

Current Perceptions on Advanced Molecular Diagnostics for Drug-Resistant *Mycobacterium tuberculosis*

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Globally, rising drug-resistant tuberculosis is a significant public health concern. Prompt diagnosis of tuberculosis and detection of drug-resistant TB within a clinically appropriate timeframe is important for the effective management of the disease. Imaging approaches Chest X-rays, CT, MRI, nuclear medicine technique as PET/CT are non-specific, plays an important role in the diagnosis and assessment of TB, but PET/CT sometimes results in false-positive or negative due to benign lesions. Currently using the point of care molecular modalities, GeneXpert MTB/RIF and line probe assays focused only on resistance-conferring mutations in specific target hotspot regions, but did not identify novel mutations, outside mutations and they may miss some locally prevalent rifampicin-conferring mutations, and not provided a large number of antibiotics/antibiotic groups that are used for DRTB treatment. Recently revolutionized high throughput next generation sequencing (NGS) technologies are offering new prospects for molecular diagnosis, for example, infectious disease pathogens like tuberculosis, influenza, and most recently SARS-CoV-2. NGS is an essential resource for the tuberculosis community either target, WGS, or NGS; a rapid method that offers a complete spectrum of *Mycobacterium tuberculosis* resistance mutations, strain typing for transmission surveillance, unlike traditional molecular or phenotypic DST. It shall be helpful for early regimen design and TB management before mutations emerge and therefore, we believe that the worldwide TB infection will be eliminated by the use of NGS.

Keywords: *Mycobacterium tuberculosis*, Molecular Diagnosis, Drug resistant TB, Next Generation Sequencing, TB treatment.

Tuberculosis (TB) is an infectious and insidious disease, its leading cause of significant morbidity and mortality globally. *Mycobacterium tuberculosis* (MTB) typically affects the lungs and virtually all organs. In 2018, the World Health Organization (WHO) estimated burden of TB disease was 10 million people and India's highest number of new TB cases accounted for 27 % of

the global burden. Besides, there are estimated to be 1.7 billion people living with latent tuberculosis and a potential source of active tuberculosis in the future. The prevalence of multidrug-resistant TB (MDR-TB) and extensively drug-resistant TB (XDR-TB) is increasing worldwide, both among new and previously treated TB cases. Early diagnosis of tuberculosis and detection

of drug-resistant TB is important for effective disease management. However, the WHO - End TB Strategy aims at drug susceptibility testing (DST) for WHO-recommended drugs and is essential for any person who has been diagnosed with TB for proper treatment¹. Currently, the molecular diagnostic assays, including the Xpert MTB/RIF (GeneXpert MTB/RIF; Cepheid, Sunnyvale, CA) and line probe assays (GenoType MTBDRplus and MTBDRs/; Hain Lifescience GmbH, Nehren, Germany) can rapidly identify the limited number of mutations in first-line and second-line antibiotic resistance genes². These assays have significantly improved the MTBC detection, DST, and supports the results turnaround time than gold-standard phenotypic culture test. Drug-resistant mechanisms are not fully recognized at the current molecular level³. However, at present none of them, can able to detect the complete spectrum of drug-resistant tuberculosis mutations for appropriately aimed treatment in all forms of TB patients².

Next-generation sequencing (NGS) technologies have recently revolutionized the area of medical microbiology and serve as a valuable diagnostic tool for infectious diseases such as tuberculosis, influenza, and most recently SARS-CoV-2⁴. These new technologies allow the simultaneous sequencing of a large number of biological samples and they can be used for the unbiased identification of infectious diseases and the characterization of multiple agents in a single sample. To date, NGS was used to discover new novel viruses Arenavirus, a new Zika Virus, Ebola Virus, and more recently a widespread novel coronavirus that early in the outbreak^{5,6}. Reports on the use of NGS are more accurate for diagnosis of TB and anti-mycobacterial drug susceptibility with higher sensitivity and specificity than existing routine diagnostic modalities including imaging procedures^{7,8}. In this review, the challenges of traditional molecular TB diagnosis, and rational genetic approaches to drug resistant prediction with emerging technologies were identified as comprehensive tools which are likely to be Next Generation Sequencing.

Drug Resistance In Tuberculosis

The emergence of drug resistant tuberculosis (DRTB) is a primary global public health concern, which in recent decades has threatened the development in tuberculosis care and

prevention¹. Drug-resistant tuberculosis is caused when tubercle bacilli elicit resistance to the anti TB drugs that are commonly used for tuberculosis treatment. DRTB is transmitted in the same manner as drug-sensitive TB, and infection is no more than drug-susceptible TB. Moreover, delay in the early drug resistance detection or long infectious periods may facilitate increased transmission and further into drug resistance development, and this may lead to substantial TB morbidity and mortality⁹. Prompt diagnosis and treatment with effective drugs are a mainstay of tuberculosis control and a life-saving intervention for tuberculosis patients. *Mycobacterium tuberculosis complex* (MTBC) develops drug resistance through the selection of naturally occurring genomic mutants. The development of drug-resistant TB is strengthened by two strategies, such as acquired and primary DRTB. The acquired or secondary resistance TB develops during their treatment due to the inadequate treatment regimen, irregular prescription practices, poor quality of medicines with low bioavailability, poor compliance, and other circumstances, such as drug malabsorption or drug-drug interactions leading to low serum concentrations¹⁰. Primary antibiotic resistance occurs in persons who have not been treated with TB before and can be infected with a resistant tubercle bacilli and direct transmission of DRTB from one person to another¹¹.

imaging techniques for TB diagnosis

The complex immune response to MTB provides a wide range of clinical manifestations, symptoms may mimic, so the radiological diagnosis of TB remains challenging¹². Chest radiography is the primary diagnosis of pulmonary TB, but due to heterogeneous presentation, non-specific X-rays not provide a decisive diagnosis¹³. The current, noninvasive techniques, such as ultrasound, computed tomography (CT), and magnetic resonance imaging (MRI) are non-specific and cannot reliably distinguish between infectious lesions and non-infectious diseases such as cancer or autoimmune diseases^{14,15}. Molecular/anatomic imaging with 18F-labelled fluorodeoxyglucose (18F-FDG) positron emission tomography (PET)/ computed tomography (CT) scan is a sensitive and non-invasive biomarker tool commonly used for the differentiation of a wide range of malignancies from benign lesions. 18F-FDG-PET/

CT is also useful for the TB staging and location of Extrapulmonary TB (EPTB), for the identification of subclinical TB, and for the monitoring of treatment responses to TB¹²⁻¹⁶. Nevertheless, inflammatory cells such as activated macrophages, lymphocytes, and neutrophils actively accumulate the tracer 18F-FDG at the site of inflammation or infection¹⁶. Some benign lesions, or such as infectious inflammatory granulomatous lesions, specifically tuberculosis and chronic infections, inflammatory pseudotumor, and sarcoidosis, have been found to also be associated with increased uptake of 18F-FDG, resulting in false-positive lung cancer diagnosis in 18F-FDG-PET/CT. However, certain forms of cancer, such as Bronchoalveolar carcinoma and carcinoid tumors, have poor 18F-FDG absorption, which may contribute to false-negative outcomes^{12, 17}. In addition to 18F-FDG, other PET probes have been examined for TB imaging, including [18F] fluoroethylcholine (18F-FEC), 11C-choline, [18F] sodium fluoride (18FNaF), 68Ga-citrate, 30-deoxy-30-[18F]fluoro-L-thymidine (18F-FLT) and radiolabelled anti-TB medicines¹².

Conventional TB Diagnosis

Globally, sputum smear examination by microscopy is the point of care testing and the cornerstone of TB diagnosis. Smear microscopy (SM) is easy to perform, inexpensive, rapid method, and highly important in regions with a high incidence of tuberculosis. However, smear microscopy has more limitations such as it lacks sensitivity, requiring at least 10000 AFB/ml to become smear positive¹⁸. Microbiological confirmation of pediatric TB is less because of the sample collection issues, the low sensitivity of smear examination results, and poor culture accessibility. Extrapulmonary TB (EPTB) is a complex and multifaceted disease that constitutes a significant percentage of the global tuberculosis burden. The initial and precise diagnosis of extrapulmonary TB remains challenging, especially, the low number of tubercle bacilli present in the tissue and other extrapulmonary samples^{19,20}. Phenotypic diagnostic techniques are still the gold standard method for culture and DST is usually labor-intensive, time-consuming, and requires biosafety measures. Growth based Drug susceptibility testing takes few weeks to fully define *Mycobacterium tuberculosis* drug resistance

profile due to slow mycobacterial growth, and this delays the initiation of patient effective treatment and contributes to the transmission of resistant TB strains. Phenotypic DST relied on the testing of a single, critical concentration (CC) of each antibiotic agent and more possible to miss the emerging low-level drug resistance detection²¹.

molecular based assays for TB Diagnosis

All molecular tests detect mutations in mycobacterial DNA that are known to cause resistance to a specific anti-TB drug. Molecular methods have significant benefits of increasing programmatic management and monitoring of DRTB, contributing earlier diagnosis, standardized testing, suitable for high throughput locations, and lower laboratory biosafety requirements²². Unfortunately, currently using molecular tests are the Xpert MTB/RIF and line probe assays including first line and second line probe assay having some difficulties, including inhibitor issues, when the biological samples are used directly³. Genotypic drug susceptibility testing for first-line drugs is more accurate for rifampicin and isoniazid but less reliable for Pyrazinamide, streptomycin, and Ethambutol^{23,24}.

GeneXpert Assay

The Xpert MTB/RIF (GeneXpert) is a point of care molecular diagnostic assay, which can rapidly identify the *Mycobacterium tuberculosis* complex and confer rifampicin resistance (surrogate marker - MDR-TB) by real-time PCR with 5 molecular beacon probes (A to E) that cover the RIF-resistance determining regions of *rpoB*¹⁰. The detection time of rifampicin resistance reduces considerably from 2 to 8 weeks (Culture is the “gold standard” for final determination) to 2 hours. It has a beneficial impact on patients who instantly begin the treatment for MDR tuberculosis²⁵. More than 95% of clinical isolates are rifampicin-resistant *MTBC* strains having a point mutation within the 81-base pair hot spot region of *rpoB* gene (codons 507–533)^{26,27}. Xpert MTB / RIF can also provide a false-positive result for MTB strains to detect rifampicin resistance due to silent mutations or low bacilli load, although this is uncommon²⁶⁻²⁸. Sometimes rifampicin-resistant Xpert MTB/RIF result has to be confirmed with a second different test. Besides, the correlation between phenotypic and molecular DST testing is sometimes challenging.

For example, MGIT DST tests may miss the low-level RIF resistance, but these are usually detected by Xpert MTB/RIF or Line probe assay. In contrast, Xpert MTB/RIF may miss some locally prevalent rifampicin-conferring mutations that can be detected by MGIT testing²⁹. In Swaziland, for example, it was pointed out that up to 30% of MDR-TB seemed to have a rifampicin-resistance mutation outside the rifampicin-resistant determining region. Furthermore, this resistance pattern is not detected phenotypically, and therefore only sequencing-based methods enable reliable detection and help in understanding about this mutation³⁰. Xpert MTB/RIF Ultra (Xpert Ultra) assay is an upgraded version with better MTB identification capabilities and rifampicin resistance detection. Xpert Ultra requires only bacilli detection of limit (LOD) 16 CFU/ml when compared to 131 bacilli/ml for the existing Xpert MTB/RIF cartridge. Additionally, Xpert Ultra includes 2 distinct PCR target genes (IS6110 & IS1081) mutation detection chemistry with a new cartridge design^{26,28,31}. The new Xpert MTB/XDR assay platform can also detect isoniazid, fluoroquinolone, and aminoglycosides resistance^{32,33}. Xpert XDR might be highly valuable for XDR-TB triaging in high drug-resistant TB settings, allowing for the fluoroquinolone based short regimen.

Line Probe Assay

The GenoType MTBDR*plus* (First Line –Line probe assay) detects *Mycobacterium tuberculosis complex* by targeting specific mutations in the rifampicin resistance determining region (RRDR) of the *rpoB* gene (codon 505 to 533), *inhA* promoter (-16 to -8 nucleotides upstream of *inhA*) and the *katG* (codon 315) regions to identify INH resistance from direct sputum smear-positive samples or indirect culture isolates. The FL-LPA has sensitivity and specificity of 96.7% and 98.8% for rifampicin resistance detection, and 90.2% and 99.2% for isoniazid resistance detection, respectively³⁴. GenoType MTBDR*sl* (SL-LPA; Second Line –Line probe assay) rapid method detects resistant to fluoroquinolone (FQ) drugs and second-line injectable drugs (SLIDs). SL-LPA includes the quinolone resistance determining region (QRDR) of *gyrA* gene (codon 85 to 96) and *gyrB* (codon 536 to 541) genes for the detection of resistance to

fluoroquinolones and the *rrs* gene (position 1401, 1402 and 1484) and the *eis* promoter region (from -37 to -2 nucleotides upstream) for the detection of resistance to SLID drugs. SL-LPA has a pooled sensitivity and specificity of 86.2% and 98.6%, respectively, for the detection of FQ resistance by direct testing, and pooled sensitivity and specificity of 87.0% and 99.5%, respectively for the detection of SLIDs resistance^{34,35}. Geographic variation in the frequency of mutations linked with RIF and isoniazid resistance may lead to variable results of line probe assays under multiple epidemiological circumstances. The *rpoB* gene encoding the β subunit of RNA polymerase showed that the majority of rifampicin resistance globally is due to point mutations. The molecular INH resistance mechanisms in MTBC are still yet to be fully understood. Recent systematic reviews have confirmed the most frequently resistance-conferring mutation regions in phenotypically isoniazid-resistant population in *katG*315 (64%) and *inhA*-15 (19%) genes. Regional differences in the prevalence of specific mutations may reduce the molecular test sensitivity rate³⁶. Whole genome sequencing (WGS) is a valuable technique for the early detection of less common 15% of INH resistance mutations missed by the presently used molecular assay and allows a reduced opportunity of acquired drug resistance and treatment failure³⁷.

The GenoType MTBDR*sl* assay was supported by the WHO as a first direct test for resistance assessment in MDR or mono-resistant rifampicin patients instead of phenotypic susceptibility methods. However, for certain mutations, the precise distinction between phenotype and genotype methods remains unclear, which interprets for this test to be complicated³⁸. SL-LPA observed that a few synonymous and non-synonymous mutations in the *gyrA* gene in MTB result in systematic error results (false-resistance) to fluoroquinolones drugs by preventing the binding of wild type (WT) probe. In addition, such mutations are detected explicitly by mutation probes, while others have been only inferred by the absence of amplicons binding to WT probes. However, these mutations are probably rare globally, and they appear in roughly 7% of MDR tuberculosis strains in some places^{34,38}. Detection of MTB hetero-resistance remains challenging

with present molecular-based DRTB assays³⁹. Conventional growth-based phenotype DST is more capable of detecting drug-resistant and susceptible MTB strains in the sample when compared to the molecular LPA and Sanger sequencing methods, which is not suitable to detect the clinically significant 1% of the resistant population³⁹⁻⁴¹. Heteroresistance tuberculosis is a forerunner for complete resistance in MTB patients, and proper diagnosis is critical for developing and customizing treatment plans for patients³⁹. Currently, accessible laboratory-based molecular assays do not detect the resistance to new anti-tuberculosis drugs such as linezolid, delamanid, and bedaquiline, which are included in DRTB regimens in India¹.

The new Fluoro-Type (FT) MTBDR automated molecular assay showed high sensitivity, and it detects rifampicin and isoniazid corresponding mutations in the same three target genes namely *rpoB*, *katG*, and *inhA* promoter genes irrespective of sputum smear grades⁴². The test can detect both known and unknown mutations within the *rpoB* gene but does not identify the exact type of mutation. The FT MTBDR testing of tubercle bacilli has high sensitivity to rifampicin resistance detection but has lower sensitivity to INH resistance detection. The TrueNAT MTB (Molbio Diagnostics, India) novel, real-time PCR based rapid assay can detect TB with good sensitivity and specificity similar to Xpert MTB/RIF. The TrueNAT MTB might be suitable in clinics with a microscopy facility^{43,44}.

Sequencing

NGS is a high throughput, massive and parallel sequencing, along with bioinformatics, and has become a potential method for the detection, identification, and analysis of human pathogens which provide new insights into virulence, antimicrobial resistance, and disease transmission^{4,45}. Several studies have highlighted in recent years that using NGS technologies for the identification of viral, bacterial, or eukaryotic pathogens in a wide variety of samples including blood, respiratory, biopsy, liquid biopsy materials, and faeces^{4,5,46-49}. The NGS technologies generate genomic information which allows clinicians to make accurate diagnostic and treatment choices. This NGS platform is also playing an important role in cancer research, for example, breast cancers have historically been diagnosed by physical

examination, mammogram, and histology and Genetics was an essential factor in the discovery of BRCA1, BRCA2, and other biomarkers⁵⁰. There are several NGS methods such as whole genome sequencing (WGS), targeted NGS, and shotgun NGS are frequently used for rapid diagnosis of MTB drug resistance to epidemiological surveillance. WGS is a technique for analyzing the entire sequence of a whole cell genome in a single run to provide more comprehensive genome data and the targeted NGS is faster, generates fewer information, and focuses on the specific genome areas for deeper analysis. Shotgun NGS, is determining the sequence of every chromosome and the entire genome through the generation of random DNA fragments overlapping ends, then a computer for assembling and organizing the fragments overlapping ends into the whole genome⁵¹. The findings of a WHO population-based, multi-country study for various NGS platforms (e.g. Illumina, Ion Torrent) and targeted methods showed that NGS was highly accurate in the diagnosis of MDR-TB or drug-resistant mutations from culture isolates⁵².

Genome sequencing is a versatile tool with the ability to improve TB infection management through accurate and rapid detection of all clinically significant mutations⁵². The feasibility of Whole Genome Sequencing (WGS) based diagnosis of drug-resistant TB can be achieved in high income and low burden TB settings, however, it remains to be assessed in low income and high burden countries⁵³. Whole Genome Sequencing (WGS) or targeted next generation sequencing (NGS) can offer a comprehensive analysis of MTB drug-resistant mutations for all anti-tuberculosis drugs⁵⁴. It can provide MTBC species identification/strain typing, both known and unknown mutation detection, including insertions, deletions, synonymous & non-synonymous mixed infections in a sample and organism evolution prediction unlikely traditional molecular or phenotypic DST^{26,55}. Targeted NGS, i.e. Ion Torrent Personal Genome Machine or Illumina (iSeq100 and MiSeq) platforms as they are the most feasible techniques for rapid analysis of the full-length MTB gene for MDR/XDR TB resistance mutations. Eight full-length genes such as *pncA*, *katG*, *inhA*, *rpoB*, *embB*, *rpsL*, *eis* and *gyrA* are frequently amplified and sequenced for panel

screening drugs as I & II line anti TB drugs^{52,55}. NGS of *Mycobacterium tuberculosis* from a direct sputum sample seems to have the potential to decrease the time in the detection of antimicrobial resistance within a clinically appropriate time frame which would allow for better patient management and significantly faster than the sequencing of *MTB* bacilli grown in MGIT tubes or culture-based phenotypic resistance. But NGS can be slower than currently used molecular rapid assays as Gene Xpert, for the detection of drug resistance². However, direct genome sequencing of primary clinical samples to provide a complete profile of drug resistance remains a substantial challenge. For a successful sequencing run, the DNA template quality is essential, however, obtaining purified DNA from the *MTB* is quite difficult due to its high content of protein and lipids in its cell wall which interferes in the quality of the DNA. The selection of the sample, bacterial (cell wall) lysis, genomic DNA extraction procedure, library preparation, and choice of sequencing platform must be carefully considered⁵⁴. WHO reviewed that WGS performed on direct sputum specimens, gave variable results with a high percentage of contamination of the human genome. Furthermore, it requires more quality and quantity of DNA isolated from *MTB* positive culture strains to produce valuable sequencing results³⁶. Recent studies have proven this, new approaches like targeted DNA enrichment used for WGS or NGS can be tested successfully from direct clinical specimens⁵⁴. Enrichment systems may play an essential role in ensuring a high-quality sequencing on time to notify the patient management⁵⁶. Metagenomic NGS (mNGS) technology has recently been developed, to and the genomic DNA sequences of microorganisms and provides unbiased detection of pathogens from direct clinical samples. This cohort study showed that mNGS (44% sensitivity) had a similar diagnostic potential for *MTB* compared to Xpert (42% sensitivity) in suspected TB patients, but far higher than conventional ways (29%) and it could be more useful for precious samples such as CSF. Overall, MNGS had a significant advantage over Xpert, traditional approaches and greatly enhanced the etiology diagnosis of *MTB* pathogen⁵⁷. The MinION long-read sequencing platform is portable, pocket-sized and

has many advantages, making it suitable for TB surveillance and antibiotic resistance in the near term. This platform is especially useful for remote locations or with limited infrastructure⁵⁸. Genetic sequencing costs will be a significant factor in deciding the feasibility of implementation as well as the speed of development. In most settings, the cost of sequencing is gradually reduced and less expensive compared to the expenses of phenotypic DST to first and second-line anti-tuberculosis drugs^{2, 59}. Whole genome sequencing produces enormous quantities of data and consequently needs expensive infrastructure for both analysis and storage. Analysis of WGS information also requires bioinformatics specialists who are generally not available in TB laboratories⁵⁸.

CONCLUSION

The NGS tool has been progressively used in its approaches to novel pathogen discovery and diagnosis of infectious diseases but is still being only in its infancy in the diagnosis of TB and DRTB in a current clinical setting. The global roll-out of the WHO endorsed conventional molecular assay has been increased the number of DR-TB cases. Despite this progress, a limited number of drugs are available in these technologies, and there are still major gaps in the diagnosis of drug resistance. We are expecting in the near term future of NGS based assays, either gene targeting or WGS will replace existing DST methods for rapidly predicting all forms of DRTB mutations including currently being used newer drugs. These powerful NGS has emerged as the gold standard diagnostic method, and it can help clinicians design early diagnosis regimens and treatment before mutations emerge, and epidemiological monitoring. Therefore, our belief is that the worldwide TB infection will be eliminated by the use of NGS.

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Conflicts of interest

There are no conflicts of interest.

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