fever, and fatigue. Other fluid specimen types include urine, blood, gastric aspirate, pleural fluid, and cerebrospinal fluid (CSF). Additionally, where sputum specimen collection is inappropriate (e.g. extra-pulmonary TB) or very difficult (e.g. paediatric pulmonary TB), solids such as tissue or stool have been described for diagnosis of MTBC. The use of sputum has limitations for a variety of reasons, including: the difficulty of collecting adequate specimens, infection risk to others if collection is performed in a poorly ventilated space, and the viscosity of sputum which makes manipulation difficult prior to the test method. In addition, patients may have a low bacterial load in their lungs (i.e. paucibacillary) or cannot produce an adequate volume of sputum for testing. This is commonly observed in people living with HIV (PLHIV) and paediatric patients, respectively. Both of these patient groups are in critical need of better TB diagnostics as they are prone to rapidly developing TB with a greater risk of mortality.(9) Sputum is also ineffective for diagnosis of cases of extra-pulmonary TB. Furthermore, the growth or presence of other commensal bacteria, and particularly the physiochemical composition of sputum, make it difficult to process prior to diagnostic test analyses.

Sputum specimens from paediatric patients are better collected via sputum induction by saline nebulization and chest percussion or, in children under 5 years, via nasopharyngeal aspirate (Table 1). Other more invasive methods can be used, such as gastric lavage, bronchoalveolar lavage, laryngeal swab, and the string test. A meta-analysis of these specimens showed sputum to be relatively poor in terms of TB recovered by culture.(10) Gastric and bronchiolar lavages are extremely invasive and cannot be routinely performed in low-resource settings. Blood and urine samples are typically used for culture-based diagnostics.(11) Sputum is also ineffective for the diagnosis of extra-pulmonary TB.

Table 1: Examples of paediatric specimens used for the diagnosis of TB. (12)

Specimen Collection Method	Problem/Benefits	Potential Clinical Application
Sputum	Not feasible in very young children; assistance and supervision may improve the quality of the specimen	Routine sample to be collected in children >7 years of age (all children who can produce a good-quality specimen)
Induced sputum	Increased yield compared with gastric aspirate; no age restriction; specialized technique, which requires nebulization and suction facilities; use outside hospital setting not studied; potential risk of transmission	To be considered in the hospital setting or an in- or outpatient basis
Gastric aspirate	Difficult and invasive procedure; not easily performed on an outpatient basis; requires prolonged fasting; sample collection advised on three consecutive days	Routine sample to be collected in hospitalized children who cannot produce a good-quality sputum specimen
Nasopharyngeal aspiration	Less invasive than gastric aspiration; no fasting required; comparable yield to gastric aspiration	To be considered in primary health-care clinics or on an outpatient basis
String test	Less invasive than gastric aspiration; tolerated well in children >4 years of age; bacteriological yield and feasibility require further investigation	Potential to become the routine sample collected in children who can swallow the capsule but cannot produce a good-quality sputum specimen
Bronchoalveolar lavage	Extremely invasive	Only for use in patients who are intubated or who require diagnostic bronchoscopy
Urine	Not invasive; excretion of MTBC well documented	To be considered with new sensitive bacteriological or antigen-based tests
Stool	Not invasive; excretion of MTBC well documented	To be considered with new sensitive bacteriological or antigen-based tests
Blood/bone marrow	Good sample sources to consider in the case of probable disseminated TB	To be considered for the confirmation of probable disseminated TB in hospitalized patients
Cerebrospinal fluid	Fairly invasive; bacteriological yield low	To be considered if there are signs of tuberculous meningitis
Fine needle aspiration	Minimally invasive using a fine 23-guage needle; excellent bacteriological yield, minimal side-effects	Procedure of choice in children with superficial lymphadenopathy

The diagnosis of extra-pulmonary TB in adults and children requires a variety of different specimen types, and these represent an even tougher diagnostic challenge in terms of low numbers of bacteria present and inhibitory substances in the specimen that affect test performance. This is illustrated in the poor sensitivity of PCR in specimens such as CSF, pleural fluid and lymph node aspirates.(13-15) The type of specimen is chosen based on clinical symptoms and the suspected site of infection, e.g. lymph node exudate for lymphatic TB or urine for renal TB disease. Other specimens include blood, CSF, ascitic fluid, pericardial fluid, and brain abscess secretion.(16) Many of these specimen types require invasive sampling techniques that are unavailable to most patients in the developing world.

TB Diagnostics Currently in Use

Culture-based systems

The most sensitive and current optimal method for the detection of active MTBC disease is via mycobacterial culture. The use of culture remains necessary for the definitive diagnosis of pulmonary TB in patients whose smear produces a negative result. The benefits of culture include identification, drug susceptibility testing (DST), and further use for molecular epidemiology using DNA fingerprinting (e.g. spoligotyping). Culture can be performed using either solid or liquid media with manual or automated culture methods. Culture can use all specimen types but typically sputum is used for the diagnosis of pulmonary TB. Sputum is chemically processed to both liquefy it and to kill other microflora prior to concentration and use of the concentrate for inoculation. Results typically take 2–8 weeks, depending on the type of method used and the number of TB cells in the inoculum. Solid culture media typically uses either Lowenstein–Jensen [LJ] media or Middlebrook 7H10 or 7H11 agar media for the isolation, culture, and DST of MTBC. Recently, blood agar has been shown to have similar sensitivity to LJ in resource-limited settings, but with a significantly shorter time to results.(17) LJ culture is inexpensive, may be stored for several weeks after preparing (if refrigerated), and is used by most laboratories in developing countries for culture or DST of TB. While solid culture can afford definitive identification of drug resistance, it is still problematic, especially for MDR-TB, due to the length of time required for the cultures to grow. Therefore solid culture results often have limited or no impact on patient management.(18) The median turnaround time for DST in a recent study by Shah *et al.* was 70 days using the standard method for solid media-based DST for MDR-TB.(19) In 2003-2004, it was estimated that 13.4 million cultures tests were performed outside of Europe, Japan, Australia/New Zealand.(20)

TB typically has a faster growth rate in liquid media than on solid agar. The use of commercial liquid culture-based systems was endorsed in 2007 by WHO, which recommends phased implementation of liquid culture where feasible, including low-income countries (see Table A3.1 in Appendix 3 and Figure 2).(21) Automated liquid culture systems are sold by several commercial vendors, including Beckton Dickson (Bactec960 MGIT [mycobacterial growth indicator tube]), bioMérieux (BacT/ALERT), and Trek Diagnostic Systems Inc. (Myco-ESP culture System II). These are fully automated systems that use either fluorimetric or colorimetric detection of mycobacterial growth and can be used for the identification of MTBC and for DST. Automated systems permit a higher throughput of specimens for testing; for example, the Bactec960 has a 960 tube capacity and is claimed to have a throughput of 8,000 specimens per year. The Foundation for Innovative New Diagnostics (FIND) has negotiated pricing of Bactec960 MGIT automated systems for introduction in high-burden countries (HBCs). The unit costs per machine are ~$40,000 with $205 per 100 MGIT tubes (tests) and $71 for supplemental reagents for the test. In parallel, FIND has worked in some countries to maintain and upgrade laboratory facilities to enable the use of the Bactec960 MGIT automated system. The Bactec960 MGIT can also produce results on drug susceptibility, but the cost and complexity of this type of instrumentation limits its use to reference laboratories and serve only a fraction of suspected TB cases.

The rapid confirmation of MTBC from mycobacterial cultures, derived from either liquid or solid media, can use the Capilia TB Neo test (TAUNS Corporation, Japan), a lateral flow test for MPB64 (a specific antigen to MTBC) that takes only 15 minutes. There are now other companies offering similar rapid speciation tests; for example, the TBc ID test (Becton Dickinson, USA).(22;23) An alternative diagnostic method that uses liquid culture, this test is predictive for MTBC by microscopically examining the cultured specimen for micro colonies whose cells are cording. This method is designed for use with the microscopic-observation drug-susceptibility (MODS) assay described later in this document.(24) In TB culture facilities in high-resource countries, the AccuProbe (Gen Probe, USA) is a nucleic acid-based test (NAT) that confirms the presence of MTBC in culture.(25)

There are several alternative, non-commercial, culture-based methods that have been described, including the MODS assay, the nitrate reductase assay (NRA), colorimetric redox indicator (CRI) method, phage-based assays, and the thin layer agar (TLA) assay. WHO has recently approved the use of MODS, NRA, and CRI for use as an interim measure leading to the eventual use of automated liquid culture and DST (or molecular assays) with an unambiguous proviso that they are used under clearly-defined programmatic and operational conditions, in reference laboratories, and follow strict laboratory protocols (Table 2 and Figure 2).(26) Other NAT-based methods associated with DST will be discussed later in the section covering molecular detection of TB.

The MODS assay was developed as a faster, cheaper, and more sensitive test than other solid or liquid culture-based tests currently in use for **TB** diagnosis.(19;24;27) The basic principle is via microscopic examination of the liquid media (Middlebrook 7H9) using an inverted light microscope to identify MTBC within wells that exhibit bacterial growth. The test involves a 24-well plate, each well containing small volumes of liquid media that are used to identify MTBC growth and in addition resistance to rifampicin and isoniazid, i.e. 4 wells per specimen. In addition, *para*-Nitrobenzoic Acid (PNB), a compound that inhibits the growth of MTBC but not of non-tuberculosis mycobacteria (NTM), can be added to one (drug free) well to help discriminate between MTBC and NTM prior to microscopic examination.(28) TB-complex bacteria grow as a tangled or corded mass of cells while non-tuberculous mycobacteria do not.(29) *M. chelonae*, an NTM also has a chorded form, but has a relatively faster growth rate by which it can be differentiated from MTBC. The time to detection is typically under 2 weeks. The incorporation of anti-TB drugs into some of the wells at the outset enables DST with clinical specimens, unlike other current methods that rely on an initial culture followed by DST.

The use of MODS is recommended for use in areas that currently use solid-based media and have the basic facilities and trained staff to safely perform TB culture. In a recent study by Shah et al., MODS detection of MTBC took a median of 9 days as opposed to MGIT (16 days) and solid culture (29 days).(19) A further study demonstrated a median of 7 days to identify MDR strains as opposed to 70 days using the conventional solid media DST assays.(19) In a recent meta-analysis, the MODS assay had a sensitivity of 98% for rifampicin resistance and a specificity of 99.4%. The mean turnaround time was about 10 days.(30)

As a culture-based method, consideration must be given to specialist training and appropriate containment facilities are still needed to protect laboratory staff and to correctly perform the tests when using MODS. The manual reading of test results with a microscope limits the throughput. A further barrier to more widespread use of MODS has been the availability of the test media, test components, and suitable low-cost inverted microscopes with which to read the plates.

Hardy Diagnostics (USA) has released a CE-marked MODS kit which contains all of the necessary reagents and drugs for performing DST MODS. The test uses a plate that employs a tightly sealed silicon lid to reduce contamination and risk of spills—a concern with the original method that used a conventional polycarbonate plate and loose fitting lid. Further improvements to MODS include the development of a low-cost microscope (31) and an automated reader for high throughput analyses of cultures.(32) The standardization of a low-cost culture method and equipment may, consequently, help improve DST for MDR-TB in intermediate level laboratories.

The principle of CRI is to use a reactive dye that indicates viable cells. For DST using CRI, TB cells are exposed to a drug (e.g. rifampicin or isoniazid) and then cultured in the presence of a dye. Colorimetric change in the media is relative to the number of viable bacteria present and sensitivity to different drugs can be established by colour change in the liquid media. The CRI assay is faster than the conventional proportion method (PM) performed on solid culture medium and is less expensive than commercial liquid culture methods and molecular line probe assays.(33)

The NRA assay is similar to the CRI test in that a colorimetric dye is used to indicate bacterial growth. However, NRA is based on using solid media and cells are cultured for 10 days before adding Griess reagent, which, by turning from pink to purple, indirectly indicates the presence growing cells.

TLA is a solid culture-based approach similar in principle to MODS. TLA is a simple, rapid, and inexpensive method allowing initial identification of *MTBC* based on colony morphology, visualized microscopically for chording. The time to results are similar to MODS (8.6 days MODS and 11.4 for TLA) and like MODS DST can be performed simultaneously with TB diagnosis. Unlike MODS, TLA is currently not recommended by WHO as an interim method for DST.(21)

FIND and the London School of Tropical Hygiene and Medicine (UK), are developing an improved colorimetric TLA assay with the potential for use in peripheral laboratories by culturing directly from sputum onto a TLA plate and generating results in 2-3 weeks. The assay will establish MTBC infection (via on-plate microscopy) in addition to concurrently determining MDR status and potential resistance to one second-line drug (typically fluoroquinolone). This method may have the potential to start DST on patients recently diagnosed with TB via smear microscopy or to double the sensitivity in diagnosing symptomatic patients whose tests are smear negative (e.g. paediatric cases, PLHIV, or patients with extra-pulmonary TB). It has been proposed that WHO may review this assay in 2012 (34) but recent contact with the WHO has shown this to be unlikely.

Two recent reviews on the suitability and performance of non-commercial assays for the diagnosis of TB both confirm that the non-commercial, culture-based methods assessed were cost-effective tools for the diagnosis of MTBC and for DST.(30;35) A consistent premise with the use of simplified, culture-based methods in lower tier laboratories is that an effective method for sterilizing and disposing of used culture media must be employed to remove the risk of laboratory associated infections and cross contamination of specimens.

Benefits: Culture media is the most sensitive diagnostic that is currently available and can also be used to determine first- and second-line drug resistance. Given that the technique is over 50 years old, many processes for the optimal culture and handling of isolates are well established. The automated Bactec system has a high throughput. Non-commercial methods have been shown to have similar or better performance than some commercial methods, and performance has been assessed by multiple evaluations. Their low cost and the potential for implementation using existing laboratory materials and equipment makes them a viable alternative to commercial culture that can be used in lower tier laboratories while more appropriate assays are being developed. TB testing closer to the patient using simplified culture techniques may improve case detection. In addition, these tests simultaneously permit DST-to-MTBC diagnosis which improves treatment decisions and may reduce further transmission of MDR-TB.

Drawbacks: Due to the need for biosafety and containment, culture-based diagnostics can only be performed in specialized facilities with highly trained laboratory technicians, which limit testing to reference Biosafety level 3 (BSL 3) laboratories and serve only 15% of the population. The contamination of automated test reagents (e.g. MGIT) can be a problem in some laboratories. Automated batched testing means that equipment failure can affect a large number of test results. The cost of culture using commercial systems is significant and requires trained staff and appropriate facilities. The preparation of an inoculum for culture from sputum is labour intensive and involves at least 7 or 8 steps prior to inoculating the test media. Culture laboratories using commercial systems are reliant on effective supply chains to ensure adequate stocks of critical reagents. Culture methods take at least 9 days and often much longer to obtain a result. Lack of standardization of some of the non-commercial methods and the need for very strict QA makes these tests difficult to scale-up in many settings. Once used for culture, all media and materials should be safely disposed of following appropriate guidelines for biohazardous and medical waste. The development of simplified culture methods for use in lower tier laboratories may increase the exposure risk to laboratory technicians, if appropriate training and effective culture disposal methods are not in place.

Phage-based TB diagnostics

The use of TB-specific bacteriophages is an alternative approach for the diagnosis of TB from sputum. There are two types of phage assay, the luciferase reporter phage assay (LRP)(36) and the phage amplified biological assay (PhaB) or mycobacteriophage-based assay (MBA), respectively.(37) These use different mycobacteriophage-based approaches to detect TB cells in sputum or from culture (i.e. for DST).

The LRP assay involves measuring the bioluminescence emitted from MTBC cells infected with a bioluminescent phage, while PhaB/MBA detects the amplified phage from MTBC cells which are the host. The assays have been used to diagnose MTBC and to assess MDR. Both assays are relatively simple to use and do not require extensive materials. A result is generated within 48 hours of processing. A recent multicentre evaluation in China reported that a prototype PhaB kit has sensitivity of 98.4% when compared to LJ culture.(38) However, the specificity was lower at 71.6%.

MBA assays are commercially available from Biotec Laboratories (FASTPlaque-TB/PhageTeK MB and assay variants to detect drug resistance, FASTPlaque-TB-MDRi**, and FAST**Plaque-Response). The FASTPlaqueTB kit

for MDR-TB diagnosis was evaluated by FIND in Peru in 2007 and other, more recent evaluations with FAST-Plaque-TB showed better performance than smear microscopy in the diagnosis of TB. The authors also noted variable performance in multiple studies and persistent contamination problems which suggest that it be used in laboratories with significant infrastructure and that appropriate training in its use was needed. The use of phage-based methods for identification of MDR from cultured cells has been described in several studies.(39-41) A meta-analysis demonstrated that while both assays had good sensitivity, specificity was problematic and contamination was also a concern.(42) This meta-analysis was updated for review by WHO in 2010.(43) After considering the evidence, WHO has not recommended the use of phage-based assays for interim coverage of DST until automated systems become available to laboratories.(21) FIND has discontinued its investments in the phage-based technology.

Benefits: Faster diagnosis of MTBC disease and DST when compared to culture and the assay may have greater sensitivity than microscopy.

Drawbacks: Low specificity and persistent problems with contamination of the test laboratory with phage can lead to unacceptable numbers of false positive test results. With some drugs phage replication may not be blocked by their presence.

Smear microscopy

As a member of the acid fast bacteria (AFB), MTBC can be stained to visually distinguish it from other non-AFB using microscopy. The use of direct (unconcentrated) sputum smear microscopy is the primary method of TB diagnosis in the developing world and has been in use for 129 years. The greatest priority for TB control in high-burden settings is for the detection of active cases of pulmonary TB that excrete infectious bacteria and for their immediate start on treatment. Although cases identified with high bacteria loads present the greatest risk of spreading infection, 17% of transmission occurs from patients with pulmonary TB whose smear tests are negative (associated with lower bacterial load).(44). Smear microscopy is also used to monitor patients on drug treatment to ensure smear conversion to negativity with effective treatment.

A global analysis of the TB diagnostics market in 2006 reported there were nearly 83 million smear tests performed in 2003–2004.(20) This excluded smear tests performed in Australia, Europe, Japan, New Zealand, and North America.

Microscopy is fast, simple, inexpensive, widely applicable, and specific for AFB diagnosis in HBCs. The resultant smear test is scored by interpreting the number of stained bacteria in each field of view in a range from smear negative (0 AFB/100 field) to smear positive 3+ (>10 AFB/field). Traditional Ziehl-Neelsen (ZN) staining uses a light or bright field microscopy at 100X magnification and a trained microscopist can read a maximum of 25-30 slides in a day. However, smear microscopy is not very sensitive in patients with low bacterial load or ineffective for the diagnosis of extra-pulmonary disease. The sensitivity can be improved by chemical or physical treatment such as concentration via centrifugation or settlement after bleach treatment but this increases processing time, biosafety risks to the laboratory technician, and requires dedicated power equipment for processing, if centrifugation is used.

The development of fluorescent dyes that bind to the mycobacterial cell wall has led to improvements in the sensitivity of smear microscopy by ~10% when compared with ZN staining. The smear preparation method for fluorescence detection is faster and the reading of slides at 40X magnification rather than 100X takes a microscopist less time to read a slide, allowing 60 slides to be screened per day as opposed to 25 using the ZN method. Until recently, the use of fluorescence microscopy (FM) in peripheral microscopy clinics was not possible due to the cost, bulb lifespan, appropriate maintenance, storage, and power requirements of mercury vapour fluorescent microscopes. The development of more robust fluorescence microscopes incorporating light emitting diode (LED) technology permits the use of FM at the periphery of TB care. FIND has partnered with Zeiss to accelerate the development and demonstration of the Primo Star iLED microscope, a lower cost LED microscope that may be used in peripheral microscopy centres. Other brands are available (e.g. the CyScope® [Partec, Germany]) in addition to units that can be attached to conventional microscopes to convert them into LED microscopes (FluoLED™ [Fraen Corp., Italy], Lumin™ [LW Scientific, USA], and ParaLens™ [QBC™ Diagnostics, USA]). The performance, specifications, and cost of LED microscope units and LED conversion kits have been reviewed.(45)

The LED-based light sources and microscopes have many advantages over traditional fluorescence microscopes including: low cost; robust light source (i.e. warm up and cool down of bulbs); reduced power requirements; greater lifespan and no risk of mercury vapour release from broken bulbs. The roll out of low-cost, high-performance microscopes also expands the utility of peripheral microscopy centres for diagnoses of other diseases such as malaria, leishmaniasis, and Chagas disease.

In spite of this, the overall sensitivity of smear microscopy is modest with low numbers of MTBC and especially with paucibacillary specimens from patients with HIV co-infection. Although LED FM has significant benefits over both ZN microscopy and conventional FM, its implementation and validation may be prone to difficulties which could hamper evaluation of its performance. For example, fluorescence-stained smears rapidly fade, and this poses challenges for blinded re-checking after a period of time.(46) Adequate training and detailed standard operating procedures are important to maximize accuracy.(47) WHO recommends that LED replace conventional fluorescent microscopes and be phased into microscopy facilities using ZN. Suitable equipment, standard procedures (including external quality assurance [EQA]), and their implementation are being developed and/or evaluated.(48)

Currently, depending on national TB programs, sputum specimens are collected on three separate days and each is analysed via smear microscopy. An increased number of specimens were thought to increase the sensitivity of the test. However, the requirement of patients to present three independent specimens to a clinic on multiple days can lead to the loss of the patients if they cannot afford to be present at the microscopy centre. In an effort to improve the efficiency of smear microscopy, several changes to the testing algorithm have been endorsed by the WHO (see Table A3.1 in Appendix 3). The WHO, based on the review by Mase *et al.* decided to review the strategy of 3 sputum smears for diagnosing pulmonary TB.(49) But by switching to two smears, the WHO needed to also change the definition of what constitutes a positive smear. In 2007 the case definition of "TB positive via smear microscopy" was changed from two positive smears out of three to one positive smear (see Table A3.1 in Appendix 3). With the implementation of effective EQA systems and documented good-quality microscopy only one smear positive is required to identify a new case of TB.

To complement this activity, in March 2010 WHO recommended that same-day microscopy on two samples result in test performance comparable to spot testing on different days (Table 2). This reduces the workload from the previous test algorithm of three specimens and reduces loss to follow-up as more patients can afford the time to be present at a microscopy centre. A recent randomized trial further demonstrated that there was insignificant change in performance using the two-spot method when compared with the three-specimen screening process.(50)

To improve detection of acid fast bacteria via smear microscopy, the sample can be concentrated. This can be performed rapidly via centrifugation, but this also requires sealed buckets to reduce the risk from aerosolization of the sample. Trained staff, infrastructure, and reliable power are required. A simple and arguably effective alternative method is to treat liquefied sputum with hypochlorite (bleach), then leave the specimen overnight to allow the cells to sediment (see Table A3.1 in Appendix 3).(51) WHO considered the bleach method and decided not to recommend or endorse the approach after reviewing evidence, summarized in a systematic review.(50) A further alternative is to concentrate MTBC cells from sputum by using modified magnetic beads that bind to the mycobacterial cell wall. This allows for a rapid and instrument-free concentration of TB cells for analysis by FM. An initial evaluation found that sensitivity was not significantly improved by this method. This method is undergoing further development.(52) Research is also being done on various approaches to improve microscopy, including the use of mobile phones,(53) an array of miniature microscopes (54) and automated reading of stained smears using image analyses software (e.g. Signature Mapping TBDx, by Guardian Technologies Intl. Inc.). However, none of these technologies is ready for WHO review or endorsement.

Benefits: Smear microscopy is a low-cost method that has been implemented as the frontline tool for TB diagnosis across the developing world. Testing can be performed in basic laboratories and is effective in picking up the most infectious cases and identifying patients with a high bacterial load of TB in sputum. The simplification of test algorithms has reduced the number of TB positive cases that are lost to follow up and, with the implementation of EQA protocols, permits faster diagnosis of MTBC. The introduction of fluorescence microscopy has increased the sensitivity of the test, and, by simplifying staining and counting procedures, it allows microscopists to prepare and analyse larger volumes of tests—from around 25 slides per day up to 60.

Drawbacks: The detection rate with microscopy is limited to TB with a relatively high bacterial load and, even with improvements in detection via fluorescence, a large number of cases remain undiagnosed. It is estimated that 17% of transmission occurs from patients with smear-negative pulmonary TB and therefore microscopic diagnosis does not detect a significant proportion of cases.(44) A shortage of trained personnel can limit the amount of testing performed. In addition, extra-pulmonary TB cannot be diagnosed with sputum smear microscopy. In a study comparing the sensitivity of smear microscopy (using three expectorated sputum specimens) to culture on a cohort of patients infected with HIV, only 55% of TB cases were identified by microscopy.(55) Microscopy also cannot determine drug sensitivity. Lastly, the effective implementation of external quality assurance systems to maintain high standards and improve diagnostic skills can be difficult in some laboratories.

Nucleic acid amplification technologies (NAAT)

The amplification of nucleic acids (DNA or RNA) for the diagnosis of TB or to detect drug resistance is a sensitive method that can produce a much faster result than by conventional culture methods. Polymerase chain reaction (PCR) is the most common method of amplification. Assays and platforms have been developed to address a variety of roles in TB diagnosis and control. In addition to commercial assays, there are many protocols for so-called "home brew" assays, especially using PCR, and these produce highly inconsistent results.(56)

The majority of these tests have not been extensively validated. The time-to-results for testing with these assays is typically 6-8 hours, although the processing times of sputum are not included. Rapid testing of this type is particularly informative for extra-pulmonary TB, especially tuberculous meningitis, a severe form of TB. In this case, microscopic smear analysis is often not informative, and culture can takes weeks for a result.

Assays are commercially available from: Roche (Amplicor, PCR, FDA-approved), Becton Dickson (BD Probe Tec, strand displacement amplification [SDA]), Genprobe (Amplified Mycobacterium tuberculosis Direct [AMTD], FDA-approved), transcription mediated amplification [TMA]), Hain Lifescience (GenoType Mycobacteria Direct, PCR) and Cepheid (GeneXpert® MTB/Rif, nested real-time PCR). These are well validated and, in some cases, endorsed by WHO (see Table A3.1 in Appendix 3). Additional NAAT products aimed at replacing smear microscopy are in development. For example, the loop mediated amplification [LAMP] method to amplify MTBC DNA with manual specimen processing and a simple colorimetric method to score positive results has been developed by Eiken (Japan) and FIND (see Table A3.2 in Appendix 3 and Figure 2).(57) The Loopamp® Tuberculosis Complex Detection Reagent Kit was released in March 2011 by Eiken, but the test has not yet been endorsed by WHO.

The sensitivity of nucleic acid assays to detect TB is high (>95%) in sputum smear positive samples with specificities of 90-100%.(58) The sensitivity of many NAATs is greatly reduced when smear negative/culture positive sputum specimens are tested. The physiology of the mycobacterial cell wall makes it challenging to extract DNA from cells and, allied with an initial low number of cells, greatly reduces the amount of available DNA for concentration, purification, and subsequent amplification. Current amplification technologies rely upon appropriate laboratory infrastructure to house the delicate equipment and the reagents are often cold chain dependent. The complexity of non- or partially automated NAATs requires highly skilled technicians. The risk of contaminating the test site with amplified DNA also requires stringent quality control procedures and a specific containment infrastructure. Ideally, a series of rooms are designed for specific NAAT activities (e.g. DNA extraction, reaction preparation, reaction amplification, and post-amplification manipulation). Many of the processes for PCR-based diagnosis can be automated, but the cost and the complexity of maintenance make this difficult to achieve in most developing country settings other than in reference laboratories.

The Cepheid Xpert MTB/RIF test, based on the GeneXpert® system is an advance in the field of MTBC diagnosis via PCR amplification of MTBC DNA. In addition to identifying MTBC, Xpert MTB/RIF test GeneXpert® can also identify common rifampicin (RIF) drug-resistant alleles to that may indicate MDR-TB. By incorporating DNA extraction and amplification in a single, sealed cartridge, this system avoids many of the necessary laboratory and biosafety infrastructure and skills requirements typically required for NAAT-based diagnosis of TB. A sputum sample is first liquefied and chemically inactivated to kill MTBC cells. An aliquot of this is placed into the cartridge, which is then sealed and inserted into the machine for analysis. The GeneXpert® automatically extracts the DNA from the sample, performs nested real-time PCR, analyses the data, and gives a result in under 2 hours. Other than initial training in the use of the device, no further training is needed. The GeneXpert® unit that has been tested for the Xpert MTB/RIF cartridge has four test modules that operate independently so that tests may be run individually on the same instrument. Larger test modules are available—e.g. fully automated

16- and 48-unit instruments. The instrument with a 48-cartridge capacity, the Infinity, is capable of processing up to 2074 tests in 24 hours.

The GeneXpert® and Xpert MTB/RIF have undergone extensive evaluation by FIND and others, with encouraging performance data regarding sensitivity for identification of MTBC and rifampicin resistance. In addition to excellent sensitivity with smear positive specimens, the device is also effective in identifying 72.5% of smear negative/culture positive samples from single tests. With further tests, the sensitivity was incrementally improved to 90.2% after three tests.(59) The results of the FIND evaluation studies were confirmed in a larger multi-country demonstration study.(59) This performance is much better than other existing PCR assays which typically have good performance only on smear positive samples.

All of the test reagents are stored in the cartridge and have a shelf life of 18 months and can be stored at up to 28° C. Although the assay was initially developed for the detection of pulmonary TB, preliminary evaluations have been performed using extrapulmonary specimens with encouraging results.(60;61) Early evidence is also promising for the use of this technology in children.(62) In the last two years there have been 37 peer-reviewed articles assessing the performance of the GeneXpert® and Xpert MTB/RIF assay.

WHO recommends that the Xpert MTB/RIF is suitable for use at district and sub-district level and should not be restricted to central/reference laboratory level. Patients in need of testing include persons with TB/HIV co-infection and cases suspected of MDR-TB. Preliminary roles for the GeneXpert® system and where it is best placed within country TB programs to give maximum benefit are still being assessed. The primary concerns with the GeneXpert® are its cost, availability of second-line treatment for MDR-TB and that it may not be suitable for use in peripheral facilities.(63) Like all sophisticated tests, the equipment is expensive and needs adequate storage of the test device and consumables within acceptable temperature ranges. A computer and continuous power supply are necessary, and an uninterrupted power supply unit is recommended. Disposal of used test cartridges may also be difficult in rural areas. The cost of the device is ~$17,500 with each test cartridge costing $16.68. Based on a volume-based price reduction agreement negotiated between FIND and Cepheid, the cartridge will drop in price to approximately $10 if sales targets are met.(64) Other costs are associated with annual maintenance ($1,600). A recent cost-effectiveness analysis has suggested that the GeneXpert® is a cost-effective method of TB diagnosis, but cost-effectiveness will clearly depend on the context and setting.(65)

WHO has released a series of test algorithms for use of Xpert MTB/RIF in HIV co-infected patients, MDR-TB and, diagnosis of TB.(64) Due to the current costs associated with each test as compared to smear microscopy, WHO has published recommendations for the roll out of the Xpert MTB/RIF and has highlighted that screening of PLHIV and cases of suspected MDR-TB are of greatest significance.(64) In areas of low MDR-TB prevalence, the Xpert MTB/RIF assay results for positive cases should be confirmed by an alternative method or a second Xpert MTB/RIF test. In areas of high MDR-TB, the specificity of assay is adequate to assume that rifampicin resistance will be a good marker of MDR-TB.(63)

Isothermal amplification technologies have great potential for nucleic acid testing for MTBC and other diseases in peripheral facilities.(66) Isothermal amplification operates at a uniform incubation temperature unlike PCR which is cyclical and the detection of amplified DNA can be simplified, thereby dispensing with the need for complex thermal cycling equipment with precision optics reducing capital costs on equipment. This creates a technology that is minimally instrumented with the potential for use in peripheral test sites, such as microscopy centres. In addition, isothermal amplification methods often have faster reaction times and similar performance and appear to be more tolerant to inhibitory compounds than PCR.

A variety of novel isothermal amplification technologies are described that have potential for high performance and rapid TB diagnostics in a simple-to-use and robust format necessary for low-resource settings. These include loop mediated amplification (LAMP, Eiken, Japan),(67) recombinase polymerase amplification (RPA, TwistDx, UK),(68) cross priming amplification (CPA, Ustar Biotechnologies, China),(69) helicase dependant amplification (HDA, BioHelix Corp. USA),(70) and Nicking enzyme amplification reaction (NEAR, Ionian Technologies, USA). Epistem (UK) has partnered with Xcelris (India) to market a rapid TB diagnostic test using their Genedrive™ technology. This is a handheld device which incorporates a rapid NAAT to identify specific MTBC markers.

It should be noted that currently there are no POC NAATs approved for use in any health-care settings in the developed or developing world.(7) In an effort to create a POC TB test kit, FIND has been collaborating with Eiken to develop a manual DNA extraction and rapid TB assay using LAMP.(67) While the assessment of this

assay is intended for review by WHO in 2012 (Table 3 and Figure 2), a recent report has described the performance of the LAMP assay kit for the detection of TB from sputum.(57) This preliminary study demonstrated that LAMP TB assay had comparable performance with other commercially available PCR–based assays and, when using unprocessed sputum, only required one hour to perform testing after receipt of the specimen.(57) The LAMP assay products can be visually detected via fluorescence under ultra-violet light.

Ustar Biotechnologies Ltd. (China) recently described their CPA TB assay to amplify TB target DNA. In their initial study, it was reported to have high sensitivities for both smear positive (96.9%) and smear negative/ culture positive specimens (87.5%).(69) The labelled amplicons are subsequently detected after the amplification reaction by using an enclosed immunochromatographic strip detection system. Unlike antigen-based rapid diagnostic tests (RDTs) where the sensitivity is dependent on the amount of target biomarker available in the specimen, NAAT detection-based RDTs have good performance. The amplification of target DNA prior to testing creates adequate levels of labelled product for detection on the strip. The CPA TB assay takes one hour and is currently undergoing trials in China to evaluate its performance in decentralized laboratories.

Benefits: The application of NAAT has created some very sensitive and specific diagnostic tools for TB. The principle advantages of NAAT is that a result can be obtained in only a few hours and the test process can be adapted for high- or low-throughput screening depending upon demand. For TB diagnostics, the Cepheid GeneXpert® format has been particularly exciting given its performance relative to smear microscopy and the added advantage of being able to diagnose MDR-TB in populations where rifampicin resistance is high. The simplicity of the almost full automation and the cartridge design significantly reduces the need for highly skilled staff, and careful design of the system allows testing outside of a large laboratory; test results are available within two hours. The development of isothermal NAAT based assays, such as LAMP, have the potential to move testing into all microscopy centres as the Loopamp® Tuberculosis Complex Detection Reagent kit format is simple to use, requires minimal equipment, and produces interpretable results without the need for an analyser. The development of fully enclosed detection devices to identify target amplicons produced by other isothermal NAATs may further improve the interpretation of test results while still preventing amplicon contamination at the test facility.

Drawbacks: The main concern, as was noted with the GeneXpert® technology, is the cost of the instrument and test cartridges. Currently, the cost of a cartridge can equal the annual per capita health budget for HBCs.(71) In addition, the annual recalibration of GeneXpert® devices requires shipping to France, which may be disruptive. There is a midterm goal to move calibration to regional facilities and a longer term goal of recalibration at or close to the test sites via calibration kits and online tools.

Adequate maintenance due to equipment failure remains unclear, although each test unit can be replaced or removed without affecting the other test units. The specificity for rifampicin resistance in populations where MDR-TB is rare requires confirmation by repeat testing and increases cost.

Although undoubtedly promising, limited performance studies have been described for the manual isothermal NAAT based tests (57;67;69) and long term stability of the reagents at elevated temperatures has yet to be reported. The performance of the LAMP assay and other isothermal NAATs on sputum specimens need to be improved as performance is currently similar to smear microscopy and, in the case of LAMP, visual interpretation of test results via colorimetric change can be difficult. Sealed cassette-based RDTs as described by BioHelix and Ustar may provide better interpretation but with an increased cost per test. In a recent review of POC tests for TB diagnosis, McNerny *et al.* noted that a limitation of the LAMP assay in the fluorescence detection format is that the current design is monoplexed and a process control to confirm specimen integrity or functionality of test reagents cannot be included in this format.(72)

The risk and rate of test site contamination via accidental release of DNA amplicons has not been assessed and needs to be fully understood. Effective decontamination materials and procedures are also needed to ensure appropriate preparation of contaminated facilities. All tests use sputum, which is far from ideal when diagnosing TB in PLHIV, paediatric patients or patients with extra-pulmonary TB. A recent evaluation of GeneXpert® in an HIV cohort had a sensitivity of only 44% with patients who had CD4 counts greater than 200 cells/ µL.(73)

Indirect detection of drug resistance via nucleic acid analysis: The conventional method of examining the phenotypic characteristics of drug resistance can takes from weeks to months depending on the culture method used to assess susceptibilities.(74) For MDR-TB control to be more effective, a rapid determination of MDR status is necessary. A variety of genotypic methods have been developed whereby common genetic elements

associated with the drug resistance of MTBC are interrogated to indicate the presence of drug-resistant alleles. These target regions are first amplified by PCR and then screened for the presence of specific mutations that have been correlated with drug-resistant phenotypes. The advantages of these include, rapid DST in days rather than weeks, no need to handle MTBC culture, and the potential for a lower cost per test.

Line probe assays (LPA) have been developed and evaluated to perform screening for alleles commonly associated with drug resistance using smear-positive sputum samples directly or to perform rapid drug resistance screening on culture isolates. Two LPA tests are commercially available: the Inno-LiPA Rif.TB line probe assay (Innogenetics, Belgium) and the GenoType MTBDR*plus* assay (Hain Lifescience, Germany). The LPA tests are performed in reference level facilities as they need dedicated rooms for DNA preparation, amplification and a biosafety level 2 (BSL2) laboratory for processing sputum, or a BSL3 laboratory, if manipulation of MTBC culture is required.

LPA tests produce information on MTBC identification with rifampicin (Inno-LiPA Rif.TB) and also isoniazid (GenoType MTBDR*plus*) resistance in 1-2 days rather than weeks as with culture DST. While the Inno-LiPA product tests only for common mutations in *rpoB* gene that are associated with rifampicin resistance; in high-burden areas, the association of these with MDR-TB is 90%. As a result, suspicion of MDR can be applied with a high degree of confidence, similar to the Xpert MTB/RIF assay. The GenoType MTBDR*plus* assay also targets the common mutations in *katG* and *inhA* genes associated with isoniazid resistance in addition to *rpoB* mutations for rifampicin resistance.

The general LPA protocol first involves DNA extraction from a sputum specimen or TB culture. The DNA targets are then amplified by PCR and hybridized to oligonucleotide sequence-specific targets immobilized on a nitrocellulose strip. The captured hybrids are developed via a colorimetric labelling to establish MTBC. The presence of wild type or mutant (drug resistant) alleles is established by identifying a pattern of the stripes to a chart indicating specific alleles. The results can be interpreted visually or via an instrument. The time-to-result using smear positive sputum is 4–5 hours rather than a minimum 60 days using culture methods. The LPA assays have been demonstrated to have appropriate performance and if the original specimen is smear positive samples can produce enough MTBC for adequate PCR amplification. Paucibacillary samples or samples with inappropriate DNA preparation will not give adequate PCR amplification and therefore poor prediction of drug resistance.

Evidence on LPA (75) was reviewed by the STAG-TB, and LPA has been recommended by WHO as a rapid tool for MDR-TB diagnosis (see Table A3.1 in Appendix 3).(76) It does not replace DST or culture confirmation of smear negative samples but complements it by reducing the overall number of culture tests required. A preliminary cost analysis in South Africa has suggested that LPA was lower in cost than using conventional culture. Costs were estimated to be 50% to 30% less depending on testing sputum or cultured cells, respectively. FIND has negotiated pricing with Hain Lifescience (Germany) for components of the GenoType® MTBDR*plus* assay ($4.75 per test). Ancillary equipment includes GT Blot ($13,150 or $19,000), TwinCubator® ($3,000), and GenoScan® ($15,500). The cost per test in country for China and India and for countries in South America is estimated to be ~$10.

Hain Lifescience are now developing a new LPA-based product to detect MTBC as well as common allelic biomarkers for resistance to ethambutol another first-line drug and also fluoroquinolones and aminoglycosides, two second-line drugs (Appendix 1). The product, GenoType® MTBDR*sl* (MTB Drug Resistance *second line*) is designed to identify common mutations in *gyrA*, *rrs,* and *emb,* which can lead to resistance to fluoroquinolones, aminoglycosides, and ethambutol.(77) The test is performed in a similar fashion to the GenoType MTBDR*plus* assay and test results are created in the same format: a striped test strip is compared to a reference strip to identify the presence of wild type or drug resistant alleles. The performance of the MTBDR*sl* is expected to be reviewed in 2012 (Table 3).

A further development for the genotypic detection of MDR-TB is the use of DNA microarrays, which are similar in principle to LPAs and use oligonucleotide sequence specific targets that represent sensitive or drug-resistant genotypes. Microarrays may offer the potential for mycobacterial identification and/or for more drug resistance alleles screened for first-line drug resistance. There is also the potential to screen for resistance to second-line drugs, such as fluoroquinolones. The oligonucleotide targets are arrayed in a matrixed grid. PCR amplification is also required to generate labelled DNAs, which are then hybridized to target probes on the microarray and the test data developed via a scanner.

The growing use of microarrays in academic and commercial research and development has led to the development of automated systems where high-throughput hybridization and processing of multiple arrays is in practice. Commercially developed assays to identify mycobacteria and/or perform genotypic DST for MDR are available from Autogenomics (INFINITI® MDR-TB Assay, USA), CapitalBio (*M. tuberculosis* Drug Resistance Detection Array Kit, China), the Engelhardt Institute of Molecular Biology at the Russian Academy of Sciences (TB Biochip, Russia), Akonni (TruArray® MDR-TB Test, Canada [analogous to the TB Biochip), and Chipron (MYCO Resist 3.0, Germany). None of these formats are currently recommended by WHO. It should, however, be noted that the CapitalBio and Engelhardt Institute assays are in demonstration studies in China and Russia, respectively, and this reflects the emergence of sophisticated diagnostic technologies for the rapid identification of MTBC and MDR-TB.

Benefits: LPA strips can use smear positive sputum as a specimen and provide a result in 1–2 days that can be used for diagnosis MTBC disease and also drug resistance, thereby allowing health-care workers to begin and administer an effective treatment regimen to patients. The DST testing of culture isolates can be performed in 1–2 days rather than several weeks. DNA microarray systems are automated to interpret and score test results reducing user training requirements to identify actionable results.

Drawbacks: Direct use of the tests is effective only with smear positive sputum. Test facilities are at the reference level only and require dedicated equipment and reagents. In general, LPAs are expensive for many low-resource settings. Molecular drug resistance screening has technical limitations: LPAs can detect only well characterized drug resistance alleles, not every drug resistance allele can be discriminated via current tests, and silent mutations (which do not confer drug resistance) can be detected by probes leading to misclassification of drug resistance. In addition, molecular tests cannot determine the proportion of drug resistant bacteria within a mixed population of cells (i.e. wild type and drug resistant). Cross contamination with amplicons generated from previous tests can be problematic especially when the tests have been employed in laboratories without appropriate staff training and quality control. Microarray technology is expensive to procure and use, and requires highly trained staff and adequate ancillary infrastructure. The equipment is delicate and adequate maintenance would be difficult in many resource-limited settings.

Immune response-based diagnostic tests

Tuberculin skin test (TST). The current WHO policies regarding immune based TB diagnostic products are listed in Table 2. Infection with MTBC can result in an immune response from the patient indicating latent TB infection (LTBI). TST is a test that can indicate TB infection, but cannot discriminate latent from active forms of TB. A small amount of TB antigen (purified protein derivative [PPD]) is intradermally administered into the forearm and re-examined 48-72 hours later. If there has been previous exposure to MTBC a palpable, raised, hardened area or swelling (an induration) is measured. The diameter of the induration can be associated with the infection; typically > 10 mm indicates LTBI. However, vaccination with the Bacillus Calmette-Guérin (BCG) vaccine in developing countries can produce a positive response. The main limitation of TST is its modest specificity in those vaccinated with BCG and its low sensitivity in populations—immuno-deficient individuals and young children—with the highest risk of progression to active TB. In addition, TST placement and reading is a subjective procedure and examination of the induration requires a return visit to the clinic.

Table 2: Current WHO policies regarding the use of immune based TB diagnostic products

Tool/Approach	Status	Method
Serological antibody detection tests	Negative recommendation by WHO in 2011: WHO strongly recommended that these commercial tests not be used for the diagnosis of pulmonary and extra-pulmonary TB.(78)	Commercially available antibody detection rapid tests and ELISA tests
Interferon-gamma release assays [in low- and middle-income countries]	WHO recommended that neither IGRAs nor TST should be used for the diagnosis of active TB disease; IGRAs are more costly and technically complex to do than TST. Given comparable performance but increased cost, replacing TST with IGRAs as a public health intervention in resource-constrained settings is not recommended.(79)	Commercially available IGRAs (QuantiFERON-TB Gold In Tube and T-SPOT.TB)

Interferon gamma release assays (IGRAs). Interferon gamma release assays (IGRAs) are whole-blood, *in-vitro* tests that can aid in diagnosing *LTBI*. The basic principle of the test is that white blood cells from persons previously infected with *MTBC* will release interferon-gamma (IFN- **IFN-ϒ**) when stimulated with MTBC antigens. The **IFN-ϒ** is then measured to establish a test result. Two different commercial tests measure either the amount of **IFN-ϒ** (QuantiFERON®-TB Gold In Tube, Qiagen, Germany), or the number of **IFN-ϒ** producing cells (T-spot®. TB, Oxford Immunotec, UK) with a result in 24 hours. The test appears to be more specific than TST (which reflects only recent exposure), because BCG vaccination does not affect the result. A recent meta-analysis compares TST and IGRAs with regard to various characteristics.(80)

Benefits: IGRAs are more specific for latent TB infection than TST, and the *in-vitro* format is more convenient in terms of logistics.

Drawbacks: IGRAs, like TST, cannot separate latent infection from active disease and should not be used for diagnosis of active TB. Children or individuals who have recently been infected do not have a strong reaction and immuno-deficiency disorders can produce an indeterminate result. The assays are relatively expensive and require a laboratory to process the results. The test specimen requires a venous blood draw.

Longitudinal, cohort studies show that both TST and IGRAs have low predictive value: a majority of those with positive results do not progress to active disease and therefore do not benefit from isoniazid preventive therapy. (81) IGRAs are accepted tests for latent TB infection and are widely used in low TB incidence settings. Several guidelines are now available,(82) however, stating that their role and value in high TB burden settings appears to be limited. WHO recently published a policy document for low- and middle-income countries based on a series of systematic reviews.(83-86) The current WHO policy (87) states that:

1. There is insufficient data and low quality evidence on the performance of IGRAs in low- and middle-income countries, typically those with a high TB and/or HIV burden.
2. IGRAs and the tuberculin skin test (TST) cannot accurately predict the risk of infected individuals developing active TB disease.
3. Neither IGRAs nor the TST should be used for the diagnosis of active TB disease.
4. IGRAs are more costly and technically complex to do than the TST. Given comparable performance but increased cost, replacing TST by IGRAs as a public health intervention in resource-constrained settings is not recommended.

This policy is only for low- and middle-income countries and is not meant to supersede guidelines that are already implemented in high-income countries (e.g. guidelines from the US Centers for Disease Control and Prevention [US CDC]). A more recent assessment of the European Centre for Disease Prevention and Control (ECDC) also concluded that IGRAs should not replace the standard diagnostic methods for diagnosing active TB.(88) A detailed comparison of TST and IGRAs is in Appendix 2.

Serodiagnostic assays

An RDT targeting an antigen or effective biomarker indicative of MTBC infection is urgently needed as no effective and simple-to-use tool exists for TB diagnosis at the POC, and the current tests are less effective in groups that are more susceptible to TB-related mortality, such as young children and PLHIV. The development of rapid, antibody based serodiagnostic tests has facilitated the effective diagnosis of HIV-1 infection and other diseases, such as malaria, hepatitis B, hepatitis C, and Chagas disease via RDT strips at peripheral health centres.

At this time, there is not an appropriate RDT for MTBC diagnosis and is urgently needed. A large variety of commercially produced TB serodiagnostic tests exist. Some are laboratory-based tests (e.g. Enzyme-linked immunosorbent assay [ELISA]) and others are in an RDT format, but none has appropriate performance; TB serodiagnostic tests should therefore not be used.(89) An updated meta-analysis published in 2011 shows that serological antibody detection tests produce highly inconsistent results and have no clinical value, (90) while another study showed that these tests are not cost-effective when compared to conventional tests for active TB. (91) Based on this evidence, WHO made the unprecedented step of not recommending the use of current, commercial TB serodiagnostic tests in individuals with suspected, active pulmonary or extrapulmonary TB, irrespective of their HIV status or age.(92)

The available evidence indicates that these current tests lack either the necessary sensitivity or specificity or both to be an effective diagnostic test. For many of these tests, false results far outnumber positive results. In many cases, low sensitivity does not detect active TB leading to increased morbidity and mortality, especially in regions with high HIV comorbidity. A low specificity test results in many patients receiving TB therapy when there is no need, thus wasting resources and allowing underlying conditions to remain undiagnosed. Unfortunately many of these tests are sold to and used in the private sector, and limiting their continued use not possible. In fact, commercial serological tests are available on the market in 17 of 22 TB HBCs, many of which have weak regulation.(93)

The future use of serodiagnostic assays has not been dismissed by WHO. In fact, the WHO policy strongly recommends more research to develop POC tests based on antibody/antigen detection. There are multiple, ongoing efforts aimed at identifying more accurate biomarkers. These efforts have the potential to develop accurate serologic assays, which could fill the POC niche.(7)

Benefits: User training is straightforward and the RDT version is low cost, rapid, and non-instrumented. The test uses a finger prick blood specimen and can be performed at the lowest tier health-care settings.

Drawbacks: Current tests have unacceptable performance and all are inappropriate for the accurate diagnosis of MTBC. More appropriate biomarkers have yet to be identified or assessed for their utility but research is actively being pursued to identify biomarkers.

Alternative antigen assays

The presence of antigen biomarkers derived from MTBC disease are currently being used in immunodiagnostic tests, but their performance is poor.(94) Lipoarabinomannan (LAM) is a major lipopolysaccharide constituent of the cell wall of MTBC. During the degradation of the bacterial cell wall, the LAM antigen enters the bloodstream and is filtered by the kidneys, passing into the urine. The detection of LAM in urine has the advantages of being able to provide a diagnostic result independent of the location of infection and specimen collection is non-invasive and easy to obtain from patients in all age groups. A sputum-based sample is only useful for the diagnosis of pulmonary TB, whereas urine-based LAM antigen testing may provide an indication of both pulmonary and extrapulmonary TB.

Two LAM ELISA-based assays have been developed by Alere (USA): Clearview® TB ELISA and Determine® TB-LAM. The former is a laboratory-based ELISA assay and the latter is in a RDT strip format. A meta-analysis of the LAM ELISA has shown performance to be suboptimal in many cases.(95) Of particular note is that LAM assay sensitivity is highest when CD4 counts decrease below 200/µL. LAM ELISAs may have utility within this population, but more evaluation is necessary to establish the performance of the assay in patients with advanced HIV infection who are suspected of having TB coinfection.(96) A recent study assessed both of Alere's LAM assays, used in testing PLHIV and who were suspected of having MTBC coinfection, with the goal of determining the potential of the Determine® TB-LAM RDT to diagnose TB.(73) In terms of performance, sensitivity was best with patients with CD4 counts of < 50 cells/µL and overall specificity was greater than 98%. These early data do suggest that in very high HIV-burden areas where advanced HIV-associated immunodeficiency is common, the Determine® TB-LAM could provide early rapid diagnosis of TB coinfection.(73)

Benefits: TB coinfection is commonly associated with HIV and a rapid test that can be used to screen for TB in PLHIV with advanced immune suppression would be of great benefit to those most vulnerable to the disease. Tests are low cost, rapid, use only 60 µL of urine, and can detect all sites of MTBC infection.

Drawbacks: Performance of the test is poor in persons without HIV infection, and in HIV-infected persons with CD4 counts $>$ 150 cells/µL. The test is also not specific for MTBC as all mycobacteria produce LAM as a byproduct of cell wall degradation.

Volatile organic compounds

An expanding field of diagnostic research has stemmed from the study of metabolic biomarkers derived from either TB or the host, which can be assessed in a variety of specimen types, including sputum (97;98), urine (99) and breath.(100-103) When TB is active, the pathogen releases volatile organic compounds (VOCs). The use of VOCs, in terms of creating diagnostic tools, has been already demonstrated in low-resource settings us-

ing trained Giant African pouch rats to identify TB from sputum. This is an apparently effective, diagnostic tool when compared to smear microscopy.(97;98) Although pouch rats cannot be considered as a global solution to improving TB diagnosis, they have been shown to be effective in diagnosing pulmonary TB and demonstrate that VOCs indicative of pulmonary TB disease are produced in sufficient amounts for diagnosis.

The VOCs in an individual's breath and urine consist of a variety of different organic compounds and gases representing a variety of compounds including oxidative stress products, volatile metabolites of MTBC (101) and nitric oxide.(104) A recent study investigating the potential of urine for VOC biomarkers indicating TB found five compounds that were indicative of TB disease.(99) The advantages of this technology include a rapid and non-invasive test that could screen all patients for active TB, and potentially other infectious and non-communicable diseases.

A POC device from Menssana Research Inc. (USA) is under development to detect TB via metabolic biomarkers that are indicative of pulmonary disease.(101) However, most current research into effective analysis methods is in early development, is laboratory-based, and uses complex equipment (including mass spectrometers and gas chromatographs) requiring highly skilled technicians. The ability of MTBC to release VOCs, which can be identified as a unique signature, offers tremendous future potential for new diagnostics although validated systems to effectively perform this in low resource settings have yet to be found.

Benefits: Non-invasive specimen collection and may also be used with PLHIV and paediatrics. Time-to-result is fast, and other ancillary test accessories may not be required.

Drawbacks: Technology is still in the early development stage and no product is undergoing demonstration. The size, complexity, and power requirements of analytical equipment may preclude testing close to the POC, and it is unclear if a pulmonary screening tool can detect cases of extra-pulmonary TB. It is also unclear if MTBC can be discriminated from NTM via VOCs.

Unmet Needs and Future Outlook for TB Diagnostics

As this and other recent reviews on the current status of TB diagnostics have highlighted, there are no suitable POC TB diagnostic tools that can be used in peripheral health-care settings and none are currently in the development pipeline of potential technologies that are under evaluation or demonstration.(7;72;105) There is, of course, an urgent need for the development of tools that perform similarly to the RDTs currently in use in the most resource-limited settings and for tools that can diagnose TB in children. The TB Research Movement by the Stop TB Partnership and WHO recently published "An international roadmap for tuberculosis research" and the following were identified as the highest-priority topics for improving TB diagnosis and case detection:

> (i) Identification of bacterial and/or host molecules that differentiate people at different stages of the disease spectrum (including predictive markers of progression from latent tuberculosis infection to active TB), and
>
> (ii) Simplification and validation of novel tools for diagnosis at the point of care.

- A high priority is studying how to combine existing and new diagnostics to optimize the detection of various forms of TB (including drug-sensitive, drug-resistant and latent TB infection) in various population settings and at all health-care levels.
- Of great importance are definition and evaluation of the performance of new diagnostic tests in terms of feasibility, cost-effectiveness, reduced diagnostic delay and impact on clinical decision-making and patient benefit.
- Particular reference is made to the need to identify combinations of methods for collecting useful specimens from children.
- Another high priority is development of a systemic marker of bacterial load in TB with various samples and methods.

- The automated nucleic acid amplification test is potentially revolutionary for TB control, but it must be decentralized to points of treatment, and its use would have to be scaled up rapidly in order to achieve an impact at population level, particularly in resource-limited settings.(106)

For new TB diagnostics to succeed, several key areas need to be addressed and strengthened. Discovery needs to be better integrated with delivery, and competition for market share should be tempered with collaboration, especially between academic and commercial groups. While academia continues to produce a myriad of potential opportunities, there is poor translation to products. Collaborative interests between academia and industry will better promote product development.

In the development of effective products there needs to be a better understanding of the health needs for the product design. Ministries of Health and the public and private health-care sectors also play an important role in informing developers as to the appropriate specifications that a product must meet to warrant effective and widespread sustained use. Development efforts must meet the real needs of TB control programs.

In the developing world, the growing economies in countries such as Brazil, China, and India have created significant increases in funding for research and product development from both the public and private sectors. As a result of these opportunities there are large numbers of biotechnology companies being created. This increases the potential to create new diagnostic products, as many companies from these countries see a TB diagnostic product as a viable pursuit given the potential market size within their region. Such small startup companies need guidance to identify accurately the many key components of a target product profile with which to focus research and development activities to meet the core needs for a product.

A recent conference in Bangalore, India, brought together academics, companies, and health-care specialists from the public and private sectors to foster collaboration, create a common understanding of the problems at hand, and identify which tools or products may be available.(107) Of particular note: developers were enthusiastic to learn about the public sector procurement system and what products are needed by national TB control programs. A similar conference is being planned for China in September 2012.

In addition to understanding the target specifications and markets that a product must meet to be of interest to TB programs, an earlier hurdle for developers is access to well-characterized specimen panels with which to guide their product development and provide initial evaluation data. Given the low case rates of TB in developed countries, it is difficult for groups to obtain sufficient numbers of adequate specimens to accurately guide product development and to perform preliminary field trials at a reasonable cost. This is starting to change with the development of repositories containing fully characterized specimens that are available to academics and developers at a reasonable cost.

WHO's Special Programme for Research & Training in Tropical Diseases (TDR) has been amassing a large specimen repositories for discovery and validation purposes since 1999, and specimen panels are available upon application.(108) Currently, there are 41,437 samples (serum, sputum, saliva, and urine) that were collected from adult patients suspected of having TB in 13 countries worldwide. In addition, a strain bank has also been recently developed at TDR to give developers access to fully characterized strains (phenotype and genetic) of MTBC with 236 strains representing all the resistance profiles. With the increase of MDR- and XDR-TB, access to these resources by developers and academic groups is very timely and appropriate. A recent review by Batz *et al.* describes in detail the benefits and gaps in this program.(7)

In the United States, the National Institute of Allergy and Infectious Diseases (NIAID) has created the Tuberculosis Clinical Diagnostics Research Consortium (CDRC). This is intended to be a resource to the TB community in several ways. First, CDRC aims to provide advice and aid to developers and small businesses to create new approaches with which to assess diagnostic tests within the current clinical algorithms and, second, they offer specimen panels to assist in development efforts of prototypes and can provide access to trial sites in Brazil, South Africa, South Korea, and Uganda for evaluation studies. These sites are chosen not only for their diverse geography and ethnicities, but also because they reflect the endemicity of MTBC in terms of association with high and low prevalence rates of MDR-TB and comorbidity with HIV.

Once diagnostic devices have been developed and partially evaluated, one of the greatest hurdles facing their eventual roll-out is the cost and time taken for sufficient evidence gathering prior to review and endorsement by WHO's STAG-TB.(109) The adequate demonstration of products and WHO endorsement are essential for uptake

by country TB programs. This may be expedited in the future by creating harmonized protocols and permitting accurate comparison in multiple settings in order to facilitate more rapid uptake by country programs once an endorsement is made. In recent years, FIND has successfully led the demonstration efforts with the Bactec MGIT test, the Capilia TB test, Hain Lifescience LPA, Zeiss Primo Star iLED, and Cepheid GeneXpert® systems being introduced and the manual LAMP TB kit and the TLA assays in development. Other academic, not for profit, private healthcare companies, and national public health organizations are also currently evaluating diagnostic devices for MTBC. Closer collaboration between these groups is needed in the global TB community so that when appropriate tests are developed their entry into public health is not delayed by repeated evaluations due to differences in study design.

APPENDIX 1:
Tuberculosis Diagnostics Currently in Use* or under Development§

Diagnostic Platform	Manufacturer	Platform Name	Description
Culture-based	Beckton Dickson	Bactec960 MGIT*	Liquid culture system. Fully automated systems that use either fluorimetric or colorimetric detection of mycobacterial growth and can be used for the identification of MTBC and for DST for both first and second line drugs.
	bioMérieux	BacT/ALERT*	
	Trek Diagnostic Systems Inc.	Myco-ESP Culture System II*	
	Hardy Diagnostics	CE-marked MODS kit*	A low cost microscopic-observation drug-susceptibility assay that uses liquid culture and microscopy to predict MTBC and identify resistance to rifampicin and isoniazid
MTBC identification from culture	TAUNS Corporation	Capilia TB Neo Test*	Antigen detection-based lateral flow strip tests for MPB64 to provide a rapid confirmation of MTBC from mycobacterial cultures derived from either liquid or solid media.
	Beckton Dickson	TBc ID*	
	Gen Probe	Accuprobe*	A nucleic acid-based test that confirms the presence of MTBC from positive culture media (in addition see LPA assays below)
Phage-based	Biotec Laboratories	FASTPlaque-TB§ FASTPlaque-TB-MDR§i FAST*Plaque-Response*§	Currently not recommended for use by WHO. Mycobacteriophage-based assay (MBA) assays.
Smear microscopy	Carl Zeiss	Primo Star iLED™*	LED microscopes for improved TB microscopy
	Partec	CyScope®*	
	QBC Diagnostics	ParaLens™*	LED conversion kits to provide fluorescence capability to conventional microscopes. Improved TB microscopy
	Fraen Corporation	FluoLED™*	
	LW Scientific	Lumin™*	

Diagnostic Platform	Manufacturer	Platform Name	Description
PCR-based NAAT	Hoffmann-La Roche	Amplicor*	Polymerase chain reaction assay to detect MTBC
	Cepheid	GeneXpert MTB/RIF*	A instrument that uses nested real time PCR to identify MTBC and common rifampicin resistance mutations
	Innogenetics	Inno-LiPA Rif.TB*	Line probe assays that use PCR generated amplicons to detect MTBC and first-line drug resistance
	Hain Lifescience	GenoType MTBDR*plus**	
	Hain Lifescience	GenoType MTBDR*sl*[§]	Line probe assay that uses PCR generated amplicons to indicate MTBC and resistance to one first-line drug resistance and two second line drugs
Isothermal based NAAT	Beckton Dickson	BD Probe Tec*	Strand displacement amplification assay to identify MTBC
	BioHelix Corporation	HDA[§]	Helicase dependant amplification assay to identify MTBC
	Eiken and FIND	Loopamp® Tuberculosis Complex Detection Reagent Kit[§]	A manually prepared Loop-mediated amplification assay designed to diagnose MTBC with a test kit that can be used in intermediate facilities or microscopy centres
	Epistem	Genedrive[§]	NAAT detection to identify MTBC
	GenProbe	AMTD*	Transcription mediated amplification assay to identify MTBC
	Ionian Technologies	NEAR[§]	Nicking enzyme amplification reaction assay to identify MTBC
	TwistDx	RPA[§]	Recombinase polymerase amplification assay to identify MTBC
	Ustar Biotechnologies	CPA[§]	Cross priming amplification assay to identify MTBC

Diagnostic Platform	Producer	Platform Name	Description
Immune response-based	Qiagen	QuantiFERON-TB Gold*	Not recommended for MTBC diagnosis. Interferon gamma release assays to identify active MTBC infection
	Oxford Immunotec	T-spot*	
Alternative antigen assays	Alere	Clearview® TB ELISA§	Not recommended for MTBC diagnosis. Laboratory based ELISA Antigen assay to detect MTBC from urine sample via LAM
	Alere	Determine® TB-LAM§	Not recommended for MTBC diagnosis. Health centre-based RDT to detect MTBC from urine sample via LAM
Volatile organic compounds	Menssana Research Inc.	No product name§	Not recommended for MTBC diagnosis. Determination of pulmonary TB from compounds exhaled in breath

APPENDIX 2:
Comparisons between Tuberculin Skin Tests (TSTs) and Interferon Gamma Release Assays (IGRAs)

Characteristic	TSTs	IGRAs
Potential advantages or benefits	Simple, low-tech test Can be done without a laboratory No equipment necessary Can be done by a trained health worker even in remote locations Effect of BCG on TST results is minimal if vaccination is given at birth and not repeated Longitudinal studies have demonstrated its predictive value and systematic reviews of randomized trials show that IPT is highly effective in those who are TST-positive	Interferon gamma release assays (IGRAs) require fewer visits than TST for test completion (follow-up visits will be needed for both tests for IPT initiation) Potential for boosting test response eliminated with IGRA IGRA interpretation is objective whereas TST interpretation is affected by inter- and intra-reader variation IGRA results can be available within 24-48 hours (but are likely to take longer if done in batches) IGRA does not have cross-reactivity with BCG IGRA has less cross-reactivity than TST with non-tuberculous mycobacteria, though data are limited in low and middle income countries
Risks or undesired effects	TST may give false negative reactions due to infections, live virus vaccines, and other factors TST may give false positive results because of BCG vaccination and non-tuberculous mycobacteria Requires an intra-dermal injection Can rarely cause adverse reactions (acute reactions,, skin blistering and ulceration) Interpretation of serial TST is complicated by boosting, conversions and reversions	IGRA requires a blood draw (which may be challenging in some populations, including young children) Risk of exposure to blood-borne pathogens Risk of adverse events with IGRA may be reduced compared to TST Interpretation of serial IGRA is complicated by frequent conversions and reversions, and lack of consensus on optimum thresholds for conversions and reversions

Appendix 2 Continued: Comparisons between TSTs and IGRAs

Characteristic	TST	IGRAs
Values and preferences	Patients may prefer to avoid visible reaction to TST Patients may prefer not to come back for repeat visit for reading the test result Patients with prior BCG may not trust TST results and may be reluctant to accept IPT Patients may self-read their TST results erroneously	Patients may prefer to avoid blood draw (for cultural or technical reasons) Patients with prior BCG may not trust TST results and prefer IGRA
Resource implications	Less expensive than IGRAs (reagent cost is substantially less than IGRA kit costs), but personnel time costs will have to be factored, along with time and cost for 2 patient visits No laboratory required Need to establish a program with trained staff to administer and read TST results Staff training is needed to minimize reading errors and variability (under reading, within and between reader variability, digit preference, etc) PPD must be stored at optimal temperatures Only standardized PPD must be used	Need to establish well equipped laboratory with electricity, that can perform ELISA or ELISPOT Need to procure equipment and supplies for IGRA performance and quality assurance (IGRA reagents cost higher than TST reagents) Need for staff training, including blood-borne pathogen training Need for cold chain for transport of kits and reagents and for storing them Need for careful handling (e.g. tube shaking) and processing of blood samples (incubation of samples within a specific time window) to ensure accuracy of tests Availability of well trained staff or staff to be trained

APPENDIX 3:
WHO Endorsements and Reviews

Since 2007, there have been nine recommendations and two endorsements from the WHO's Strategic and Technical Advisory Group (STAG-TB) for new diagnostic procedures or test algorithms (see Table A3.1 and Figure 2). Their utility, in terms of improved TB diagnosis and also the identification of multidrug resistant forms of MTBC (MDR-TB), is discussed where pertinent. In particular, the type of facility where the tests can be effectively performed is highlighted. It is apparent that there are currently no effective rapid POC tests for TB. Although several rapid antibody tests are on the market in many high burden countries, there is strong evidence that these serological tests are inaccurate (90) and not cost-effective.(110) In July 2011, WHO issued a strong, negative recommendation against currently available commercial serological tests.(92) WHO welcomes research and development using serological methods, and they are urgently needed since many suspected cases are widespread in periurban and rural areas where access to POC tests would have the greatest impact. While the current pipeline suggests that tests are expected by 2015 (Figure 2), there are several candidates in the pipeline that may be reviewed earlier by WHO (Table A3.2).

Table A3.1: TB diagnostic products and modifications to TB test algorithms that have been recently endorsed by WHO's Strategic Technical Advisory Group of experts (STAG). Adapted from *HIV, Hepatitis C Virus (HCV), and Tuberculosis Drugs, Diagnostics, Vaccines, and Preventative Technologies in Development* (34).

Tool/Approach	Status	Test location	Method
New smear positive case definition	WHO endorsed 2007 http://www.who.int/tb/dots/laboratory/Proposal%20for%20a%20revision%20of%20the%20case%20definition%20of%20Sputum.pdf	Peripheral	Microscopy: Faster time to diagnosis.
Reduction in number of specimens examined	WHO endorsed 2007 http://www.who.int/tb/dots/laboratory/policy/en/index2.html	Peripheral	Microscopy: Reduced burden on laboratories with faster time to diagnosis.
Liquid culture	WHO endorsed 2007 http://www.who.int/tb/laboratory/use_of_liquid_tb_culture_summary_report.pdf	Reference	Culture: MTBC and first/second line DST.
Rapid speciation	WHO endorsed 2007 http://www.who.int/tb/laboratory/use_of_liquid_tb_culture_summary_report.pdf	Reference	Immunoassay: MTBC identification from culture.
Molecular line probe assay (LPA)	WHO endorsed 2008 http://www.who.int/tb/laboratory/lpa_policy.pdf	Reference	DNA-based detection of MTBC and rifampicin and isoniazid resistance.
Front loaded Microscopy [same-day smear diagnosis]	WHO endorsed 2009 www.who.int/entity/tb/laboratory/whopolicy_same-day-diagnosis_bymicroscopy_july10.pdf	Peripheral	Microscopy: to allow for same day diagnosis by testing both smears in one patient visit
Light emitting diode (LED) microscopy	WHO endorsed 2009 www.who.int/entity/tb/laboratory/egmreport_microscopymethods_nov09.pdf	Peripheral	Microscopy: Improved performance.
Non-commercial rapid culture methods (MODS, NRA and CRI)	WHO endorsed 2010 http://whqlibdoc.who.int/publications/2011/9789241501620_eng.pdf	Reference	Culture using Microscopically Observed Drugs Susceptibility (MODS), Nitrate Reductase Assay (NRA) and Colorimetric Redox Indicator (CRI): MTBC detection and first line DST.
Xpert MTB/RIF (GeneXpert)	WHO endorsed 2010 http://whqlibdoc.who.int/publications/2011/9789241501545_eng.pdf	Peripheral	DNA-based detection of MTBC and Rifampicin resistance

Table A3.2: The TB diagnostic pipeline with products and the anticipated time for their performance review via the WHO STAG-TB. Adapted from *HIV, Hepatitis C Virus (HCV), and Tuberculosis Drugs, Diagnostics, Vaccines, and Preventative Technologies in Development* (34).

Pipeline Tools	Status	Test location	Method
TLA DST	Under development/demonstration. Estimated WHO review, 2012.	Microscopy Centre	Culture: Simultaneous MTBC and first line DST testing.
Genotype® MTBDRsl	Under development/demonstration. WHO review, 2012.	Intermediate	DNA-based detection of second line drug resistance.
Manual NAAT	Under development/demonstration. WHO review, 2012.	Microscopy Centre	DNA-based detection of MTBC using loop-mediated isothermal amplification (LAMP).
Determine© TB LAM	Under development/demonstration. Estimated WHO review, 2013.	Peripheral	Immunoassay: mycobacterial disease Dx from urine.
Sensititre®, improved second line DST	Under development/demonstration. Estimated WHO review, 2013.	Reference	Liquid culture: MTBC in addition to first and second line DST.

Drug-resistant TB is becoming more widespread and is found in all of the countries surveyed. In 2010, among the estimated 12 million episodes of TB, it is believed that 650,000 were Multidrug-resistant TB (MDR-TB). (9) Drug resistance initially emerges as a result of inadequate treatment or mismanagement of therapy and is passed from person to person in the same way as drug-sensitive TB. MDR-TB is a form of TB that is resistant to the most important first-line drugs, isoniazid and rifampicin. In their 2011 Global TB Control report, WHO noted that less than 5% of TB patients are tested for MDR-TB, typically due to a lack of laboratory capacity, but new diagnostics tools could improve identification of MDR-TB.(9) It can take two years to treat MDR-TB with drugs that are more toxic and much more expensive.(111) If the drugs administered to treat MDR-TB are mismanaged, further resistance can occur. Extensively drug-resistant TB (XDR-TB) is a form of TB caused by bacteria resistant to all of the most effective drugs (i.e. MDR-TB plus resistance to any fluoroquinolone and any of the second-line anti-TB injectable drugs: amikacin, kanamycin or capreomycin).(112)

In their recent reviews on TB biomarkers and diagnostics, Wallis *et al.* and the TAG pipeline report note the following key points for effective technologies for the diagnosis and treatment of TB.(1;34)

1. The tuberculosis diagnostics pipeline has rapidly grown, with development of several promising technologies.
2. In the existing pipeline there still is not a simple, rapid, inexpensive POC test.
3. Accurate, rapid tests are also needed for smear-negative and childhood tuberculosis, as are tests for latent tuberculosis with increased predictive value for progression to disease.
4. Several diagnostics and diagnostic strategies have been endorsed by WHO and are being introduced into clinical use and national tuberculosis control programs.
5. Governments in all countries, especially industrialized countries, have to increase funding for tuberculosis research and control.

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