

## Diagnosis of TB: From conventional to modern molecular protocols

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### 1. ABSTRACT

Tuberculosis (TB) is an infectious disease caused by different strains of Mycobacterium tuberculosis complex. TB is a curable infection if diagnosed correctly and timely. Late diagnosis and improper treatment may lead to relapse or its escalation to MDR / XDR-TB. TB with HIV co-infection is difficult to diagnose by conventional set-up. Also, detection of child TB and extrapulmonary TB has its own set of problems and is not straightforward to diagnose.

The increasing complexity of TB due to the advent of new circulating strains and invasion to the regions where it was non-existent or thought to be eradicated is putting a severe strain on the health management services and making it an unmanageable pandemic. This increasing complexity has led to the spurt in the development of TB diagnostics platforms. This review focuses on the new emerging technologies that have changed the diagnostics landscape.

## 2. INTRODUCTION

Tuberculosis (TB) is a transmissible disease caused by the bacterium *Mycobacterium tuberculosis* (MTB). The bacterium normally targets the lungs causing pulmonary TB (PTB). It can also infect and spread to the other parts of the body like kidney, spine and brain causing extra pulmonary TB (EPTB) there. It usually spreads through the air by means of respiratory droplets through the sneeze or cough of an infected person. For diagnosis of pulmonary TB (PTB) which is more common, samples from respiratory tract like sputum, induced sputum, broncho alveolar lavage or lung biopsy is usually done. Diagnosis of extrapulmonary TB (EPTB) depends on the site of infection, e.g. stool sample for intestinal TB to detect *Mycobacterium avium* as in the case of AIDS patients; other samples may include biopsies, aspirates, pus, urine, and sterile body fluids like cerebrospinal, synovial, pleural, pericardial, and peritoneal fluids (1-3).

Tuberculosis continues to be the major cause of mortality despite all sorts of developments in medicine including diagnostics and medicines. It poses continued threat to the health programs and services in resource poor set-ups. Co-infection with HIV is the new factor that is responsible for the escalation of TB (9% of global TB incidence) (4). Majority of infection in human results in an asymptomatic latent infection and about one in ten latent infection progresses to active TB if left untreated, killing more than 50 percent of those infected. The situation becomes aggravated further due to its co-infection with HIV and incomplete treatment worsening it further to relapse cases and emergence of drug resistance in the form of multi drug resistant (MDR) and extensively drug resistant (XDR)-TB. MDR-TB develops due to bacterial resistant to first line TB drugs like rifampicin (RIF) and isoniazid (INH), while XDR-TB occurs due to bacterial resistant to fluoroquinolone (FLQ), amikacin (AMK), kanamycin (KAN) and Capreomycin (CPM). Tuberculosis is difficult to diagnose due to the late onset of symptoms, so that a person acts as a reservoir of TB bacilli with potential to infect others. Similarly, co-infection with HIV makes TB difficult to detect via conventional sputum smear microscopy (SSM) / X-ray. Late diagnosis coupled with incomplete treatment escalates it to MDR and XDR-TB. Although MDR-TB is curable via second line anti-TB drugs, therapy is ineffective, marred with toxic effects and require almost 2 years of treatment which is certain to have its side effect (1-3).

20<sup>th</sup> century has seen spurt of development in TB diagnostics and medicine from conventional sputum microscopy to Nucleic acid amplification test (NAAT) based molecular and biosensing based protocols, culminating into genome sequencing. This has generated a ray of hope for the timely and accurate diagnosis and treatment, hence eradication of TB.

However, it is still the giant killer and major cause of mortality and morbidity. Main cause of concern is the emergence of TB bacilli in the regions where it is thought to be completely eradicated. Irrespective of the evolution of fancy and improvised technologies for TB diagnosis SSM and culture based (Lowenstein-Jensen (LJ) media) are still the most reliable and preferred diagnostics of choice for detecting AFB causing active TB, particularly for resource limiting set up of developing countries (2-5).

Timely detection and effective treatment are essential for managing tuberculosis. However, the rate of global case detection is considerably low. The diagnosis of pediatric TB and EPTB is particularly difficult and there are, as yet, no rapid screening tests that can help finding active cases in the target population. Tuberculosis diagnosis is primarily based on the culture method and non-culture methods. Recently, it is complemented by cutting edge molecular and genome sequencing techniques that have increased the sensitivity of TB diagnosis manifold and also provide epidemiological information about its prevalence, INTRODUCTION of new strains or cause of relapse etc (2-3).

This systematic review has incorporated a decadal growth in TB diagnostics from conventional SSM to NAAT based molecular and sequencing based protocols. The review also highlights the TB diagnostics in varied set-ups focusing on challenges of customized diagnostics. In addition, review elucidates on currently available and upcoming protocols and barriers to their successful uptake. The review further focuses on the advantage and disadvantages of each technique / protocol and intends to complement earlier reviews and reports, stimulating discussion and informing potential opportunities for market intervention to improve access to effective TB diagnostics.

### 2.1. Search strategy and selection criteria

The references for this review were identified through searches of PubMed for articles published from 2005 to 2016 by the use of terms "Tuberculosis", "diagnostics", "DST", "SSM", "NAAT". Relevant articles published during the aforementioned period were identified through searches from secondary sources (published reports, peer reviewed papers, WHO policies, UNAIDS landscape report, and developer's website).

### 3. SPUTUM SMEAR MICROSCOPY (SSM)

Time tested diagnosis for active tuberculosis (TB) involves detection of *Mycobacterium tuberculosis* complex bacilli in specimens from the respiratory tract for PTB or specimens from other bodily sites for EPTB. Microscopic analysis of sputum smear detects AFB by

**Table 1.** Imaging techniques

1.	SSM	Developed by Robert Koch (1870)	Imaging	Sputum	Bacterial load indicator, Rapid, economical	Fails to differentiate between tubercular and non-tubercular, bias of technician, cannot detect EPTB	Lower, could be increased by fluorescent staining or by incubating with CB-18 (20-30%)	Low
2.	TBDx	Signature mapping medical sciences, USA	Imaging	Sputum	High-throughput (200 slides), robotic loading of stained slides, output as digitized images	High tier infrastructure and trained manpower required, a limitation in low economical set-up	High	High
3.	CellScope	UC Berkeley / CITRIS / Banatao Instit.	Fluorescence Microscope	Blood sample	High resolution enlarged digitized image	Lack of access in under developed countries, Insufficient studies to validate its utility	Sufficient study / data required	Sufficient study / data required
4.	CXR algorithm CAD4TB		Imaging	Chest X-ray	Rapid detection of active TB particularly in HIV co-infection, can be interfaced with other such platform, detects both active and passive TB	Trained expertise required	High	High
5.	Easy portable DCXR	(Delft Imaging Systems)	Imaging	Chest X-ray	Capacity to hold 50,000 DCXR images, can be operated from a laptop	Trained expertise required	High	High
6.	riView-TB	Advenio (Chandigarh, India)	Imaging, CAD4TB enabled automated image analysis software similar to DCXR	Chest X-ray	Easy to interpret images, simultaneous diagnosis of pneumonia and silicosis to complement PTB diagnosis	Prior training in clinical radiography required	High	High

Ziehl-Neelsen (Z-N) staining (6). Sputum test is good indicator of bacterial load hence the infectivity. It is the most common, rapid and economical diagnostic method for active PTB diagnosis but it has lower sensitivity, when bacterial load is less than 10,000 bacilli / ml of sputum (Table 1). However, sensitivity can be improved by fluorescent staining (auramine-rhodamine) coupled with LED microscopes but it fails to distinguish between tuberculous and nontuberculous bacteria. Other limitations include prevalent epidemiological factors in the region of interest and skill of laboratory technician (7-8) (Table 1). Laserson *et al.* (2005) (9) in their study of Vietnamese immigrants have observed that incubation of sputum with detergent C18-Carboxypropylbetaine (CB-18) for 24 hours improves the sensitivity of AFB staining by 20-30% when compared to direct auramine and Z-N staining (9). Sputum analysis cannot detect EPTB. There might be a possibility that bacilli resides in cavities of lungs and may not be there in sputum. Decontamination process of sputum for mycobacterial culture may lead to the loss of bacilli from the sputum.

In addition, HIV patients and children might find it difficult to produce sputum. These constraints severely limit the utility of this very simple and economical diagnostics method<sup>10</sup>.

TBDx developed by signature mapping medical sciences, USA is an automated high-throughput system with the capacity of 200 slides. It utilizes robotic loading of stained slides. Results are rapid in few minutes in the form of high resolution digital image (8, 11-12) (Table 1).

#### 4. IMAGING BASED DIAGNOSIS: FUTURE OF AUTOMATED MICROSCOPY

Priority based treatment depending on the severity of symptoms have the potential to discriminate amongst those at risk from the people bearing symptoms of PTB but do not actually have TB. Chest X-ray (CXRs) serves as a potent algorithm in mathematical analysis of TB epidemiology. CXR integrated with user friendly, robust interface has evolved into an automated

algorithm to quantify abnormalities from digital CXR (DCXR) images. DCXR can be utilized for both active and passive TB diagnosis. It can be interfaced with other such platforms like as Xpert® MTB/RIF for rapid detection of active TB particularly in HIV co-infection setup. It is an economical tool with high sensitivity and specificity and could be a tool of need in low resource set-up (Table 1). Delft Imaging Systems (Netherlands) have come up with a software to score CXR image at the rate of one per minute. Fourth version of Computer aided detection for tuberculosis (CAD4TB) (Delft Imaging Systems) can also be integrated with DCXR platform (13-14). EasyPortable DCXR (Delft Imaging Systems) fits the requirement of limited resource set up. It includes an X-ray detector, CAD4TB software and Picture Archive and Communication System (PACS) with a capacity to hold 50,000 DCXR images and can operate from a laptop (2) (Table 1).

Advenio (Chandigarh, India) has launched riView-TB, a CAD4TB enabled automated image analysis software similar to DCXR. This platform provides easy to interpret images without prior training in clinical radiography. It is slated to be improvised for simultaneous diagnosis of pneumonia and silicosis to complement PTB diagnosis (2) (Table 1).

CellScope, a mobile cell microscope (CITRIS, University of California, Berkeley, San Francisco) is another innovation in the series of next generation microscopy. It is a fluorescence microscope that gives output in the form of high resolution enlarged digitized fluorescent images of blood samples to detect rod shaped bacterium (8, 15) (Table 1).

## 5. SAMPLE COLLECTION

A good quality sample in sufficient amount is a prime requirement for accurate diagnosis. This becomes a limitation with children and HIV patients who cannot cough up enough sputum. Lung flute from Medical Acoustics (USA) allows the patient to collect their own sample when they are not able to expectorate enough volume. It consists of a plastic device held via lips to exhale air into it. This generates vibrations in the lungs that soften and liquefy the sputum enabling patient to cough out more sputum (16).

Deton Corp. (USA) has come up with one liter bag to collect microdroplets presumed to contain MTB bacilli. These microdroplets are used for diagnosis (3). DNA Genotek Inc. (Ottawa, Canada) has developed OMNIgene® SPUTUM to liquefy and decontaminate the sputum. This facilitates its transport without cold chain for further diagnosis via microscopy, culture, Xpert® MTB/RIF and other molecular diagnostic protocols. prepIT® MAX technology from DNA Genotek Inc. (Ottawa, Canada) enables release of nucleic acid from MTBC cells via chemical lysis (3).

PrimeStore Molecular Transport Medium® (PS-MTM®) from Longhorn Vaccines & Diagnostics (USA) provides a NAAT enabled solution to inactivate, lyse and stabilize MTB DNA without cold chain. The genetic material produced can be utilized for range of other TB tests. PS-MTM® has further diversified into range of products like PrimeSwab™ and PS-MTM® for sample preparation, PrimeSwab™ and PS-MTM® for DNA extraction (PrimeExtract™) and PrimeMix® for real time PCR assay. Molzym GmbH & Co. KG (now Molzym) has launched the MTB-DNA Blood kit (25 to 50 reactions) that removes excess human genomic DNA and concentrates MTB DNA (3).

## 6. LATENT TUBERCULOSIS AND SKIN TESTING

Tuberculosis may remain latent in an infected patient before reactivation. This is one amongst the most challenging aspect in TB diagnostics. The advent of ELISPOT assay has provided promise to test for latency. This test involves the quantification of interferon production from whole blood or peripheral blood mononuclear cells (PBMC) stimulated with either purified protein derivative (PPD) or specific antigens (17-19). Currently available QuantiFERON®-TB Gold Test (Cellestis Ltd, WA, USA) for latent and active TB utilizes ESAT-6 or CFP-10 antigens (absent from BCG vaccine) (Table 4). Although the assay got the FDA approval it is yet to be commercialized. MPB skin patch test is another test with very high specificity and sensitivity (20-21). This test is free from reader bias, do not give false positive results due to booster phenomena, free from the effect of prior BCG vaccination and require only one patient visit to give sample and get result (22) (Table 4).

### 6.1. Culture based drug resistance testing (DST)

Increasing global burden of TB has diversified the diagnostic protocols for TB detection. Various phenotypic methods that include nitrate reductase assay (NRA / Griess method), thin-layer agar (TLA), colour test (Color Test), microscopic observation drug susceptibility assay (MODS), colorimetric redox indicator (CRI) method and phage-based assays can be directly set on the clinical samples and do not have to undergo sample preparation like decontamination process (2, 23-24). These methods can detect MDR-TB including resistance to the second line drugs like INH and RIF. Among different phenotypic methods available, MODS is widely validated and has perfect synergy with conventional drug susceptibility test for INH, RIF and MDR-TB (100%, 97% and 99%). MODS is rapid, economical and works well with all types of primary clinical samples and isolates. MODS depend on light microscopy. However, it requires a requisite expertise that involves extensive manpower training (25-27). The effectiveness of MODS for HIV

**Table 4.** Culture based phenotypic assays

S.No.	Assay	Manufacturer	Method	Material	Advantage	Disadvantage	Sensitivity % (95% CI)	Specificity % (95% CI)
1.	MODS	MODS (Hardy diagnostics)	DST	Sputum	Directly set on the clinical samples and do not have to undergo sample preparation like decontamination process, detect MDR-TB including resistance to the second line drugs like INH and RMP; Handling risk is entirely avoided, Safer as performed in closed system	Daily observation of culture, risk of laboratory transmission, requirement of inverted microscope, require extensive manpower training	High	High
2.	NRA	To be marketed	DST on LJ medium With KNO <sub>3</sub> (ability of MTB to reduce nitrate to nitrite)	Sputum	Simple to perform with good results, inexpensive reagents, advantage in resource limited settings	Culture time is 3-4 times of MODS, Require mature MTB culture to open to add specific reagent	High	High
3.	TLA	To be marketed	DST on solid medium	Sputum	Rapid, Accurate, Standard microscope Required which is Easily available, cost effective	Contamination rate is higher, data on DST is lacking	Low	Low
4.	CRI	To be marketed	Indirect, done on MTB isolates grown from conventional culture	Sputum	Less equipment and infrastructure, Consumables that are readily available	Biosafety level 3 required, additional staff skill	High	High
5.	TKME DIU M® SLC-L	Salubris Inc. USA	Colorimetry	Sputum	Rapid, does not require centrifugation step, no media preparation required, lower contamination	Lack of automation, Disadvantageous in terms of turn-around time, Future studies in differential settings are required	Better sensitivity over LJ for MTB detection	High
6.	Accuprobe	Gen-Probe Inc	Chemiluminescent acridinium ester-labelled (AE)-DNA Probes (AE-MAC) probe	Sputum	Simple and rapid, Readily available In market, Validated	Labor intensive	High	High

co-infection is not yet established. Further, this test is only approved for INH and RIF leaving other first and second line TB drugs. TLA has also shown the similar promise for the detection of MDR/XDR-TB colour test, MTB complex and resistance to INH, RIF and ciprofloxacin (CFP) in cultures (27), details given in Table 4.

TK MEDIUM® SLC-L (Salubris Inc. USA) has developed a liquid media that provides an advantage over conventional decontamination method using sodium hydroxide and N-acetyl cysteine. It does

not require centrifugation step thus completing the whole process in around 25 minutes. It shows better sensitivity over LJ for MTB detection (2) (Table 4). Gonzalo *et al* (2014) (27) have used Biphasic media assay (BMA) having two media L-J and Kirchner medium in tubes with 66mg/ml of pyrazinamide (PZA) to grow PZA susceptible and resistant strains of MTB (27). Reproducible results have shown the majority of strains to be susceptible to PZA while few were resistant. BMA might be a useful low cost, culture based alternative for DST for detecting PZA resistance particularly where MDR-TB is prevalent.



## 6.2. Phage amplification technique (PAT)

Phage Amplification Technique (PAT) is a bacteriophage based test that can detect MTB in sputum. Non-pathogenic mycobacteria (sensor cells) are used as a negative control. The phage replicate, infect and lyse the sensor cells leaving zones of clearing (holes) in the agar conforming that sputum contains MTB. This diagnostic protocol is rapid (2 days), sensitive, and requires less infrastructure. However, it can only be applied to sputum samples and requires expert manpower (28).

## 7. GENOTYPIC METHODS

Genotypic methods target the nucleic acid of the mycobacterium. Ribosomal rRNA, which has universal occurrence, abundance, and conserved specific sequences can be a very useful genetic marker. Accuprobe (Gen-Probe Inc) detection method targets rRNA. This method can detect *M tuberculosis* complex, *M avium*, *M intracellulare*, *M avium* complex, *M gordonae*, and *M kansasii*. Accuprobe is simple and rapid with accuracy around 90%. However, it covers a limited range of species (Table 4). Peptide nucleic acids (PNA) based assay provides another approach to differentiate *M. tuberculosis* complex from NTM cultures<sup>29</sup>. PCR based restriction enzyme analysis of the *hsp65* also provides an economical alternative for mycobacterium detection from culture. However, technique is complicated and labor intensive (22,30). Similarly, sequencing 16sRNA can also provide rapid detection of mycobacterium but it is labor intensive (22, 31).

## 8. IMMUNOLOGICAL PROTOCOLS

Detection of antibodies like lipoarabinomannans (LAM, cell wall component of Mycobacteria), A60, 38Kd and 16 Kd against MTB is a simple and economical method for EPTB detection. However, inconsistency in results as covered by meta-analyses and systematic reviews hampers its commercialization (3,32-33).

Determine™ TB LAM Ag rapid assay (Alere Inc.) is an immune-chromatographic strip that detects LAM antigen in urine. Surprisingly, a 'negative result' does not rule out the possibility of TB. Test is also vulnerable to 'false positives' due to contamination with dust or faeces. For test to be accurate, the CD4 counts/mm<sup>3</sup> should not be less than 100 and best results are obtained with CD4 counts around 200 counts/mm<sup>3</sup> (3, 32-35). It detects NTMs in addition to MTBC along with the site of mycobacterial infection (Table 5).

### 8.1. TB biomarker assays

The T-cell activation marker-tuberculosis (TAM-TB) assay is a flow cytometry based protocol

that detects fluorescently stained TB antigen-specific CD4 T-cells counterstained with an antibody to CD127. It measures the level of CD127 in the CD4 cells challenged with TB antigens (36) (Table 5).

### 8.2. Interferon-Gamma Release Assays (IGRA)

This test utilizes the ability of MTB antigens like Early Secretory Antigen Target 6 (ESAT-6) and Culture Filtrate Protein 10 (CFP-10) to stimulate the host for the production of interferon –gamma (IFN-γ). These antigens are absent in NTM or in BCG vaccine hence can differentiate between latent TB infection, BCG immunization and NTM infection. This procedure is simple and rapid. The commercially available assays for IFN-γ are; the Quantiferon-TB Gold (Cellestis) and T Spot-TB test (Oxford Immunotech). Interгам Rapid Immuno Suspension Array, IRISA-TB (Antrum Biotech Ltd, South Africa) detects IFN- γ in pleural, pericardial, ascitic, and cerebrospinal fluid. Testing IFN-γ has higher sensitivity for pleural TB detection as compared to the Xpert MTB / RIF (8,37) (Table 5).

## 9. VOLATILE ORGANIC CHEMICALS (VOCs)

Analysis of metabolic pathways for TB detection is another breakthrough in technology that is rapid, safe and useful for TB diagnosis in children and HIV patients. Maiga *et al* (2012) (6) have developed Rabbit urease breath test to analyze the sensitivity and specificity of urease based detection of *M. tuberculosis* (6). This test involves administration of urea labeled with isotope as substrate. Urea metabolized to <sup>13</sup>C-CO<sub>2</sub> can be detected in exhaled breaths using IR spectrometers. Signal shows good relation with bacterial load that served well for diagnostic purposes and treatment monitoring (6). The test is under clinical trial to evaluate its efficacy.

BACTEC TB 460 radiometric system from Becton Dickinson, Sparks, MD, USA facilitates rapid detection of *M. tuberculosis* thus expediting evidence based therapy (6). Liquid crystal display (LCD) based TB Breathalyser from Rapid Biosensor Systems Ltd takes less than 4 minutes to detect active TB bacilli based on the MTB specific antigen in sputum and gives output as TB positive or negative (2).

## 10. NUCLEIC ACID AMPLIFICATION TESTS (NAAT)

PCR is commonly used for nucleic acid amplification tests (NAAT). NAAT can detect single copy of nucleic acid in a clinical sample. However, presence of PCR inhibitors in samples can significantly compromise the sensitivity. Also, processing of samples may lead to the loss of nucleic acids (38-42). This technique is further complemented by fluorescent probe based detection or melts curve analysis,

**Table 5.** Immunological biomarkers

S.N.	Assay	Manufacturer	Method	Material	Advantage	Disadvantage	Sensitivity % (95% CI)	Specificity % (95% CI)
1.	Determine™ TB LAM Ag Rapid assay	Alere Inc.	Immunochromatographic strip	Urine	Simple and economical method, detects TB in HIV positive patients, EPTB, NTMs, MTBC along with the site of mycobacterial infection	Inconsistency in results, negative result does not rule out the possibility of TB, Test is also vulnerable to 'false positives' due to contamination with dust or faeces, test to be accurate, the CD4 counts/mm <sup>3</sup> should not be less than 100	Increased when combined with sputum microscopy	High
2.	TB Rapid Screen	Tashima Inc Global BioDiagnostics, USA, with FIND, Switzerland	One-step Chromatographic immunoassay, which specifically detect the antibodies to <i>Mycobacterium tuberculosis</i> in human serum or plasma	Blood	Rapid, simple and easy to use, Suitable to use in peripheral laboratories, use low-cost fluorescence reader	Limited to the qualitative detection Of antibodies to <i>M. tuberculosis</i> in human serum and plasma, Qualitative test, Negative result does not rule out TB, Need confirmation from other tests	High	High
3.	TAM-TB assay	Ludwig-Maximilians University of Munich (LMU), Germany	Flow cytometry, fluorescently stained TB antigen-specific CD4T-cells	Blood	Quick and efficient diagnosis, Paediatric TB	Need optimization for TB detection in HIV kids and reduced cost	High	High
4.	Intergam Rapid Immuno Suspension array, IRISA-TB	Antrum Biotech Ltd, South Africa	Measures IFN- $\gamma$ in pleural, pericardial, ascitic, cerebrospinal fluid	IFN- $\gamma$ In pleural, pericardial, ascitic, and cerebrospinal fluid	Rapid assay, simple and flexible format for simple or multiple assays, Test can be manual or automated	Antigen- specific T- cell IGRAs were limited by suboptimal accuracy and the inability to isolate sufficient mononuclear cells to perform the assay	High	High
5.	T-SPOT®TB	Oxford Immunotec, UK	ELISPOT / IGRA assay	Blood	Rapid, not influenced by previous BCG vaccination	Lower sensitivity for patients with prior BCG, diagnostic value of this test in detecting active TB approached 90% in sensitivity, specificity, limitation in detecting pediatric TB	High relative to TST	High relative to TST
6.	QuantiFERON®-TB Gold Test	Cellestis Ltd, WA, USA	ELISPOT assay	ESAT-6 or CFP-10	Results are not subject to reader bias, no booster phenomena (i.e., increased sensitivity on subsequent test, leading to false-positive results), prior BCG vaccination and require only one patient visit	Not tested for infants, Kids, Pregnant women	High	High

i.e. real time PCR (RT-PCR). PCR based protocol can differentiate between wild type and mutated sequence. Other methodologies include ligase chain reaction (LCR), strain displacement amplification (SDA), loop-mediated isothermal amplification (LAMP) and transcription mediated amplification (TMA) (43-45). These molecular techniques simultaneously detects resistance genes as well, e.g. DNA probe and DNA sequencing of MTB gene such as catalase (katG) or RNA polymerase (rpoB).

Mutations in katG and rpoB lead to the resistance to INH and RIF respectively. NAAT based methods are more suited for academic / research purposes than for commercial clinical purpose.

### 10.1. Commercial NAATs based protocols

COBAS® TaqMan® MTB Test (Roche Diagnostics) is a RT-PCR based high throughput detection protocol (100 samples/day) that targets the

gene responsible for 16S RNA. This protocol requires liquefied, decontaminated and concentrated human respiratory samples like sputum and bronchial alveolar lavages. This test is highly specific to the MTBC (2) (Table 6).

The RealTime MTB from Abbott Molecular is also an RT-PCR based assay. It can be interfaced with fully automated DNA extraction platform. It detects MTBC from sputum or bronchoalveolar lavage (46) (Table 6).

GenoXtract®96 with the FluoroCycler® 96 from Hain Lifescience is a 96 well format extraction and amplification platform provided with the the GenoLyse® and FluoroLyse® for nucleic acid extraction for LPAs or MTB assay (2). The Fluorotype® MTB (Hain Lifescience) assay provides PCR based amplification of MTBC DNA. The amplicons are detected via molecular beacons and melt curve analysis (47). Fluorotype® MDRTB is an improvised version of Fluorotype® MTB that detects RIF and INH resistance in addition to MTBC detection (2) (Table 6).

Hain Lifescience has also come up with a multiplexed assay that detects MTBC and also RIF and INH resistance like the previous case. This protocol utilizes a novel PCR technique known as linear-after-the-exponential (LATE) PCR coupled with 'lights on' or 'light off' probe technology for precise identification of mutations. This protocol creates specific fingerprints for each allelic variant of drug resistant mutations (48) (Table 6).

Tosoh Bioscience (Japan) has developed TRCRapid® MTB assay that targets MTBC 16S RNA. The protocol includes amplification and transcription-reverse transcription concerted (TRC) reaction (49). NanoBioSys Inc. (Republic of Korea) has reported a microfluidic chip (LabChip G2-3) based sample processing and amplification protocol (2, 50). Detection is via RT-PCR and takes approximately 30 minutes (Table 6).

Seegene (Republic of Korea) provides a highly multiplexed RT-PCR based NAAT assay. This provides a range of Anyplex™ series of assays that includes the Anyplex™ MTB/NTM for MTBC and NTM; the Anyplex™ plus MTB/NTM/MDR-TB for MTBC, NTM and genotyping RIF and INH resistance; and the Anyplex™ II MTB/MDR/XDR-TB that detects MTBC, genotypes MDR via typing for INH and RIF resistance alleles and also 13 alleles linked with XDR-TB (7 alleles for FLQ and 6 for injectable drugs). This platform detects MTBC and also XDR if the sample is MTBC and MDR positive (50-51) (Table 6).

Xiamen Zeesan Biotech Co. Ltd (China) has developed MeltPro® Drug-Resistant TB Testing Kits as

RT-PCR enabled detection of resistant alleles to RIF and INH. It is undergoing further refinement to detect alleles resistant to ethambutol (EMB), streptomycin (STR) and fluoroquinolones (FLQs) (52-53) (Table 6).

### 10.2. Xpert MTB/RIF assay for the diagnosis of PTB

Xpert® MTB/RIF (Cepheid, Sunnyvale, CA, USA) is modular / single cartridge based NAAT platform to diagnose MTB. It involves semi-nested RT-PCR based amplification of *rpoB* and genotypes RIF resistant alleles. Melt curve analysis of amplicons generate fuoremetric fingerprints for five molecular probes in real time, which discriminate allelic variant in each probe region. This platform is accurate and sensitive and suits to the requirement of low income, limited resource set-up. This platform can process 16 to 1000 clinical samples (INFINITI® Xpert®). WHO has approved Xpert® MTB/Rif for the diagnosis of PTB, EPTB, HIV associated TB and TB meningitis in adults and children (2) (Table 6).

Xpert MTB/RIF assay provides a rapid and automated molecular test for pulmonary TB with the sensitivity of 89%, and specificity of 99% (54). However, this test is yet to be approved for the detection of non-respiratory clinical samples (55). Truenat MTB (Molbio Diagnostics, India) is a chip enabled RT-PCR operated on a portable device. Results are out in an hour. Nucleic acid is extracted from sputum utilizing a nano-particle based procedure. It shows the specificity similar to the Xpert MTB/RIF (56) (Table 6). EasyNAT Diagnostic Kit detects MTB complex. It is an isothermal amplification kit and results are qualitative in terms of visual bands. For thinned and concentrated sputum the sensitivity is comparable to culture on LJ medium (57-58) (Table 6). The VereMTB assay (Veredus Laboratories, Singapore) assay is more for academic / research purpose. It is a chip enabled platform and combines PCR with microarray technology to detect *M. tuberculosis*, *Mycobacterium avium*, *Mycobacterium intracellulare*, *Mycobacterium simiae / kansasii / scrofulaceum*, *Mycobacterium abscessus / chelonae*, *Mycobacterium xenopi*, and *M. fortuitum* in addition to RIF and INH (8,59) (Table 6). GeneXpert® Omni Cepheid Inc. is a mobile operated device that uses the cartridges already in use with the Xpert® platforms (e.g. MTB/RIF). This platform offers miniaturized and durable assay. It can store upto 20,000 results (2) (Table 6).

### 10.3. Xpert MTB/RIF assay for the diagnosis of extrapulmonary tuberculosis (EPTB)

Globally, PTB is more common as compared to the EPTB, which is nearly 25% of all TB cases. However, EPTB has higher prevalence in children and in HIV-infected individuals (62-63). EPTB diagnosis is marred with the lack of accuracy, pace and requisite expertise,



**Table 6.** NAAT based molecular diagnostics

S. No.	Assay	Manufacturer	Method	Material	Advantage	Disadvantage	Sensitivity,% (95% CI)	Specificity % (95% CI)
1.	*B-SMA RT	(LabCorp) USA	PCR+ DST MTB detection, Resistance to PZM	Sputum	Under development	Under development	Under development	Under development
2.	iCubate System	iCubate, USA	Multiplex PCR	Sputum	Highly integrated Single platform for multiple assays for resistance, TB, NTB bacilli	For research use only	NA	NA
3.	COBAS® TaqMan® MTB Test	Roche Diagnostics	RT-PCR	Sputum and bronchial alveolar lavages	Ease to use, Rapid, Reduced contamination, Enhanced quality, Automated, High throughput	Only sputum, BAL, liquefied decontaminated and concentrated using NALC-NaOH methods, false negative results due to PCR inhibitors	High	Highly specific to the MTBC
4.	RealTime MTB	Abbott Molecular	RT-PCR	Sputum and bronchial alveolar lavages	Interfaced with fully automated DNA extraction platform, detection of 8-members of MTBC, No cross reactivity with NTB	Not suited for resource limited peripheral laboratories	High	High
5.	GenoXtract® 96	Hain Lifescience	LATE-PCR amplification, molecular beacon and melt curve analysis	Pulmonary and extrapulmonary samples: stool, blood, urine, serum, swabs or cell pellets	Efficient extraction, multifunctional, flexible processing, user friendly, RIF and INH resistance in addition to MTBC detection	Not suited for resource limited peripheral laboratories	High	High
6.	TRC Rapid® MTB Assay	Toho Bioscience, Tokyo, Japan	Transcription-reverse transcription concerted (TRC) reaction	Respiratory and non-respiratory	Rapid technique for the detection of MTBC in respiratory, smear positive and smear negative non-respiratory specimens, feasible for peripheral laboratories	Cross-reactions are possible when a high number of M. avium and M. marinum isolates are present	High for respiratory samples than non-respiratory	High for respiratory samples than non-respiratory
7.	Lab Chip G2-3	NanoBioSys Inc. (Republic of Korea)	RT-PCR	Sputum	Ultra fast cost-effective quantitative analysis, takes 30 minutes only, Easy-to-use, User-friendly software	Trained expertise, infrastructural set-up required	High	High
8.	Anyplex™ series	Seegene (Republic of Korea)	Multiplex RT-PCR with DPO™ and TOCE™ technologies	Respiratory, non-respiratory samples	Automated liquid handling workstation from nucleic acid extraction to PCR setup, Quick and accurate RT-PCR optimized to multiple pathogen detection, Specialized in multiple tests, Analyze up to 94 samples	Limitations regarding detection of INH resistance	High	High
9.	MeltPro® Drug-Resistant TB Testing Kits	Xiamen Zeesun Biotech Co. Ltd (China)	RT-PCR	Sputum	High accuracy, short testing turnaround time, low unit price, excellent tool for MDR/XDR-TB	Significant heterogeneity for the detection of KAN resistance	High	High
10.	MFqRT-PCR	Qiagen, Germany	Multi-Fluorescence quantitative RT-PCR	Sputum, culture isolates	Efficient, accurate, reliable, easy-operated method for detection of RIF and INH resistance, distinction of MTC and NTM of clinical isolates	False negative and false positive results. Further studies are required to optimize this method and evaluate its performance	High	High
11.	Xpert® MTB/RIF	Cepheid, Sunnyvale, CA, USA	RT-PCR Melt curve analysis	Respiratory clinical samples	Suits to the requirement of low income, limited resource setting, diagnosis of PTB, EPTB, HIV associated TB and TB meningitis in adults and children	Test yet to be approved for the detection of non-respiratory clinical samples	High	High
12.	Truenat MTB	Molbio Diagnostics, India	RT-PCR	sputum	Portable device, rapid, quick and accurate	Expensive, Sample care needed, Large studies are required to obtain better estimates of the performance	High	High

## Diagnostic approaches in tuberculosis

13.	EasyNAT Diagnostic Kit	Ustar Biotechnology	Isothermal amplification	Sputum	Useful in low-resources setting, simple and easy-to-use, equipment-free, compatible with other amplification technologies, such as PCR, LAMP, SDA, HAD, Rapid, results are qualitative in terms of visual bands	Low detection rate for the smear- negative, culture- positive sputum samples, length of time needed to get results, technical complexity, high costs and biosafety requirements	Low, Acceptable for smear positive cases but low for smear negative cases	High
14.	Elken's Loopamp <sup>TM</sup> MTB	Elken (Japan)	LAMP	Sputum	High tier laboratory to the peripheral laboratory, robustness of equipment, reagent stability	Manual preparation of samples, pre-treated sputum resulted in poorer performance	High for smear positive, low for smear negative specimens	High
15.	Genedrive <sup>®</sup> MTB assay	Epistem (UK)	PCR amplification, genotyping, sequence analysis	Sputum	High tier laboratory to the peripheral laboratory, robustness of equipment, reagent stability	Failed to meet the performance standards recommended by WHO for SMeplacement, Epistem is working on modifications to improve the assay	Low	Low
16.	True lab <sup>TM</sup> Real Time microPCR	Mobio Diagnostics (India)	Nanotechnology, Micro Electro-mechanical systems (ME-MS) PCR	Sputum	Fully automated, integrated system, robust, Affordable, GPRS and bluetooth enabled	Switching on the analyzer before finishing chip loading may render chip unusable, High stringency for chip usage	High	High
17.	EasyNAT <sup>TM</sup> TB	Ustar Biotechnologies (China)	RT-PCR coupled with fluorescent probe	Sputum /FNAB peripheral tuberculosis lymphadenitis	Promising diagnostic algorithm for suspected childhood TB lymphadenitis, suits to the limited resource set up of peripheral laboratories requiring centrifuges, water bath etc to perform the assay	Restricted due to the length of time needed to get results, technical complexity, high costs and biosafety requirements	Low	High
18.	Alere <sup>TM</sup> q	Alere Inc. USA	NEAR-isothermal amplification of TB DNA	Sputum /Blood	Highly integrate automate, Portable, Walk away operation, No complex procedures, minimal qualification and training requirement, Rapid case detection	Tedious multi-step process	High	High
19.	GeneXpert <sup>®</sup> Omni	Cepheid Inc.	PCR amplification	Sputum	Portable, Proven cartridge technology, Durable, Low power consumption, Integrated battery, cloud-based connectivity	Short cartridge life, Requirement of stable electric supply, Annual calibration required, Temperature ceiling is critical, unreliable in HIV positive condition	High	High
20.	EOS CAPE-TB System	Wave 80 Biosciences (USA)	Genotyping RIF and FLQ resistance alleles	Sputum	Multiple tests can be run in parallel, single analyzer to generate results that can be used with multiple processors	NA	NA	NA
21.	PCR(XCR <sup>TM</sup> )	Fluorocentric Inc. (USA)	PCR amplification and isothermal testing	Sputum	Battery operated device for rapid amplification (10 minutes), real time detection of individual XCR <sup>TM</sup> assays, no primer dimer, low cost reagents and portable instrumentation, high multiplex and simple sample preparation, resistance to PCR inhibitors	NA	NA	NA
22.	TOP-PCR	Episcience/ Frontiers Laboratory	NAAT	Sputum	TB rule out <sup>®</sup> test, minimum background noise and maximum specificity in its output	Require prospective validation with clinical outcomes	Variable	Variable

which usually requires invasive procedures. Absolute culture specificity can be achieved by complementing the routine diagnostic protocols with antigen tests or NAATs. In case of TB meningitis the low culture yield can be enhanced in terms of sensitivity by replicating the CSF examination or by complementing it with NAAT (62-63). High performance liquid chromatography (HPLC) based detection of tuberculostearic acid in CSF is highly sensitive but requires expert manpower and costly laboratory set up (22,64).

### 10.4. Semi-automated NAATs for use in peripheral laboratories

The biggest challenge in molecular diagnostics of TB is to shift the set up from the higher tier laboratory to the peripheral laboratories. This may represent a diversity of issues that may range from the availability of sufficient infrastructure, robustness of equipment, reagent stability, user expertise in addition to improved performance over SSM (65-66). Four different platforms like the Loopamp™ MTBC assay from Eiken (Japan); Genedrive® MTB assay from Epistem (UK); Truelab™ RealTime micro PCR System from Molbio Diagnostics (India); and the EasyNAT™ TB assay from Ustar Biotechnologies (China) are sighted with potential for delivery (2).

Eiken's Loopamp™ MTB provides a (LAMP) based amplification assay with better sensitivity over SSM. This assay involves manual preparation of samples which are subjected to RT-PCR. Genedrive® MTB assay provides for MTBC diagnostics only with future plan of including genotyping MDR-TB through RIF resistance. This assay involves manual preparation of samples followed by RT-PCR. Truelab™ RealTime micro PCR involves semi automated DNA extraction and amplification for MTBC detection. Truelab™ QUATTRO is an improvisation over Truelab™ RealTime micro PCR in being fully automated and integrated system. EasyNAT™ TB suits to the limited resource set up of peripheral laboratories requiring, centrifuges, water bath etc to perform the assay (2) (Table 6).

Alere™ q platform is highly integrated and automated NAAT for diagnosis and assessing the viral load of HIV-1 and 2 (67). It is a cartridge based system, where cartridge is equipped with the reagents to liquefy and inactivate the sample. This is followed by extraction of MTBC-DNA and amplification. This assay involves nicking enzyme amplification reaction (NEAR), an isothermal amplification of TB-DNA (2) (Table 6).

GenePOC Diagnostics (Canada) offers an automated platform that uses RT-PCR and fluorescence detection for TB diagnostics (2). TBDx system from Keck Graduate Institute (KGI; USA) provides a low cost instrumentation where all process starting from

sample processing occurs on single cartridge platform. Nucleic acid preparation is simplified as it does not require chaotropic salts and organic solvents (2). The Northwestern Global Health Foundation (USA) in partnership with Quidel Inc. (USA) is coming up with an integrated MTBC assay. The assay involves lysing MTBC cells via heat block followed by purifying DNA using immiscible oil interface (68) (Table 6).

Point-of-Need (PON) Qiagen offers isothermal nucleic acid amplification assay for the detection of PTB and MDR-TB. Q-POC™ platform, under development from QuantuMDx (UK) offers diagnosis for PTB. The assay involves coughing the sputum in a cup that contains reagents for its liquefaction and decontamination. This is followed by concentration of MTBC cells via paramagnetic bead concentration step and quick nucleic acid amplification (4 minutes) via isothermal asymmetric PCR (2).

EOSCAPE-TB System from Wave 80 Biosciences (USA) offers detection of MTBC via genotyping RIF and FLQ resistant alleles. Using this platform multiple tests can be run in parallel and single analyzer generates results that can be used with multiple processors (2) (Table 6).

PCR (XCR™) assay from Fluorocentric Inc. (USA) offers a RT-PCR based assay that works via proprietary primer design algorithms. It is powered by a battery operated device for rapid amplification (10 minutes) and real time detection of individual XCR™ assays (2) (Table 6). Co-diagnostics (USA) and Thisis (a spinoff company from Boston University School of Medicine, USA) offers a RT-PCR based platform for detection of MTBC via 16S RNA gene. This platform can be integrated with variety of other RT-PCR platforms. It is claimed to detect MTBC at LOD of 4 cfu and can genotype MTBC into five geno-groups using totally optimized PCR (TOP) (Table 6). Scanogen, though not strictly a NAAT assay detects MTBC specific DNA from body fluids using single molecule scanning technique without PCR amplification. This platform claims to have minimum background noise and maximum specificity in its output (2).

### 10.5. Spoligotyping

Spoligotyping is very useful technique in providing epidemiological information on the causative strain. It utilizes amplification of polymorphic direct repeat sequences in *M. tuberculosis* genome. Higher cost of laboratory infrastructure and requirement of trained expertise are biggest limitations (5,69).

### 10.6. Line Probe Assay (LPA)

LPAs or reverse blot hybridization assays are a relatively low-cost tool and have been developed

for a variety of areas around MTB diagnostics, including MTBC-specific assays, speciation of NTMs and assays that genotype first or second-line drug resistance alleles. DNA extraction can be manual or automated and DNA is amplified by asymmetric PCR to create a population of biotin-labelled single-stranded (ssDNA) amplicons. Oligonucleotide capture probes with sequences specific to the target amplicons are printed as an array of stripes on a nitrocellulose strip. The amplification mixture is then exposed to a strip under conditions to optimize the specific hybridization of amplicons to their sequence match probe printed on the nitrocellulose. Detection of hybridization of amplicons to the probe stripe is achieved via a streptavidin-linked enzyme that creates a colorimetric reaction visualized as defined stripes that corresponds to the presence of TB and a sensitive or resistant genotype (22). The developed LPAs are read manually with comparison to a printed score chart or can be scanned and automatically analysed by attendant software. The methodology requires open tubes containing amplified DNA, which has potential to contaminate the workspace thus compromising all future test data. The automated workstations alleviate this to some degree by providing controlled conditions that contain reaction mixtures (2).

Commercially available LPAs for the detection of TB, MDR/XDR-TB are: INNO-LiPA Rif. TB (Innogenetics, Belgium) for RIF, GenoType®MTBDR/MTBDRplus for RIF and INH and Geno-Type®MTBDRsl (both Hain Lifescience, Germany) for FLQ, injectables, second line drugs and ETM (22). INNO-LiPA Rif. TB is highly sensitive when isolates from culture are used. However, the accuracy goes down when it is applied directly on clinical samples. GenoType®MTBDR/MTBDRplus can be applied for both culture isolates and clinical samples. GeneXpert MTB/ RIF utilizes heminested RT-PCR to amplify specific sequence of the *rhoB* gene of *M. tuberculosis*. The amplicons are then probed with molecular beacons for mutations within RIF resistance determining region (6, 70-72) (Table 2).

MTBDRsl v1.0. and MTBDRsl v2.0. can detect resistance to FLQ, KAN, STR and XDR-TB. MTBDRsl v2.0. is equipped with 27 probes to detect resistance to second line drugs and also targets new gene regions for improved detection of FLQ and KAN (73). LPAs show high sensitivity and give better results when TB culture or SSM positive samples are used as a substrate (Table 2). These are economical when compared to culture based DST (3). However, it is practically impossible to determine accurately resistant genotypes for many drugs like pyrazinamide (PZA). Silent mutations in drug sensitive strains can be misinterpreted as drug resistance leading to avoidable treatment regimens (2).

## 10.7. Microarray-based platforms

Microarray based platforms acts as portable LPA. Microarrays have high potential for MTB diagnosis, speciation to analyze variation in drug resistant alleles. The probes utilized in microarray can be printed as defined spots and due to their small size more spots can be printed to analyze amplified DNA. The INFINITI® PLUS Analyzer platform from Autogenomics is one such platform. INFINITI® High Throughput System (HTS) and MDR-TB BioFilmChip® for the detection of MTBC and first-line drug (RIF, INH and PZA) are high-throughput array based platform (Table 3). CapitalBio has launched twin microrray based platforms, i.e. Mycobacterium Identification Array Kit for MTB diagnostics and NTM speciation and Drug Resistance Detection Array Kit for MTBC diagnosis and for genotyping RIF and INH resistance (2, 74) (Table 3).

The TruArray® MDR-TB microarray is fluorescent probe based single tube multiplexed asymmetric PCR reaction. It can detect MTB and *M. avium* along with resistant alleles to RIF and INH (2). VereMTB™ Detection Kit from Veredus Laboratory is solely for research purpose. This array based platform can detect MTBC through *IS6110*. In addition the array can detect MTBC, speciate eight NTMs (*M. avium*, *M. simiae*, *M. intracellulare*, *M. Kansaii*, *M. abscessus*, *M. scrofulaceum*, *M. chelonae*, *M. xenopi*) and probe alleles associated with RIF and INH resistance (74) (Table 3).

HYDRA 1K developed by Stanford University (USA) together with Insilixa Inc. (USA) is an array based platform to detect PTB MDR / XDR-TB through the genotyping of mutations. This uses complementary metal-oxide semiconductor (CMOS) technology for digital imaging on the array. PCR amplification happens in the array area so that binding with amplicon with probes is measured in real time that saves lot of time and energy. CMOS enables this platform to have melt curve analysis of amplicon binding at each probe spot. It is developed to detect PTB, MDR / XDR-TB. However, higher cost of instrument and other peripherals makes this technique less likely to commercialize (2, 74) (Table 3).

## 11. WHOLE GENOME SEQUENCING

Whole genome sequencing (WGS) is recently applied to analyze molecular evolution of *M. tuberculosis* Beijing strain and appearance of an Iberico-American strain in Tibet. WGS provides greater resolution when compared to mycobacterial interspersed repetitive unit-variable-number tandem repeats (MIRU-VNTR, the existing protocol for molecular genotyping of circulating strains) (75-77).

**Table 2.** Line probe assay (LPA)

S.No.	Assay	Manufacturer	Method	Material	Advantage	Disadvantage	Sensitivity % (95% CI)	Specificity % (95% CI)
1.	INNO-LiPA Rif. TB	Innogenetics, Belgium	LPA	Sputum	Economical, reliable, widely applicable assay, valuable tool for routine diagnostics, simple, rapid	Accuracy goes down when it is applied directly on the clinical sample	Highly sensitive when isolates from the culture are used	High
2.	GenoType®MTBDR/MTBDR +	Hain Life Sciences, Germany	LPA	Sputum, broncho-alveolar lavage, bronchial aspirate, pleural effusion, lymph node sample	Can be applied for both culture isolates and clinical samples	Contaminate the work space, may have the potential to compromise all future test data, cannot be used for direct testing of smear –negative paucibacillary sputum samples	High	Modest and variable
3.	GenoType®MTBDR sl	Hain Life Sciences, Germany	LPA	Sputum	Can be applied for both culture isolates and clinical samples, sensitive detection, efficient diagnosis, flexible processing, efficient results	Contaminate the work space, may have the potential to compromise all future test data, further research is required on test accuracy in different settings, cannot be used to rule out XDR-TB	Highly specific tool for XDR detection in direct specimens for HIV co-infected TB patients	High
4.	LiPA pyrazinamide	Nipro	LPA	Sputum/ culture based	High throughput, identify the major clinical isolates of Mycobacterium species, detect mutations associated with resistance to INH, RIF, PZA and FQ, rapid, reliable detection of DR-TB NTM	Automated sequencing is required for which there is no LiPA probes, modern molecular instrumental set-up is required	High	High
5.	REBA MTB-MDR	YD-Diagnostics	LPA reverse hybridization assay	Sputum	Rapid detection of RIF, INH, FQ, +SLID, simple, economical, no costly equipment is required	Underestimate the true number of resistant strains because of limitations of knowledge regarding the limited knowledge regarding the genotype markers of drug resistance	High	High



**Table 3.** Microarray based diagnostic platforms

S.No.	Assay	Manufacturer	Method	Material	Advantage	Disadvantage	Sensitivity, % (95% CI)	Specificity, % (95% CI)
1.	INFINITI® PLUS	Autogenomics	Microarray platform	Clinical samples	High-throughput array, high performance genetic marker detection with varying options in automation and throughput	Can only be used with human nasopharyngeal aspirates, negative results do not preclude infection	NA	NA
2.	VereMTB assay	Veredus Laboratories, Singapore	PCR-Micro array	Sputum / culture based	Rapid detection, differentiation, identification of MTBC, Resistance to RIF, INH non-MTBC species, diagnosing patients after treatment failure and relapse	More for academic / Research purpose	High	High
3.	VereMTB™ Detection Kit	Veredus Laboratory	Array based platform	Sputum / culture based	Detect MTBC, speciate eight NTMSS	Research purpose only	High	High
4.	HYDRA1K	Stanford University (USA) together with Insilix Inc. (USA)	Array based platform	Sputum / culture based	Miniaturization Saves time and energy	Higher cost of instrument	NA	NA
5.	TruArray® MDR-TB microarray	Akonni Biosystems	Fluorescent probe based Single tube multiplexed asymmetric PCR reaction	Bacterial culture / sputum	Miniaturize, Bench top test, single integrated workflow approach, accurate, rapid, broad coverage and field ready	Research purpose only	High	High
6.	Twin Microarray	CapitalBio	Mycobacterium identification array kit for MTB, NTM speciation, drug resistance, MTBC diagnosis, genotyping RIF, INH resistance	Clinical specimens	Both MTC and NTM rapid system, sensitive, reliable, fast, spinal tuberculosis	Infrastructure and trained personnel required reduces its clinical utility in poverty-stricken zones, Additional large-scale studies are needed to evaluate its diagnostic performance in spinal tuberculosis	High, variable for different clinical specimens	High, variable For different clinical specimens

QIAxcel automated genotyping system from Qiagen provides high-throughput genotyping of MTBC hence providing epidemiological informations like prevalence or introduction of a particular genotype. This protocol targets variable sized regions of MTB genomic DNA using

MIRU-VNTR approach. This platform is further improvised to detect RIF and INH resistant alleles (80-81).

Unlike other bacterial pathogens that require drug resistance through plasmid acquisition, transposon or phage mediated elements, *M. tuberculosis* acquire drug resistance through chromosomal mutations particularly, single-nucleotide polymorphisms (SNPs) (80-81). This makes identification of drug resistant alleles difficult particularly for those lying outside of drug resistant hotspots, e.g. RIF resistance-determining region for RIF or the alleles that lie outside of *rpoB* and associated with resistance (77). Newer approaches

to detect drug resistance using Next-generation-sequencing (NGS) involves PZA resistance via deletions, STR, INH and screening allelic variation associated with resistance to novel drugs (80,82-85). NGS is very useful in determining whether the case of relapse is because of infection with new strain or incomplete treatment (86). WGS is already applied to the patients with MDR-TB (87-88). McKinsey has even listed NGS as one of the 12 technologies that will transform our lives.

### 11.1. New sequencing protocols

MiSeq from Illumina, USA facilitates focused applications like targeted gene sequencing, metagenomics, small genome sequencing, targeted gene expression, amplicon sequencing and HLA typing. Only 10ng of DNA is required as starting material. MiSeq gives output of 15 Gb with 25 M sequencing reads of 2x300 bp read lengths). Personal Genome Machine® (PGM™) System (Life Technologies Incorporated; USA) is the conjugation of semiconductor sequencing technology with natural biochemistry to provide real-time chemical information as digital data in less than 3 hours. This technique is accessible to all kind of set-ups. Use of simple natural sequencing chemistry eliminates the requirement of costly optics and complicated chemistries to measure DNA extension. 454 FLX Junior (Roche Diagnostics; Switzerland) provides rapid and robust platform to sequence and assemble genome of any size without the need for supplementary Sanger data. It provides the output having double (1000 bp) the read length of the Titanium kits, hence an improvisation over current GS FLX. The applications like transcriptome and amplicon sequencing enable an all inclusive picture of gene space of organisms. GeneReader from Qiagen can complement available protocols for resistance determination. It can detect allelic variants at 1% allelic frequency. Data from liquid biopsy pipeline show 100% conformity with PCR-based variant detection methods. GnuBIO from Bio-Rad Laboratories is a novel low cost desktop sequencer. It comes up with a highly integrated platform that incorporates all the functions of DNA sequencing like target selection, DNA amplification, DNA sequencing and analysis into a single, integrated system. Single user interface with single step process ensures rapid genomic results in hours. GridION™ and the Min-ION™ from Oxford Nanopore Technologies, UK facilitates rapid assembly of genome data for a MDR isolate, which elucidates the distribution and underlying mechanism of drug resistance. It enables the selection of specific DNA molecules for sequencing by reversing the driving voltage across individual nanopores (89).

## 12. ASSAYS BEING DEVELOPED / EVALUATED UPCOMING/PIPELINE TECHNOLOGIES

There is a co-evolution of sort between TB bacilli invading to newer regions and newer

techniques / protocols as a counter offensive. Many such existing protocols are improvised and upgraded in response to the challenges and threat posed by rapidly emerging and evolving drug resistant TB bacilli. Following protocols / platforms are under development to spearhead the fight of humanity against TB:

A combine of nano- and biosensing technology is being evaluated for its use as a portable, rapid on-the-spot biosensor for MTB detection. For instance, detection of trans-renal DNA opens up new avenues for TB detection at molecular level. This technique is not yet commercialized due to the problems in the development of TB detection / readout assays.

High-resolution melting (HRM) curve analysis coupled with closed-tube RT-PCR provides a good screening method with a positive predictive value (PPV) of 100% and negative predictive value (NPV) of 99.9%, for high throughput screening of large number of specimens in any TB laboratory. Improvised amplification techniques in conjunction with 'molecular beacon' approach (LATE-PCR) offer future advancements, particularly in drug resistance analysis (90-91).

IR spectrophotometer based detection of isotopic urea in exhaled breath is another future technique that holds promise. It utilizes MTB urease as a bacterial virulence factor (6).

A biophotonic detection platform is being developed that utilizes reporter enzyme fluorescence to detect  $\beta$ -lactamase produced by MTB. This innovative technology is now being adapted for point of care (POC) use.

Becton Dickinson is in the process of designing an automated platform that will do the staining of AFB in sputum in addition to imaging. This platform will provide the sensitivity similar to the fluorescent microscopy at the rate of processing around 40 slides per day with slide scanning algorithm for scoring (2).

Xiamen Zeesan Biotech Co. Ltd (China) has developed MeltPro® Drug-Resistant TB Testing Kits as RT-PCR enabled detection of resistant alleles to RIF and INH. It is undergoing further improvisation to detect alleles resistant to ethambutol (EMB), streptomycin (STR) and fluoroquinolones (FLQs) (52-53, 92).

Genedrive® MTB assay is upgraded to include genotyping MDR-TB through RIF resistance in addition to MTBC diagnosis. This assay involves manual preparation of samples followed by RT-PCR115 (93-94).

The Northwestern Global Health Foundation (USA) in partnership with Quidel Inc. (USA) is coming

up with an integrated MTBC assay. The assay involves lysing MTBC cells via heat block followed by purifying DNA using immiscible oil interface (68).

Q-POC™ platform, under development from QuantuMDx (UK) offers diagnosis for PTB.

The assay involves coughing the sputum in a cup that contains reagents for its liquification and decontamination. This is followed by concentration of MTBC cells via paramagnetic bead concentration step and quick nucleic acid amplification (4 minutes) via isothermal asymmetric PCR (2).

Tangen Biosciences Inc. (USA) is going to launch a fully integrated TB detection platform. This platform will have provision for sputum collection in a cup to capture MTB cells followed by lysis and isothermal amplification via LAMP. It comes up with the storage capacity of 10,000 results (2).

### 13. CONCLUSIONS

Accurate and timely diagnosis of TB is the only requirement for its control and management. Currently, TB diagnosis and treatment is plagued by factors, like inability to diagnose correctly latent and active TB; lack of precise diagnostic protocols for EPTB, pediatric TB and TB associated with HIV; prolonged diagnostic protocol aggravating the otherwise curable infection and escalating it to MDR/XDR-TB; absence of readily available testable biomarkers; Inaccessibility to the POC diagnostic facilities in the peripheral laboratories. These are further challenged by the rapidly emerging resistant strains of TB bacilli in the regions where it is thought to be eradicated or where it was non-existent historically. Need of the hour is the tools and techniques that are accessible, accurate, rapid, economical and most importantly rapid as time lag in diagnosis aggravates the infection. Although TB diagnostics have seen tremendous upsurge in the newer technologies and protocols from imaging, molecular NAAT based, immunological, culture based phenotypic methods, array based to genome sequencing protocols. Unfortunately, they offer promise in some aspect but lack or have severe limitation in other aspects, which limits their applicability on 'one-shoe-fit-all' basis. Another, problem with newer protocols is the reluctance of user groups to embrace it either due to lengthy approval mechanism, lack of WHO approval in Toto or simply the cost and expertise involved. There is urgent need to evolve the mechanism to accept the newer protocols on the emergency basis. As an extension to the problem, many small companies with promising diagnostic kits (in terms of good performance on heterogeneous set-ups) do not have sufficient funds to market their products or scale up their production. Also, most of the newer technologies are accepted in the developed

countries but there is a need to evolve the technologies compatible to the requirement of resource limited developing countries. There is a dire need of all inclusive diagnostic protocols with applicability in wide spectrum of patients and set-ups with high sensitivity and specificity. A dynamic understanding of existing and forthcoming technologies is key in facilitating access to appropriate TB diagnostic tools through market-based interventions. As such, this review is intended to be a living document, updated as the TB diagnostics market evolves, to highlight potential.

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### 15. REFERENCES

1. Drobniewski, F., V. Nikolayevsky, Y. Balabanova, D. Bang, and D. Papaventsis: Modern laboratory diagnosis of tuberculosis. *Lancet Infect Dis* 3, 141–147 (2003) DOI: 10.1016/S1473-3099(03)00544-9
2. UNITAID: Tuberculosis Diagnostics Technology and Market Landscape, 4th edition. World Health Organization. (2015)
3. World Health Organization: Non-commercial culture and drug susceptibility testing methods for screening patients at risk for multidrug-resistant tuberculosis: policy statement. WHO/HTM/TB/2011.9. Geneva, Switzerland: WHO (2011)
4. Lessem, E: The tuberculosis diagnostics pipeline. *HIV & TB Pipeline Report*. (2016)
5. Kent, P.T., and G.P. Kubica: Centers for Disease Control. Public Health Mycobacteriology: A Guide for the Level III Laboratory. Atlanta, GA, Centers for Disease Control (1985)
6. Maiga, M., A. Abaza, and W.R. Bishai: Current tuberculosis diagnostic tools and role of urease breath test. *Ind J Med Res* 135, 731-6 (2012)
7. Master, R.N: Clinical microbiology procedures handbook. American Society for

- Microbiology. *J Bacteriol* 191, 4714–4721 (1992)
8. McNerney, R., J. Cunningham, P. Hepple, and A. Zumla: New tuberculosis diagnostics and rollout International. *J Infect Dis* 32, 81–86 (2015)  
DOI: 10.1016/j.ijid.2015.01.012
9. Laserson, K.F., N.T. Yen, C.G. Thornton, V.T. Mai, W. Jones, D.Q. An, N.H. Phuoc, N.A. Trinh, D.T. Nhung, T.X. Lien, N.T. Lan, C. Wells, N. Binkin, M. Cetron, and S.A. Maloney: Improved sensitivity of sputum smear microscopy after processing specimens with C18-carboxypropylbetaine to detect acid-fast bacilli: a study of United States-bound immigrants from Vietnam. *J Clin Microbiol* 43, 3460–2 (2005)  
DOI: 10.1128/JCM.43.7.3460–3462.2005
10. Jaiswal, S., S. Jay Prakash, B. Sharma: Standard Diagnostic Procedure for Tuberculosis: A Review. *Res Rev J Life Sc* 1–10 (2013)
11. Lewis, J.J., V.N. Chihota, M. van der Meulen, P.B. Fourie, K.L. Fielding, A.D. Grant, S.E. Dorman, and G.J. Churchyard: Proof-of-concept” evaluation of an automated sputum smear microscopy system for tuberculosis diagnosis. *PLoS One* 7, e50173 (2012)  
DOI: 10.1371/journal.pone.0050173
12. Divekar, A., C. Pangilinan, G. Coetzee, T. Sondh, F.Y. Lure, and S. Kennedy: Automated detection of tuberculosis on sputum smeared slides using stepwise classification. *Proc SPIE Med Imag Conf* (2012)  
DOI: <https://doi.org/10.1117/12.910484>
13. Hogeweg, L., C. Sanchez, P. Maduskar, R. Philippsen, A. Story, R. Dawson, G. Theron, K. Dheda, L. Peters-Bax, and B. van Ginneken: Automatic detection of tuberculosis in chest radiographs using a combination of textural, focal, and shape abnormality analysis. *IEEE Trans Med Imag* 34, 2429–42 (2015)  
DOI: 10.1109/TMI.2015.2405761
14. Muyoyeta, M., P. Maduskar, M. Moyo, N. Kasese, D. Milimo, R. Spooner, N. Kapata, L. Hogeweg, B. van Ginneken, and H. Ayles H: The sensitivity and of using a computer aided diagnosis program for automatically scoring chest X-rays of presumptive TB patients compared with Xpert MTB/RIF in Lusaka Zambia. *PLoS One* 9, e93757 (2014)  
DOI: 10.1371/journal.pone.0093757
15. Tapley, A., N. Switz, C. Reber, J.L. Davis, C. Miller, J.B. Matovu, W. Worodria, L. Hunag, D.A. Fletcher, and A. Cattamanchi: Mobile digital fluorescence microscopy for diagnosis of tuberculosis. *J Clin Microbiol* 51, 1774–8 (2013)  
DOI: 10.1128/JCM.03432-12
16. Fujita, A., K. Murata, and M. Takamori: Novel method for sputum induction using the Lung Flute in patients with suspected pulmonary tuberculosis. *Respirol* 14, 899–902 (2009)  
DOI: 10.1111/j.1440-1843.2009.01584.x
17. Arend, S.M., A.C. Engelhard, G. Groot, K. de Boer, P. Andersen, T.H. Ottenhoff, and J.T. van Dissel: Tuberculin skin testing compared with T-cell responses to *Mycobacterium tuberculosis*-specific and nonspecific antigens for the detection of latent infection in persons with recent tuberculosis contact. *Clin Diagn Lab Immunol* 8, 1089–96 (2001)  
DOI: 10.1128/CDLI.8.6.1089-1096.2001
18. Doherty, T.M., A. Demissie, J. Olobo, D. Wolday, S. Britton, T. Equale, P. Ravn, and P. Andersen: Immune responses to the *Mycobacterium tuberculosis*-specific antigen ESAT-6 signal subclinical infection among contacts of tuberculosis patients. *J Clin Microbiol* 40, 704–06 (2002)  
DOI: 10.1128/JCM.40.2.704-706.2002
19. Lalvani, A., A.A. Pathan, H. Durkan, K.A. Wilkinson, A. Whelan, J.J. Deeks, W.H. Reece, M. Latif, G. Pasvol, and A.V. Hill: Enhanced contact tracing and spatial tracking of *Mycobacterium tuberculosis* infection by enumeration of antigenspecific T-cells. *Lancet* 357, 2017–21 (2001)  
DOI: 10.1016/S0140-6736(00)05115-1
20. Nakamura, R.M., M.A. Velmonte, K. Kawajiri, C.F. Ang, R.A. Frias, M.T. Mendoza, J.C. Montoya, Honda, S. Haga, and I. Toida: MPB64 mycobacterial antigen: a new skin-test reagent
21. Through patch method for rapid diagnosis of active tuberculosis. *Int J Tuberc Lung Dis* 2, 541–46 (1998)
22. Nakamura, R.M., L. Einck, M.A. Velmonte, K. Kawajiri, C.F. Ang, C.E. Delasllagas, and C.A. Nacy CA: Detection of active tuberculosis by an MBP64 transdermal patch: a field study. *Scan J Infect Dis* 33, 405–07 (2001)  
DOI: 10.1080/00365540152029846



23. Drobniewski, F., V. Nikolayevskyy, H. Maxeiner, Y. Balabanova, N. Casali, I. Kontsevaya, and O. Ignatyeva: Rapid diagnostics of tuberculosis and drug resistance in the industrialized world: clinical and public health benefits and barriers to implementation *BMC Med* 11, 190 (2013)  
DOI: 10.1186/1741-7015-11-190
24. Balabanova, Y., F. Drobniewski, V. Nikolayevskyy, A. Kruuner, N. Malomanova, T. Simak, N. Ilyina, S. Zakharova, N. Lebedeva, H.L. Alexander, R. O'Brien, H. Sohn, A. Shakhmistova, and I. Fedorin: An integrated approach to rapid diagnosis of tuberculosis and multidrug resistance using liquid culture and molecular methods in Russia. *PLoS One* 4, e7129 (2009)  
DOI: 10.1371/journal.pone.0007129
25. Leung, E., J. Minion, A. Benedetti, M. Pai, and D. Menzies: Microcolony culture techniques for tuberculosis diagnosis: a systematic review. *Int J Tuberc Lung Dis* 16, 16–23 (2012)  
DOI: 10.5588/ijtld.10.0065
26. Minion, J., E. Leung, D. Menzies, and M. Pai: Microscopic-observation drug susceptibility and thin layer agar assays for the detection of drug resistant tuberculosis: a systematic review and metaanalysis. *Lancet Infect Dis* 10, 688–98 (2010)  
DOI: 10.1016/S1473-3099(10)70165-1
27. Toit, K., S. Mitchell, Y. Balabanova, C.A. Evans, T. Kummik, V. Nikolayevskyy, and F. Drobniewski: The colour test for drug susceptibility testing of *Mycobacterium tuberculosis* strains. *Int J Tuberc Lung Dis* 16, 1113–8 (2012)  
DOI: 10.5588/ijtld.11.0609
28. Gonzalo, X., F. Drobniewski, S. Hoffner, and J. Werngren: Evaluation of a biphasic media assay for pyrazinamide drug susceptibility testing of *Mycobacterium tuberculosis*. *J Antimicrob Chemother* 69, 3001–3005 (2014).  
DOI: 10.1093/jac/dku230
29. Washinton, W., A. Stephen, J. William, E. Koneman, G. Procop, P. Schreckenberger, and G. Woods: Koneman's Colour Atlas and Textbook of Diagnostic Microbiology. *Lippincott Publishing* 1066–117 (2006).
30. Drobniewski, F.A., P.G. More, and G.S. Harris: Differentiation of *Mycobacterium tuberculosis* complex and nontuberculous mycobacterial liquid cultures by using peptide nucleic acid-fluorescence *in situ* hybridization probes. *J Clin Microbiol* 38, 444–47 (2000)
31. Telenti, A., F. Marchesi, M. Balz, F. Bally, E.C. Bottger, and T. Bodmer: Rapid identification of mycobacteria to the species level by polymerase chain reaction and restriction enzyme analysis. *J Clin Microbiol* 31, 175–78 (1993)
32. Kunnath-Velayudhan, S., H. Salamon, H.Y. Wang, A.L. Davidow, D.M. Molina, V.T. Huynh, D.M. Cirillo, G. Michel, E.A. Talbot, M.D. Perkins, P.L. Felgner, X. Liang, and M.L. Gennaro: Dynamic antibody responses to the *Mycobacterium tuberculosis* proteome. *Proc Natl Acad Sci USA* 107, 14703–8 (2010)  
DOI: 10.1073/pnas.1009080107
33. Steingart, K.R., L.L. Flores, N. Dendukuri, I. Schiller, S. Laal, A. Ramsay, P.C. Hopewell, and M. Pai: Commercial serological tests for the diagnosis of active pulmonary and extra-pulmonary tuberculosis: an updated systematic review and meta-analysis. *PLoS Med* 8, e1001062 (2011)  
DOI: 10.1371/journal.pmed.1001062
34. Kroidl, I., P. Clowes, J. Mwakyelu, L. Maboko, A. Kiangi, A. Rachow, K. Reither, J. Jung, A. Nsojo, E. Saathoff, and M. Hoelscher: Reasons for false-positive lipoarabinomannan ELISA results in a Tanzanian population. *Scand J Infect Dis* 46, 144–8 (2014)  
DOI: 10.3109/00365548.2013.853133
35. Nicol, M.P., V. Allen, L. Workman, W. Isaacs, J. Munro, S. Pienaar, F. Black, L. Adonis, W. Zemanay, Y. Ghebrekristos, and H.J. Zar: Urine lipoarabinomannan testing for diagnosis of pulmonary tuberculosis in children: a prospective study. *Lancet Glob Health* 2, e278–84 (2014)  
DOI: 10.1016/S2214-109X(14)70195-0
36. Sun, D., S. Dorman, M. Shah, Y.C. Manabe, V.M. Moodley, M.P. Nicol, and D.W. Dowdy: Cost utility of lateral-flow urine lipoarabinomannan for tuberculosis diagnosis in HIV infected African adults. *Int J Tuberc Lung Dis* 17:552–8 (2013)  
DOI: 10.5588/ijtld.12.0627
37. Portevin, D., F. Moukambi, P. Clowes, A. Bauer, M. Chachage, N.E. Ntinginya, E. Mfinanga, K. Said, F. Haraka, A. Rachow, E.



- Saathoff, M. Mpina, L. Jugheli, F. Lwilla, B.J. Marais, M. Hoelscher, C. Daubenberger, K. Reither, and C. Geldmacher: Assessment of the novel T-cell activation marker-tuberculosis assay for diagnosis of active tuberculosis in children: a prospective proof-of-concept study. *Lancet Infect Dis* 2014;14:931-8 (2014)  
DOI: 10.1016/S1473-3099(14)70884-9
38. Meldau, R., J. Peter, G. Theron, G. Calligaro, B. Allwood, G. Symons, H. Khalfey, G. Ntombenhle, U. Govender, A. Binder, R. van Zyl-Smit, and K. Dheda: Comparison of same day diagnostic tools including Gene Xpert and unstimulated IFNgamma for the evaluation of pleural tuberculosis: a prospective cohort study. *BMC Pulm Med* 14, 58 (2014)  
DOI: 10.1186/1471-2466-14-58
39. Hur, M., H.W. Moon, Y.M. Yun, T.Y. Kang, H.S. Kim, K.M. Lee, S.H. Kang, and E.H. Lee: Detection of tuberculosis using artus M. tuberculosis PCR Kit and COBAS AMPLICOR Mycobacterium tuberculosis Test. *Int J Tuberc Lung Dis* 15,795– 798 (2011)  
DOI: 10.5588/ijtld.10.0367
40. Kim, J.H., Y.J. Kim, C.S. Ki, J.Y. Kim, and N.Y. Lee: Evaluation of Cobas TaqMan MTB PCR for detection of Mycobacterium tuberculosis. *J Clin Microbiol* 49, 173–176 (2011)  
DOI: 10.1128/JCM.00694-10
41. Ling, D.I., L.L. Flores, L.W. Riley, and M. Pai M: Commercial nucleic-acid amplification tests for diagnosis of pulmonary tuberculosis in respiratory specimens: meta-analysis and meta regression. *PLoS One* 3, e1536 (2008)  
DOI: 10.1371/journal.pone.0001536
42. Mitarai, S., M. Okumura, E. Toyota, T. Yoshiyama, A. Aono, A. Sejimo, Y. Azuma, K. Sugahara, T. Nagasawa, N. Nagayama, A. Yamane, R. Yano, H. Kokuto, K. Morimoto, M. Ueyama, M. Kubota, R. Yi, H. Ogata, S. Kudoh, and T. Mori: Evaluation of a simple loop-mediated isothermal amplification test kit for the diagnosis of tuberculosis. *Int J Tuberc Lung Dis* 15,1211–1217 (2011)  
DOI: 10.5588/ijtld.10.0629
43. Wang, J.Y., L.N. Lee, H.L. Hsu, P.R. Hsueh, and K.T. Luh: Performance assessment of the DR. MTBC Screen assay and the BD ProbeTec ET system for direct detection of Mycobacterium tuberculosis in respiratory specimens. *J Clin Microbiol* 44, 716–719 (2006)  
DOI: 10.1128/JCM.44.3.716-719.2006
44. Boehme, C.C., M.P. Nicol, P. Nabeta, J.S. Michael, E. Gotuzzo, R. Tahirli, M.T. Gler, R. Blakemore, W. Worodria, C. Gray, L. Huang, T. Caceres, R. Mehdiyev, L. Raymond, A. Whitelaw, K. Sagadevan, H. Alexander, H. Albert, F. Cobelens, H. Cox, D. Alland, and M.D. Perkins: Feasibility, diagnostic accuracy, and effectiveness of decentralised use of the Xpert MTB/ RIF test for diagnosis of tuberculosis and multidrug resistance: a multicentre implementation study. *Lancet* 377, 1495–1505 (2011)  
DOI: 10.1016/S0140-6736(11)60438-8
45. Pietzka, A.T., A. Indra, A. Stoger, J. Zeinzinger, M. Konrad, P. Hasenberger, F. Allerberger, and W. Ruppitsch: Rapid identification of multidrug-resistant Mycobacterium tuberculosis isolates by rpoB gene scanning using high resolution melting curve PCR analysis. *J Antimicrob Chemother* 63,1121–1127 (2009)  
DOI: 10.1093/jac/dkp124
46. Ribeiro, F.K., V. Dettoni Vdo, R.L. Peres, S.A. Vinhas, T.R. C6, R. Dietze, and M. Palaci: Evaluation of a commercial test based on ligase chain reaction for direct detection of Mycobacterium tuberculosis in respiratory specimens. *Rev Soc Bras Med Trop* 37, 431–435 (2004)  
DOI: 10.1590/S0037-86822004000600001
47. Chen, J.H., K.K. She, T.C. Kwong, O.Y. Wong, G.K. Siu, C.C. Leung, K.C. Chang, C.M. Tam, P.L. Ho, V.C. Cheng, K.Y. Yuen, W.C. Yam: Performance of the new automated Abbott Real-Time MTB assay for rapid detection of Mycobacterium tuberculosis complex in respiratory specimens. *Eur J Clin Microbiol Infect Dis* 34, 1827-32 (2015)  
DOI: 10.1007/s10096-015-2419-5
48. Hofmann-Thiel, S., and H. Hoffmann: Evaluation of Fluorotype MTB for detection of Mycobacterium tuberculosis complex DNA in clinical specimens from a low-incidence country. *BMC Infect Dis* 14, 59 (2014)  
DOI: 10.1186/1471-2334-14-59
49. Rice, J.E., A.H. Reis Jr, L.M. Rice, R.K. Carver-Brown, and L.J. Wangh: Fluorescent signatures for variable DNA sequences. *Nucleic Acids Res* 40, e164 (2012)  
DOI: 10.1093/nar/gks731

50. Takakura, S., S. Tsuchiya, Y. Isawa, K. Yasukawa, T. Hayashi, M. Tomita, K. Suzuki, T. Hasegawa, T. Tagami, A. Kurashima, and S. Ichiyama: Rapid detection of *Mycobacterium tuberculosis* in respiratory samples by transcription-reverse transcription concerted reaction with an automated system. *J Clin Microbiol* 43, 5435–9 (2005)  
DOI: 10.1128/JCM.43.11.5435-5439.2005
51. Lee, S.H., S.W. Kim, S. Lee, E. Kim, D.J. Kim, S. Park, E.J. Lee, S.Y. Lee, J.S. Lee, C.S. Lim, W.K. Kim, and K.H. In: Rapid detection of *Mycobacterium tuberculosis* using a novel ultrafast chip-type real-time polymerase chain reaction system. *Chest* 146, 1319–26 (2014)  
DOI: 10.1378/chest.14-0626
52. Hwang, I.T., Y.J. Kim, S.H. Kim, C.I. Kwak, Y.Y. Gu, and J.Y. Chun: Annealing control primer system for improving specificity of PCR amplification. *Biotechniques* 35, 1180–4 (2003)
53. Hu, S., G. Li, H. Li, X. Liu, J. Niu, S. Quan, F. Wang, H. Wen, Y. Xu, and Q. Li: Rapid detection of isoniazid resistance in *Mycobacterium tuberculosis* isolates by use of real-time-PCR-based melting curve analysis. *J Clin Microbiol* 52, 1644–52 (2014)  
DOI: 10.1128/JCM.03395-13
54. Zhang, T., S. Hu, G. Li, H. Li, X. Liu, J. Niu, F. Wang, H. Wen, Y. Xu, and Q. Li: Evaluation of the MeltPro TB/STR assay for rapid detection of streptomycin resistance in *Mycobacterium tuberculosis*. *Tuberculosis (Edinb)* 95, 162–9 (2015)  
DOI: 10.1016/j.tube.2014.12.004
55. Steingart, K.R., I. Schiller, D.J. Horne, M. Pai, C.C. Boehme, and N. Dendukuri: Xpert1 MTB/RIF assay for pulmonary tuberculosis and rifampicin resistance in adults. *Cochrane Database Syst Rev* 1, CD009593 (2014)
56. Weyer, K., F. Mirzayev, G.B. Migliori, W. Van Gemert, L. D'Ambrosio, M. Zignol, K. Floyd, R. Centis, D.M. Cirillo, E. Tortoli, C. Gilpin, J. de Dieu Iragena, D. Falzon, and M. Raviglione: Rapid molecular TB diagnosis: evidence, policy making and global implementation of Xpert MTB/RIF. *Eur Respir J* 42, 252–271 (2013)  
DOI: 10.1183/09031936.00157212
57. Nikama, C., M. Kazia, C. Nairb, M. Jaggannathb, M. Manoj, R. Vinaya, A. Shetty, and C. Rodrigues: Evaluation of the Indian TrueNAT micro RT-PCR device with GeneXpert for case detection of pulmonary tuberculosis. *Int J Mycobacteriol* 3, 205–10 (2014)  
DOI: 10.1016/j.ijmyco.2014.04.003
58. Ou, X., Y. Song, B. Zhao, Q. Li, H. Xia, Y. Zhou, Y. Pang, S. Wang, Z. Zhang, S. Cheng, C. Liu, and Y. Zhao: A multicenter study of crosspriming amplification for tuberculosis diagnosis at peripheral level in China. *Tuberculosis (Edinb)* 94, 428–33 (2014)  
DOI: 10.1016/j.tube.2014.04.006
59. Bholla, M., N. Kapalata, E. Masika, H. Chande, L. Jugheli, M. Sasamalo, T.R. Glass, H.P. Beck, and K. Reither: Evaluation of Xpert® MTB/RIF and Ustar EasyNAT™ TB IAD for diagnosis of tuberculous lymphadenitis of children in Tanzania: a prospective descriptive study. *BMC Infect Dis* 16, 246 (2016)  
DOI: 10.1186/s12879-016-1578-z
60. Moore, D.A., C.A. Evans, R.H. Gilman, L. Caviedes, J. Coronel, A. Vivar, E. Sanchez, Y. Piñedo, J.C. Saravia, C. Salazar, R. Oberhelman, M.G. Hollm-Delgado, D. LaChira, A.R. Escombe, and J.S. Friedland: Microscopic-observation drug-susceptibility assay for the diagnosis of TB. Laboratories V. VereMTB product sheet. Rapid detection, differentiation and identification of MDR-TB. Singapore: Verdus Laboratories; 2014. *N Engl J Med* 355, 1539–50 (2006)  
DOI: 10.1056/NEJMoa055524
61. Jones, B.E., S.M. Young, D. Antoniskis, P.T. Davidson, F. Krammer, and F. Barnes: Relationship of the manifestations of tuberculosis to CD4 cell counts in patients with human immunodeficiency virus infection. *Am Rev Respir Dis* 148, 1292–1297 (1993)  
DOI: 10.1164/ajrccm/148.5.1292
62. Nelson, L.J., and C.D. Wells: Global epidemiology of childhood tuberculosis. *Int J Tuberc Lung Dis* 8, 636–647 (2004)
63. Marais S., G. Thwaites, J.F. Schoeman, M.E. Török, U.K. Misra, K. Prasad, P.R. Donald, R.J. Wilkinson, and B.J. Marais: Tuberculous meningitis: a uniform case definition for use in clinical research. *Lancet Infect Dis* 10, 803–812 (2010)  
DOI: 10.1016/S1473-3099(10)70138-9
64. Pai, M., L.L. Flores, N. Pai, A. Hubbard, L.W. Riley, and J.M. Colford Jr: Diagnostic

- accuracy of nucleic acid amplification tests for tuberculous meningitis: a systematic review and meta-analysis. *Lancet Infect Dis* 3, 633–643 (2003)  
DOI: 10.1016/S1473-3099(03)00772-2
65. Elias, J., J.P. Coning De, S.A. Vorster, and H.F. Joubert: The rapid and sensitive diagnosis of tuberculous meningitis by the detection of tuberculostearic acid in cerebrospinal fluid using gas chromatography mass spectrometry with selective ion monitoring. *Clin Biochem* 22, 463–67 (1989)  
DOI: 10.1016/S0009-9120(89)80099-2
66. Denking, C.M., and M. Pai: Point-of-care tuberculosis diagnosis: are we there yet? *Lancet Infect Dis* 12, 169–70 (2012)  
DOI: 10.1016/S1473-3099(11)70257-2
67. Niemz, A., and D.S. Boyle: Nucleic acid testing for tuberculosis at the point-of-care in high-burden countries. *Expert Rev Mol Diagn* 12, 687–701 (2012)  
DOI: 0.1586/erm.12.71
68. Jani, I.V., B. Meggi, N. Mabunda, A. Vubil, N.E. Siteo, O. Tobaiwa, J.I. Quevedo, J.D. Lehe, O. Loquiha, L. Vojnov, and T.F. Peter: Accurate early infant HIV diagnosis in primary health clinics using a point-of-care nucleic acid test. *J Acquir Immune Defic Syndr* 67, e1–e4 (2014)  
DOI: 10.1097/QAI.0000000000000250
69. Sur, K., S.M. McFall, E.T. Yeh, S.R. Jangam, M.A. Hayden, S.D. Stroupe, and D.M. Kelso: Immiscible phase nucleic acid purification eliminates PCR inhibitors with a single pass of paramagnetic particles through a hydrophobic liquid. *J Mol Diagn* 12, 620–8 (2010)  
DOI: 10.2353/jmoldx.2010.090190
70. Kamerbeek, J., L. Schouls, A. Kolk, M. van Agterveld, D. van Soolingen, S. Kuijper, A. Bunschoten, H. Molhuizen, R. Shaw, M. Goyal, and J. van Embden: Simultaneous detection and strain differentiation of *Mycobacterium tuberculosis* for diagnosis and epidemiology. *J Clin Microbiol* 35, 907–14 (1997)
71. Ling, D.I., A.A. Zwerling, and M. Pai: GenoType MTBDR assays for the diagnosis of multidrug-resistant tuberculosis: a metaanalysis. *Eur Respir J* 32, 1165–74 (2008)  
DOI: 10.1183/09031936.00061808
72. Marras, S., H. El-Hajj, M.T. Taylor, and W. McMillan: Rapid and sensitive detection of *Mycobacterium* DNA using Cepheid SmartCycler® and Tube Lysis System. *Clin Chem* 47, 1917–8 (2001)
73. Morgan, M., S. Kalantri, L. Flores, and M. Pai: A commercial line probe assay for the rapid detection of rifampicin resistance in *Mycobacterium tuberculosis*: a systematic review and metaanalysis. *BMC Infect Dis* 5, 62 (2005)  
DOI: 10.1186/1471-2334-5-62
74. Tagliani, E., A.M. Cabibbe, P. Miotto, E. Borroni, J.C. Toro, M. Mansjo, S. Hoffner, D. Hillemann, A. Zalutskaya, A. Skrahina, and D.M. Crillo: Diagnostic Performance of the new version of GenoType MTBDRsl (V2.0.) Assay for detection of resistance to Fluoroquinolones and Second Line Injectable Drugs: a Multicenter study. *J Clin Microbiol* 53, 2961–9 (2015)  
DOI: 10.1128/JCM.01257-15
75. Lazzeri, E., F. Santoro, M.R. Oggioni, F. Iannelli, and G. Pozzi: Novel primer-probe sets for detection and identification of mycobacteria by PCR-microarray assay. *J Clin Microbiol* 50, 3777–9 (2012)  
DOI: 10.1128/JCM.02300-12
76. Mokrousov, I., W.W. Jiao, K. Wan, and A. Shen: Stranger in a strange land: Ibero-American strain of *Mycobacterium tuberculosis* in Tibet, China. *Infect Genet Evol* 26, 323–6 (2014)  
DOI: 10.1016/j.meegid.2014.06.011
77. Merker, M., C. Blin, S. Mona, N. Duforet-Frebourg, S. Lecher, E. Willery, M.G. Blum, S.R. Gerdes, I. Mokrousov, E. Aleksic, C.A. Begues, A. Antiriens, E.A. Kopec, M. Ballif, F. Barletta, H.P. Beck, C.E. Barry, M. Bonnet, E. Borroni, I.C. Herrero, D. Cirillo, H. Cox, S. Crowe, V. Crudu, R. Dier, F. Drobniewski, M.F. Dufaux, S. Gangneux, S. Ghebremichael, M. Hanekom, S. Hoffner, W.W. Jiao, S. Kalon, T.A. Kohl, I. Kontsevaya, T. Lilebaek, S. Maeda, V. Nikolayevskyy, M. Rasmussen, N. Rastogi, S. Samper, E.S. Padilla, B. Savic, I.C. Shamputa, A. Shen, L.H. Sng, P. Stakenas, K. Toit, F. Varaine, D. Vukovic, C. Wahl, R. Warren, P. Supply, S. Niemann, and T. Wirth: Evolutionary history and global spread of the *Mycobacterium tuberculosis* Beijing lineage. *Nat Genet* 2015;47:242–9.  
DOI: 10.1038/ng.3195

78. Jamieson, F.B., S. Teatero, J.L. Guthrie, A. Neemuchwala, N. Fittipaldi, and C. Mehaffy: Whole-genome sequencing of the *Mycobacterium tuberculosis* Manila sublineage results in less clustering and better resolution than mycobacterial interspersed repetitive-unit-variable-number tandem-repeat (MIRU-VNTR) typing and spoligotyping. *J Clin Microbiol* 52, 3795–8 (2014)  
DOI: 10.1128/JCM.01726-14
79. Gauthier, M., F. Bidault, A. Mosnier, N. Bablishvili, N. Tukvadze, S. Somphavong, P. Paboriboune, O. Ocheretina, J.W. Pape, G. Paranhos-Baccala, and J.L. Berland: Highthroughput mycobacterial interspersed repetitive-unit-variable-number tandem-repeat genotyping for *Mycobacterium tuberculosis* epidemiological studies. *J Clin Microbiol* 53, 498–503 (2015)  
DOI: 10.1128/JCM.01611-14
80. Matsumoto, T., Y. Koshii, K. Sakane, T. Murakawa, Y. Hirayama, H. Yoshida, M. Kurowawa, Y. Tamura, T. Nagai, I. Kawase: A novel approach to automated genotyping of *Mycobacterium tuberculosis* using a panel of 15 MIRU VNTRs. *J Microbiol Methods* 2013;93:239–41.  
DOI: 10.1016/j.mimet.2013.03.022
81. Martinez, E., N. Holmes, P. Jelfs, and V. Sintchenko: Genome sequencing reveals novel deletions associated with secondary resistance to pyrazinamide in MDR *Mycobacterium tuberculosis*. *J Antimicrob Chemother* 70, 2511–4 (2015)  
DOI: 10.1093/jac/dkv128
82. O'Malley, T., and E. Melief: Isolation and characterization of compound-resistant isolates of *Mycobacterium tuberculosis*. *Meth Mol Biol* 1285, 317–28 (2015)  
DOI: 10.1007/978-1-4939-2450-9\_19
83. Ioerger, T.R., T. O'Malley, R. Liao, K.M. Guinn, M.J. Hickey, N. Mohaideen, K.C. Murphy, H.I.M. Boshoff, V. Mizrahi, E.J. Rubin, C.M. Sassetti, C.E. Barry III, D.R. Sherman, T. Parish, and J.C. Sacchettini: Identification of new drug targets and resistance mechanisms in *Mycobacterium tuberculosis*. *PLoS One* 8, e75245 (2013)  
DOI: 10.1371/journal.pone.0075245
84. McGrath, M., N.C. Gey van Pittius, P.D. van Helden, R.M. Warren, and D.F. Warner: Mutation rate and the emergence of drug resistance in *Mycobacterium tuberculosis*. *J Antimicrob Chemother* 69, 292–302 (2014)  
DOI: 10.1093/jac/dkt364
85. Ramasubban, G., D. Lakshmipathy, U. Vetrivel, L.T. Kulandai, H.N. Madhavan, R. Sridhar, and N. Meenakshi: Wholegenome sequencing of streptomycin-resistant *Mycobacterium tuberculosis* isolate VRFCWCF MRTB 180 reveals novel and potential mutations for resistance. *Genome Announc* 2, e00919-14 (2014)  
DOI: 10.1128/genomeA.00919-14
86. Shekar, S., Z.X. Yeo, J.C. Wong, M.K. Chan, D.C. Ong, P. Tongyoo, S.Y. Wong, and A.S. Lee: Detecting novel genetic variants associated with isoniazid-resistant *Mycobacterium tuberculosis*. *PLoS One* 9, e102383 (2014)  
DOI: 10.1371/journal.pone.0102383
87. Guerra-Assuncao, J.A., R.M. Houben, A.C. Crampin, T. Mzembe, K. Mallard, F. Coll, P. Khan, L. Banda, A. Chiwaya, R.P. Pereira, R. McNerney, D. Harris, J. Parkhill, T.G. Clark, and J.R. Glynn: Recurrence due to relapse or reinfection with *Mycobacterium tuberculosis*: a whole-genome sequencing approach in a large, population-based cohort with a high HIV infection prevalence and active follow-up. *J Infect Dis* 211, 1154–63 (2015)  
DOI: 10.1093/infdis/jiu574
88. Outhred, A.C., P. Jelfs, B. Suliman, G.A. Hill-Cawthorne, A.B. Crawford, B.J. Marais, and V. Sintchenko: Added value of whole-genome sequencing for management of highly drug-resistant TB. *J Antimicrob Chemother* 70, 1198–1202 (2015)
89. Witney, A.A., K.A. Gould, A. Arnold, D. Coleman, R. Delgado, J. Dhillon, M.J. Pond, C.F. Pope, T.D. Planche, N.G. Stoker, C.A. Cosgrove, P.D. Butcher, T.S. Harrison, and J. Hinds: Clinical application of whole-genome sequencing to inform treatment for multidrug-resistant tuberculosis cases. *J Clin Microbiol* 53, 1473–83 (2015)  
DOI: 10.1128/JCM.02993-14
90. Rodriguez, J.G., C. Pino, A. Tauch, and M.I. Murcia: Complete Genome Sequence of the Clinical Beijing- Like Strain *Mycobacterium tuberculosis* 323 Using the PacBio Real-Time Sequencing Platform. *Genome Announc* 3, e00371-15 (2015)  
DOI: 10.1128/genomeA.00371-15



91. Chen, X., F. Kong, Q. Wang, C. Li, J. Zhang, and G.L. Gilbert: Rapid detection of isoniazid, rifampin and ofloxacin resistance in *Mycobacterium tuberculosis* clinical isolates using high-resolution melting analysis. *J Clin Microbiol* 49, 3450–7 (2011)  
DOI: 10.1128/JCM.01068-11
92. Casali, N., A. Broda, J. Rice, R. Mistry, C. Volpe, L. Wangh, J. Lewington, and F. Drobniewski: A novel single-tube PCR assay for rapid identification of MDR-TB. London, UK: 22nd European Congress of Clinical Microbiology and Infectious Diseases, 1– 4 April (2012) (Abstract P1919)
93. Pang, Y., H. Dong, Y. Tan, Y. Deng, X. Cai, H. Jing, H. Xia, Q. Li, X. Ou, B. Su, X. Li, Z. Zhang, J. Li, J. Zhang, S. Huan, and Y. Zhao: Rapid diagnosis of MDR and XDR tuberculosis with the MeltPro TB assay in China *Sci Rep* 6, 25330 (2016)
94. Shenai, S., D.T. Armstrong, E. Valli, D.L. Dolinger, L. Nakiyingi, R. Dietze, M.P. Dalcolmo, M.P. Nicol, W. Zemanay, Y. Manabe, D.J. Hadad, P. Marques-Rodrigues, M. Palaci, R.L. Peres, M. Gaeddert, S. Armakovitch, B.A. Nonyane, C.M. Denking, P. Banada, M.L. Joloba, J. Ellner, C. Boehme, D. Alland, and S.E. Dorman: Analytical and clinical evaluation of the Episteme Genedrive assay for detection of *Mycobacterium tuberculosis*. *J Clin Microbiol* 54, 1051-7 (2016)  
DOI: 10.1128/JCM.02847-15

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**Abbreviations:** CXRs, Chest X-ray; CAD4TB, Computer aided detection for tuberculosis; DST, Drug susceptibility test; PAT, Phage Amplification Technique; PNA, Peptide nucleic acids; LAM, lipoarabinomannans; TAM-TB, T-cell activation marker-tuberculosis; IGRA, Interferon-gamma release assays; ESAT-6, Early secretory antigen target 6; LAMP, loop-mediated isothermal amplification; LCR, ligase chain reaction; LPA, line probe assay; WGS, Whole genome sequencing; MIRU-VNTR, mycobacterial interspersed repetitive unit-variable-number tandem repeats; SNP, single-nucleotide polymorphism.

**Key Words:** *Mycobacterium tuberculosis* complex, MDR, XDR-TB, extrapulmonary TB, EPTB, pulmonary TB, PTB, sputum smear microscopy (SSM), Nucleic acid amplification tests, NAAT, Review

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