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Direct targeted next-generation sequencing for diagnosis of drug-resistant tuberculosis from clinical samples – An update

Radha Gopalaswamy [a,*,1], Bhargavi Subramanian [b,1], Manisha Parthasarathy [c], Chandrasekaran Padmapriyadarsini [a], Sivakumar Shanmugam [a,**]

[a] ICMR- National Institute for Research in Tuberculosis, Chennai, India
[b] SRM Institute of Science and Technology, India
[c] Vellore Institute of Technology, India

A B S T R A C T

Timely detection of drug resistance is a pre-requisite to tuberculosis management globally. While phenotypic drug susceptibility testing (pDST) by liquid or solid method takes time, current genotypic DST assays can be performed directly from clinical specimens but target only a limited number of resistance variants. Targeted Next Generation Sequencing (tNGS) is a rapid, cost-effective method using direct sample to perform the sequencing than compared to whole genome sequencing (WGS) which requires culture. tNGS provides comprehensive drug resistance profiling with turnaround time of 3–4 days when done directly from clinical samples. For respiratory samples with rifampicin resistance, tNGS could be used for the rapid detection of additional drug resistance including newer and repurposed drugs like Bedaquiline, Delamanid, Pretomanid, Linezolid and Clofazimine for which no rapid molecular tests are currently available. A variety of clinical samples can be used and there are wide choices available for DNA extraction. The targets for tNGS could be amplified using commercial kits or in house primers. tNGS could be performed using different platforms like Illumina, Oxford Nanopore Technology and/or Ion torrent and diverse bio-informatic pipeline options. Positioning of a tNGS with portability system in the current TB diagnostic algorithm and its use in the clinical management of patients' needs further evaluation and efforts.

1. Introduction

Globally, 10.8 million people developed tuberculosis (TB) in 2023 with 8.2 million newly diagnosed cases of TB. Despite increased diagnosis and treatment, TB caused an estimated 1.25 million deaths. Bacteriological confirmation is followed by detection of drug resistance by rapid molecular methods, culture, or sequencing for management of TB.[1] The current TB diagnostic pipeline shows several TB tests (non-molecular); molecular tests including nucleic acid amplification technology (NAAT); targeted next-generation sequencing (tNGS) and culture-based phenotypic drug susceptibility testing (pDST). The target for rapid diagnostic testing by initial WHO-endorsed molecular test is currently set at 100% for the year 2027. In 2023, molecular tests were used as an initial diagnostic test in only 48% of the newly diagnosed TB cases. Also, even among bacteriologically confirmed TB cases only 79% were tested for rifampicin resistance. There is hence an augmented need for drug resistant -TB (DR-TB) testing among confirmed TB cases and their enrolment into appropriate DR-TB treatment regimen.[1] Point-of-care (POC) low-complexity NAATs or Loop-mediated isothermal amplification (TB-LAMP) is used as an initial diagnostic test with or without drug resistance at the peripheral level. The moderate-complexity NAATs as follow-on tests at the intermediary level for detection of additional drug resistance are recommended. The moderate and high complexity NAATs showed detection of resistance to first and second-line drugs, but failed to cover the newer and repurposed drugs like Bedaquiline (BDQ), Linezolid (LZD), Clofazimine (CFZ), Delamanid (DLM), and Pretomanid (PTM) also fail to detect resistance to Ethambutol (EMB), Pyrazinamide (PZA), Cycloserine (CS) and Para-amino salicylic acid (PAS). Phenotypic DST (pDST) is the only approved standard test for knowing the drug resistance pattern to these newer and repurposed drugs.[2] As an alternative, next-generation sequencing (NGS) became available at higher reference laboratories for the detection of drug resistance with a variety of platforms available. NGS includes both whole genome sequencing (WGS) as well as tNGS as options for the detection of drug resistance with the identification of single nucleotide polymorphisms in genes conferring drug resistance.[3] Apart from these WHO endorsed tests, Clustered regularly interspaced short palindromic repeats (CRISPR)-Cas systems based on PCR

* Corresponding author.
** Corresponding author.
E-mail addresses: radhagopalaswamy@gmail.com (R. Gopalaswamy), shanmugam.sk@icmr.gov.in (S. Shanmugam).
[1] Both authors contributed equally.

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combined with LAMP or recombinant polymerase amplification (RPA) are available for amplifying patient sample. Cas-mediated *trans*--cleavage activity produce a fluorescent signal upon recognition of the TB genome offering promise to improve TB diagnosis.[4,5] By utilizing these advanced diagnostic approaches, we can strengthen our ability to promptly identify DR-TB cases and implement targeted treatment strategies, ultimately minimizing the DR strains, and improving patient's outcomes and quality of life. The current review focuses on recent advances in tNGS about sample types, DNA extraction methods, genes analyzed and platforms for sequencing and bioinformatic pipelines.

2. Molecular diagnostic tests for tuberculosis

Rapid and precise detection of *M. tuberculosis* (Mtb) and its susceptibility to various anti-TB drugs is essential in initiating early and appropriate treatment for TB patients. WHO-endorsed rapid diagnostic tests form the primary approach for TB diagnosis, employing molecular or biomarker-based techniques.[2,6] The amount of recently endorsed nucleic acid amplification tests (NAATs) by the WHO has continuously increased. According to WHO, there is an initial test for diagnosis of TB and then a follow-on test for drug resistance. In the most recent guideline update, three additional categories of tests – low, moderate and high complexity NAATs were incorporated as primary assessments for TB diagnosis, along with concurrent identification of resistance to Rifampicin (RIF) and/or Isoniazid (INH), fluoroquinolones (FQs), second line injectables (SLIDs) and PZA.[2,6] Molecular tests are preferred owing to low limit of detection and overall cost as initial test. Different countries adapt different diagnostic test with overall cost differences remaining the same. Typically, in a high TB burden country like India, various genotypic and phenotypic TB diagnostic tests are used which differ in their turnaround time, cost and drugs in which resistance is detected (Table 1).

Overall, most of the molecular tests focus on finding specific genes of Mtb. These tests use amplification techniques like PCR/RTPCR to identify the presence of Mtb; it can detect mutations in genes associated with DR by nucleic acid hybridization/RTPCR.[7] Altogether, molecular diagnostic tests have enhanced sensitivity and specificity, a critical aspect of the effectiveness of these tests lies in the choice of sample type – primarily includes sputum for pulmonary samples.[8,9] Apart from sputum, Broncho Alveolar Lavage (BAL) Fluid, gastric aspirate, nasopharyngeal aspirate and stool can be used to detect pulmonary TB.[2,10,11] In extrapulmonary TB, various sample types like fluids (cerebro spinal, pleural, pericardial, peritoneal, synovial), lymph nodes, lymph node aspirates, tissues, urine are used for diagnosis of TB.[2]

3. Revolutionizing tuberculosis diagnostics: next-generation sequencing (NGS)

Next-Generation Sequencing (NGS) is a high-throughput method for simultaneous sequencing of multiple genes to compare with reference sequence libraries. NGS applications encompass targeted NGS (tNGS) and WGS with the former focusing on specific targets of genomic regions while in WGS the entire genome is sequenced including the drug-resistance genes. Both techniques have similar steps starting from the (a) preparation of DNA from bacterial cultures or clinical specimen, (b) library preparation involving multiplexing of samples, (c) use of sequencing platform where multiple DNA fragments are sequenced in parallel and (d) bioinformatics approach to map the individual reads to the reference genome. However, the practical integration of WGS into clinical settings is presently constrained due to the necessity of an initial TB culture step to obtain a substantial bacterial load suitable for successful sequencing.[12] On the contrary, tNGS leverages the specific

Table 1
List of genotypic and phenotypic tests used for diagnosis of TB/DR-TB in a typical high TB burden setting.

S. No.	Type of Test	Turnaround time	Limit of detection	DR detected	Target gene	Cost (INR)
PHENOTYPIC TESTS						
1.	Smear	3–4 h	5000-10,000 bacilli/ml	None	NA	50
2	LJ solid culture	3 weeks for smear-positives; 4–8 weeks for smear-negatives	1000 bacilli/ml	None in the primary culture	NA	250
3	MGIT liquid culture	8–10 days for smear-positives; 2–6 weeks for smear-negatives	100 bacilli/ml	None in the primary culture	NA	350
4	MGIT-DST	LC-DST (All drugs) – 13 days LC-DST (PZA) – 21 days	Not applicable	All drugs included in DST	NA	5000
GENOTYPIC TESTS						
5	Xpert MTB/RIF	Within 2 h	131 CFU/ml	RIF	*rpoB*	1500
6	Xpert MTB/RIF Ultra	80 min	16 CFU/ml	RIF	IS6110 and IS1081; *rpoB*	1500
7	Xpert MTB/XDR	90 min	Not applicable	INH, FQs and SLIDs	*inhA, katG, fabG1, oxyR-ahpC, gyrA, gyrB, rrs, eis*	1700
8	Truenat MTB-RIF	55 min (MTB) 60 min (RIF)	100 CFU/ml	RIF	MTB- *nrdB* RIF-Dx - *rpoB*	800
9	Truenat MTB plus - RIF	35 min (MTB) 60 min (RIF)	30 CFU/ml	RIF	MTB plus – *nrdZ* RIF-Dx - *rpoB*	800
10	Line Probe Assay	2–3 days	161 CFU/ml	FL- RIF and INH SL- FQs and SLID	FL - *rpoB, katG, inhA* SL – *gyrA, gyrB, rrs, eis*	1400
11	Whole Genome sequencing (Illumina)	2–3 weeks (culture) 2–3 days (direct)	Not Available	All drugs	*rpoB, katG, inhA, kasA, ahpC, ndh2, pncA, panD, rpsA, clpC1, embB, gyrA, gyrB, rpsL, rrs, gidB, rrs, eis, rrs, tlyA, folC, thyA, dfrA, ribD, alr, ddl, ald, cycA, rplC, rrl*, Rv0678, *pepQ*, Rv1979c, *atpE, ddn, fgd1, fbiA, fbiB, fbiC, fbiD*	10000
12	tNGS (ONT)	2–3 days (direct)	Not Available	All drugs included in the primers for tNGS	*rpoB, katG, inhA, kasA, ahpC, ndh2, pncA, panD, rpsA, clpC1, embB, gyrA, gyrB, rpsL, rrs, gidB, rrs, eis, rrs, tlyA, folC, thyA, dfrA, ribD, alr, ddl, ald, cycA, rplC, rrl*, Rv0678, *pepQ*, Rv1979c, *atpE, ddn, fgd1, fbiA, fbiB, fbiC, fbiD*	5000

NAAT- Nucleic acid amplification technology; MGIT-mycobacterial growth indicator tube; LPA-Line probe assay; MGIT-DST – Liquid culture drug susceptibility testing; CFU – colony forming units; MTB- Mtb; RIF- Rifampicin; INH – Isoniazid; FQs- Fluoroquinolones; SLIDs – second line injectable drugs; WGS – whole genome sequencing; tNGS – targeted next generation sequencing; ONT- Oxford Nanopore technology.

amplification of regions within the *Mycobacterium tuberculosis* complex (MTBC) genome associated with DR (Fig. 1). The tNGS technique facilitates rapid outcomes directly derived from clinical samples, exhibiting high sensitivity compared to WGS. Additionally, tNGS shows a reduced turnaround time (TAT) and offers enhanced simplicity in result interpretation.[3]

3.1. Targeted NGS (tNGS): A precise and rapid tool for identifying DR-TB

Focusing on tNGS aimed at identifying DR-TB represents an emerging domain of diagnostic technology. The genome of Mtb consists of 4.4 million base pairs (bp), encoding approximately 4000 genes with about 1% of the same involved in conferring drug resistance. Hence, the full genetic sequence of the organism has restricted practicality for clinicians primarily concerned with determining the effectiveness of a drug in treating a particular DR strain of Mtb.

tNGS is more rapid, cost-effective, manageable, and requires less sample input to analyze sequences for drug resistance than compared to WGS which is time-consuming and expensive.

tNGS operates through the creation of targeted, high-speed genomic panels, encompassing exclusively the genes recognized for their role in DR.[13,14] These assays exemplify several dimensions, encompassing the preference and size of the targeted regions, the level of multiplexing within the PCR processes, and the utilization of distinct sequencing platforms such as Illumina,[15–24] Ion Torrent,[25–27]and Oxford Nanopore Technologies.[28–30] These assays can concurrently identify resistance to multiple drugs and possess the capability to integrate novel data relevant to genetic indicators of DR as they become more prominent. Currently, WHO has endorsed the use of tNGS for DR-TB detection using Deeplex Myc-TB (Genoscreen), NanoTB® (Oxford Nanopore Technologies), and TBseq® (ShengTing Biotech).[31] Apart from the variety of tNGS kits, there is a wide choice in the sample type, DNA extraction as well as NGS platforms used in the published literature.

3.2. Sample types

Sample types used so far for tNGS include Mtb culture from LJ slope and MGIT 960 tubes; respiratory specimen including sputum, bronchoalveolar lavage fluid (BALF), bronchial washes; stool; tissues including lung tissue, and lymph node tissue. For all sample types processing of the samples is done inside the BSL-3 laboratory for decontamination of the samples. For sputum, BALF, and bronchial washes, processing of the samples using the N-acetyl-L-cysteine-sodium hydroxide method.[17,18,32] or simple sodium hydroxide method.[30] is followed to remove the contaminating flora and liquefies the sputum specimen. Stool is used as an alternative sample for pulmonary specimen and taken for direct DNA extraction in a previous study.[22] Formalin-fixed, paraffin-embedded (FFPE) blocks of tissue including bone and joint, lung, pleura and lymph node was included in the tNGS study.[26]

3.3. DNA extraction

All the DNA extraction was performed inside the BSL3 facility irrespective of the type of method used. Typically, DNA extraction for mycobacteria includes lysis which is done mechanically or chemically and followed by the precipitation of DNA. In previous studies, both mechanical and chemical lysis have been effectively used in different procedures. Several methods are available for DNA extraction, and they can be used per the manufacturer's instructions. Table 2 summarizes the kits used in previous studies for tNGS. In some commercial kits including Deeplex Myc-TB, lysis is exclusively done by mechanical disruption using beads or combined with chemical lysis.[18,20,22,28,30,32] For other kits, a chemical lysis with a lysis buffer and/or proteinase K is utilized.[15,19,25,26] For the separation of DNA from the lysate either conventional alcohol precipitation or cartridge-based method of separation using silica resin or magnetic beads (Table 2).

3.4. Amplification of targets for targeted sequencing

Amplification of targets for sequencing is done using either conventional in-house primers or commercial kits as per the manufacturer instructions. In previous studies, Deeplex Myc-TB kit (Genoscreen) that covers 18 regions associated with drug resistance (*rpo*B, *kat*G, *fab*G1, *ahp*C, *inh*A, *pnc*A, *emb*B, *gid*B, *rps*L. *gyr*A, *gyr*B, *eth*A, *eis*, *rrs*, *tly*A, *rpl*C, *rrl*, Rv0678) along with species identification and genotyping of *M.tb* isolates was used.[15,18–20,22,24,27,28,33] For the custom panels, studies focused on different Mtb drug resistance gene ranging from first- and second-line drugs to additional newer and repurposed drugs. However, the number and length of the amplicons (ranging from 300 to 3000bp) was variable among the different studies. Ion Ampliseq (Illumina) custom panel was used where genes related to tuberculosis antimicrobial resistance can be customized to generate amplicons of variable number to attain the drug resistance pattern.[26] Conventional panels using either only first and second line drugs were done and/or SNPs for lineage predictions were used in different studies.[16,17,23,25,29,30] Inclusion of newer and repurposed drugs like BDQ, LZD, CFZ, DLM, and PTM into the tNGS panel would be more useful in getting a comprehensive drug resistance profile.

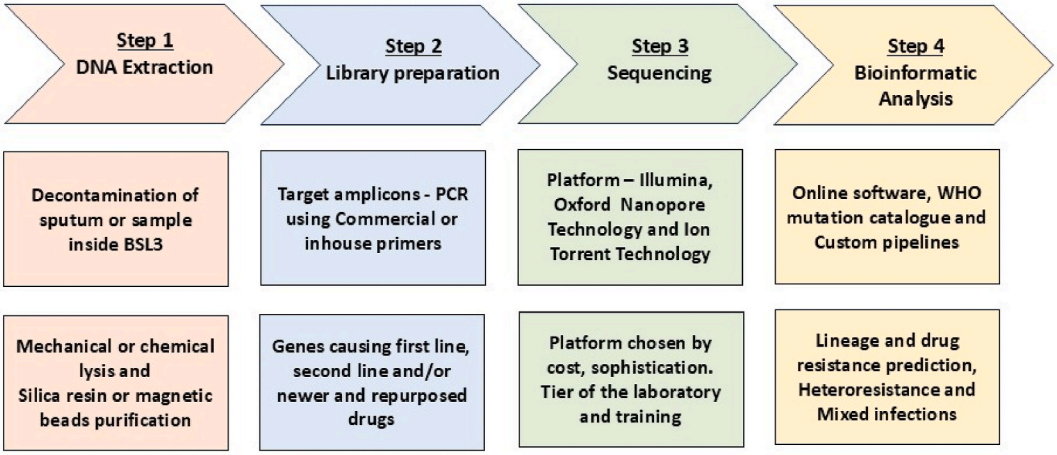

Fig. 1. – Overview of tNGS.

Table 2
– DNA extraction methods/kits used for tNGS in Mtb.

S. No	Methods	Company	Lysis	Purification	References
1.	EZ1 Virus DSP Kit	Qiagen	Automated lysis	Magnetic purification (Platform - EZ2 Connect MDx or the EZ1 Advanced XL)	30
2.	Deeplex Myc-TB kit	GenoScreen	Bead lysis	Isopropanol or ethanol precipitation	18,28,32
3.	Maxwell 16 FFPE DNA kit	Promega	Combination of mechanical disruption and chemical lysis	Magnetic bead-based technology	20
4.	High salt CTAB	NA	Chemical (containing CTAB and high salt)	Isopropanol or ethanol precipitation	17,23,29
5.	MP fast DNA prep kit	MP Biochemicals, Solon, OH	Bead-beating	Spin column method	22
6.	Maxwell 16 Low Elution Volume DNA Purification System	Promega	Chemical lysis (buffers and reagents)	Automated purification (magnetic particles)	19
7.	FFPE DNA kit	(Taipu Biosciences Co., Ltd., Beijing, China).	Chemical lysis (buffers and reagents)	Spin column method	26
8	QIAsymphony DSP Virus/Pathogen Mini kit	Qiagen	Chemical lysis (buffers and reagents)	Automated purification (magnetic particles)	25
9.	GeneLEAD VIII system	(Diagenode, Belgium)	Chemical lysis (buffers and reagents)	Automated purification (magnetic particles)	15

3.5. *Sequencing platforms and bioinformatic pipelines*

Globally, sequencing platforms like ion torrent, Illumina or Oxford Nanopore technologies are available with kits adapted for targeted sequencing.

Ion torrent sequencing system works on detecting pH change due to proton release by a semiconductor (Thermo Fisher Scientific Inc). Here, typically 200 bp fragment reads are performed on a chip and run time is between 3 and 24 h generating a data of up to 15 Gb and options to multiplex up to 386 samples. While Ion torrent have short run time and low error rates, the major disadvantage is the cost and poor performance on the homopolymer regions.[13,14] Studies using Ion torrent sequencer had customized primer sets used with Ion AmpliSeq NGS panels (Therno Fisher Scientific) and compared with phenotypic DST. In these studies, bioinformatic pipeline (Table 3) with either comparison with published literature on Mtb WGS mutations or pipeline like CLC work bench (Qiagen) was being used.[25–27]

With the Illumina platform, in a flow cell, fluorescent dyes are added to bases in a cartridge and different fluorescent chemistry is used to read the bases. There is a choice of different platforms among the bench top sequencers for tNGS namely iSeq, MiniSeq, MiSeq and NextSeq series. MiSeq has an average run time of 4–55 h while iSeq and MiniSeq have less than 24 h as maximum run time. MiSeq generates data comparable to ion torrent sequencer though run time is much higher, while NextSeq can generate huge data owing to the choice of multiplexing up to 386 samples. While iSeq, MiniSeq and NextSeq have comparable fragment lengths (2 x 150bp), MiSeq have option for up to twice the fragment length (2 x 300bp). While iSeq and MiniSeq have low cost and run time, it is advantageous but NextSeq offers high throughput and all of these are used in tNGS studies for Mtb.[15–17,22,24] In a previous study where both iSeq and MiniSeq was used, customized primers could not be added with iSeq and Illumina provided this capability on a development prototype cartridge. However, optimization was needed for successful sequencing of read 2 resulting in poor variant calling compared to MiniSeq.[16] In studies with tNGS, MiSeq is more frequently used owing to fragment size choice and low error rate, though run time is bit higher.[16,18–21,23] For the Illumina platforms, either Deeplex Genoscreen services in combination with Deeplex Myc-TB primer panel or custom bioinformatic pipeline from previous different studies were used with in house primers (Table 3).

In Oxford Nanopore technology, flow cells contain nanopores embedded in a electro-resistant membrane. As DNA passes through the pores the changes in electrical current is aligned to base calling algorithm to identify the base. ONT offers different platforms namely MinION, GridION and PromethION systems. While MinION generates data comparable to MiSeq and Ion torrent, other two are high throughput with a proportionate comparable cost per run. MinION has the advantage of cost, run time, read length and sequencing time which makes it most preferred for tNGS of Mtb compared to other systems. Though, ONT has higher error rate due to long reads, it is quite overridden while amplicons are small and could be advantageous for a tNGS run (both Deeplex Myc-TB or conventional primers) as indicated in previous studies.[23,28–30] As shown in Table 3, bioinformatic pipeline includes primarily custom pipeline or BacterioChek-TB.[23,28–30]

Validation of tNGS assays showed that with great bacillary load identified as higher smear grade or higher CFUs/cTs on baseline PCRs.[18,25,30] Lineage and SNP predictions from direct specimens by tNGS were comparable to WGS or phenotypic DST wherever available.[15,16,20,21,25,27,30] Studies comparing two platforms namely WGS by Illumina and tNGS by Oxford nanopore technology shows great promise for the use of portable tNGS system for lineage prediction and drug resistance diagnosis.[23,28–30] However, for genes like *rpo*B and *emb*B, the coverage was less compared to WGS and it is recommended to make multiple overlapping amplicons for genes more than 1 kb in size.[30] In another study, when Deeplex Myc-TB tNGS was replicated in four independent runs using serially diluted, purified, pre-quantified genomic DNA. The resistance detection and reads were higher with higher bacillary load (10^4 and 10^3 copies) when WGS was used for comparison.[19] In one previous study, tNGS was performed with sputum and BALF, both samples showed results compared to MGIT culture with BALF better than sputum for direct tNGS and drug resistance prediction.[17] Unlike pulmonary samples, FFPE specimens being smear negative and/or extrapulmonary, are critical and tNGS have shown promising results for detection of drug resistance in comparison to WGS.[26] With stool sample too, tNGS proved to be sensitive and median coverage depth was 2-fold higher when the bacillary load was high.[22] In a previous study comparing tNGS with Xpert MTB/RIF, tNGS (48.6%) showed better sensitivity over MTB/RIF (39.4%) with higher positivity over MTB/RIF among presumptive TB sputum samples.[34] There is a pertinent need to compare other existing molecular tests to tNGS in very diverse field setting. The comparison would also help corelate the bacillary load and compare the analyzable results yielded by tNGS to refine the DR-TB diagnostic algorithm.

While turn around time (TAT) for tNGS from direct sputum per se is only 2–3 days compared to cultures where it takes 6–8 weeks in total. However, as indicated in many studies, practically depending on collection of samples for a batch of run and ease of executing the prior steps in different laboratories, it is typically more than 3 days. In summary, both Illumina and Oxford Nanopore technology can be adapted for tNGS and various sample types can be optimized to achieve successful runs with good coverage and depth for lineage predictions and drug resistance diagnosis.

Table 3
List of Sequencing platforms and bioinformatics pipeline used in tNGS for M.tb

Primers used	Company	Platform	Bioinformatic pipeline	References
Deeplex 18 drug resistance-associated genes (*rpoB*, *ahpC*, *fabG1*, *katG*, *inhA*, *pncA*, *embB*, *gyrA*, *gyrB*, *rrs*, *eis*, *tlyA*, *gidB*, *rpsL*, *ethA*, *rv0678*, *rrl*, *rplC*)	Illumina	NextSeq 500	Deeplex Genoscreen	15,22
		MiSeq	Deeplex Genoscreen	18–20
		MiniSeq	Deeplex Genoscreen	24
		iSeq100	Deeplex Genoscreen	32
	Oxford Nanopore technology	MinION	In house pipeline –Published literature	28
In-house primers ***rpo*B, *kat*G, *rps*L *eis*, *rpl*C, *mab*A** *gyr*A, *pnc*A, *inh*A, *fur*A, *tly*A, *gyr*B, *rrs*, *rps*A, *emb*B, *rrl*, *ubi*A	Illumina	MiSeq	BacterioChek-TB	29
In-house primers *katG*, *inhA*, *ahpC*, *kasA*, *rpoB*, *rpoC*, *embB*, *embA*, *embC*, *embR*, *rrs*, *rpsL*, *pncA*, *rpsA*, *panD*, *ethA*, *ethR*, *inhA*, *gyrA*, *gyrB*, *rrs*, *tlyA*, *thyA*, *folC*, *rib*, *rrl*, *rplC*, Rv0678	Illumina	NextSeq 500	Custom pipeline from previous study	17,44
In-house primers *inh*A promoter, *kat*G, *rpo*B, *gyr*A, *eis* promoter and *rrs*	Illumina	MiSeq iSeq100	ReSeqTB	16
In-house primers *rpo*B, *inh*A, *kat*G, *pnc*A, *emb*B, *rrs*, *gyr*A, *gyr*B and *eis*) and two regions containing phylogenetic-determining SNPs (one region for lineages 1, 2 and 5 and another region for lineages 3, 4 and 6	Oxford Nanopore technology	MinION	Custom pipeline	29
In-house primers *katG, Rv1910c - furA intergenic region, mabA-inhA promote, rpoB, embB, ubiA, pncA, rpsL, rrs, rrs, tlyA, rrs, eis, whiB7, gyrA, gyrB*	Oxford Nanopore technology	MinION	BacterioChek-TB	23
In-house primers (*rpo*B, *kat*G, *mab*A, *inh*A, *emb*B, *gyr*A, *gyr*B, *eth*A, *rrs*, *rps*L, and *pnc*A) and/or promoter regions (*oxy*R-*ahp*C, *mab*A-*inh*A, *emb*C-A, *pnc*A, and *eis*)	Oxford Nanopore technology	MinION	Custom pipeline from previous study	30,45
In-house primers *rpo*B, *kat*G, *inh*A-*fab*G promoter, *inh*A, *emb*B, *rps*L, *rrs*, *eis* promoter, *tly*A, *gyr*A, *gyr*B	Thermo Scientific	Ion Proton Sequencer	Custom pipeline	26
In-house primers *rpo*B, *kat*G, *fab*G1, *inh*A, *emb*B, *pnc*A, *gyr*A, *gyr*B, *rrs*, *eis*, *rps*L, *atp*E, Rv0678, *pep*Q, Rv1979c, *rrl*, *rpl*C, *ddn*, *fgd*1, *fbi*A, *fbi*B, and *fbi*C	Thermo Scientific	Ion Gene Studio S5 Prime	WHO Catalogue of mutations	27
In-house primers *rpo*B, *kat*G, *inh*A, *emb*B, *pnc*A, *rps*L, *eis*, *gyr*A	Thermo Scientific	Ion Chef instrument	Ion Report v.5.2 software and CLC Genomics Workbench 11 (Qiagen)	25

4. Challenges and limitations of tNGS for tuberculosis diagnosis

tNGS is a powerful tool for MTB-DR diagnosis and recently endorsed by WHO to diagnose DR-TB.[31] WHO recommended the use of tNGS as a follow-on test for RR-TB cases for additional detection of INH, PZA, EMB, FQ, Streptomycin (STR), Amikacin (AMK), LZD, CFZ and BDQ over pDST. Comparison of tNGS with other existing molecular methods can help identify sample types which are suitable for tNGS. With all drugs except PZA and EMB, pDST was the reference standard while for PZA and EMB, a composite standard of pDST and WGS was used to calculate the sensitivity and specificity. A pooled sensitivity of 95% was estimated for INH, STR, EMB, LFX and MOX while PZA, LZD, CFZ and BDQ showed sensitivity of 90%, 69%, 70% and 68% respectively.[35,36] A systematic review on studies comparing tNGS with other molecular test, pDST and WGS showed that sensitivity of drugs in tNGS was comparable to WGS whenever tNGS produced an analyzable result. With the advantage of using direct samples over culture, tNGS was considered a superior test for comprehensive drug resistance profiling within 1–2 days of sample collection.[35,37]

However, it has certain challenges and limitations, including.

(i) tNGS requires specialized laboratory equipment, including NGS platforms and PCR machines, which can be costly to obtain and maintain in resource-limited settings, and these instruments are essential for DNA extraction, amplification, sequencing, and required data analysis. With the idea of integrating tNGS into

routine TB diagnosis, there are challenges in importing technology and supply chain management; requirement of a specialized infrastructure like existing LPA to ensure separate rooms for amplification and library preparation; choice of samples and batching; human resources; establishment of laboratory workflow and laboratory protocol; extensive training requirement to aid decentralization, internet and UPS requirement to support the run, data management and storage requirements, and further scalability.[36,38]

(ii) tNGS depends on prior knowledge of the specific genetic mutations associated with DR in MTB; therefore, it is necessary to have a comprehensive and up-to-date database of known resistance mutations, and if a new or rare mutation is responsible for resistance, it may not be detected using this method. The catalogue of mutations is routinely updated by WHO and a second version is released with additions to mutation databases in LZD, BDQ and DLM.[39] Previous studies have evaluated the updated version for understanding mutations and their correlation to pDST specifically with reference to FQs, LZD and BDQ.[40,41] However, evidence is required from large data set particularly across countries not well represented in the current version with focus on drugs like PTM and CS to further update the catalogue.[42]

(iii) The analysis of NGS data involves complex bioinformatics techniques to process and interpret the vast amount of genetic information generated; thus, skilled bioinformaticians required to handle data analysis, variant, and mutation interpretation accurately. A possible challenge is the validation of resistance mutations by confidence grading before the information is made available to clinicians.[38,39,43] Timely reporting is necessary to achieve sustainability in using tNGS as a comprehensive rapid diagnostic tool for DR-TB.

In high TB burden countries, there are different tiers for TB testing with initial Diagnosis tests like smear and/or NAAT carried out at Tier 1

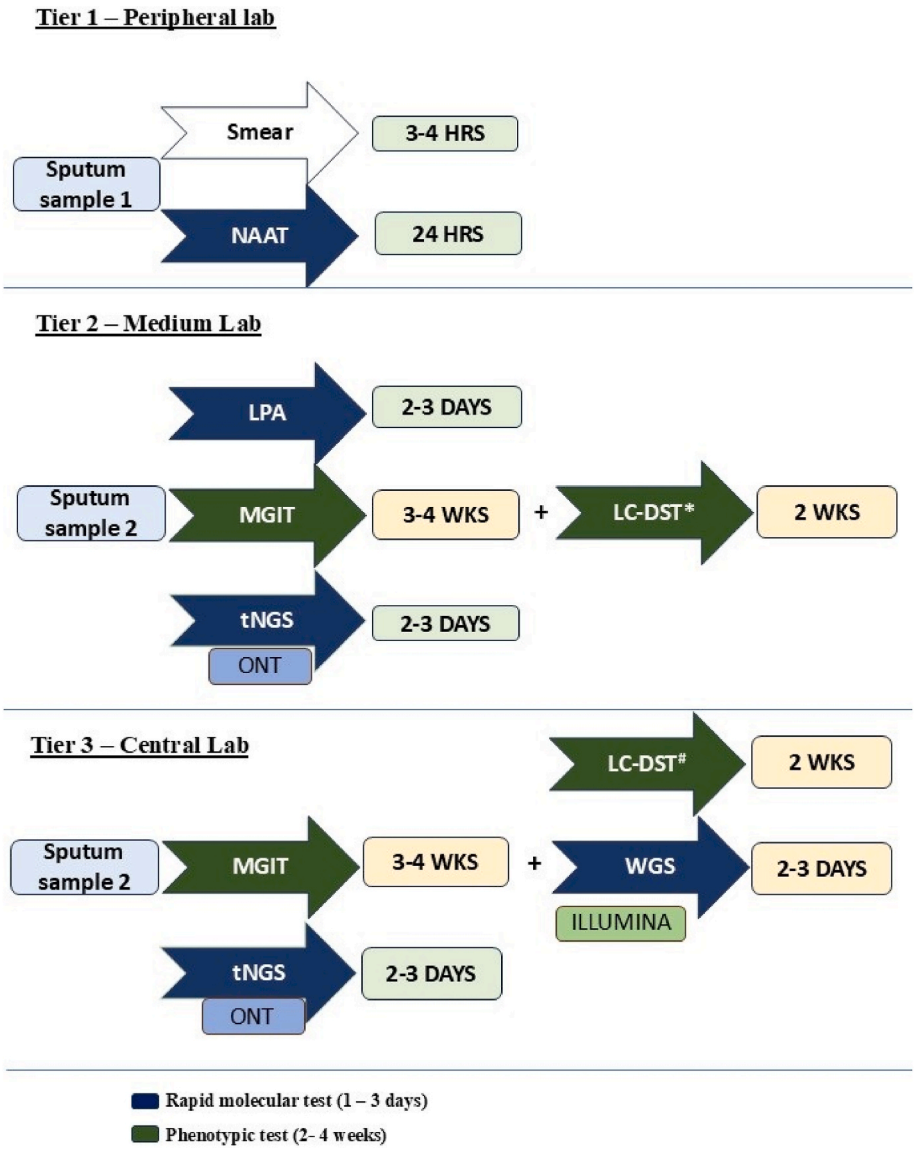

Fig. 2a. Flowchart of TB testing different tiers of laboratories with DR-TB diagnosis.
* denotes DST for FL and SL drugs.
# denotes DST for newer and repurposed drugs.
Genotypic tests are indicated in green while phenotypic tests are indicated in blue
LPA-Line probe assay; NAAT- Nucleic acid amplification technology; MGIT-mycobacterial growth indicator tube; LC-DST – Liquid culture drug susceptibility testing; WGS – whole genome sequencing; tNGS – targeted next generation sequencing; ONT- Oxford Nanopore technology; HRS – Hours; WKS- weeks.

or peripheral labs (Fig. 2a). The follow-on resistance testing is done using LPA and/or LC-DST for first- and second-line drugs at Tier 2 or medium level labs. NGS as well as DST for newer and repurposed drugs at Tier 3 or Central labs where sophisticated laboratory set up is available. With the introduction of a simple portable device for tNGS by ONT, there are possibilities of introduction of the same at Tier 2 labs using direct samples in addition to Tier 3 labs where cultures can be used for WGS or direct samples for tNGS testing. In a previous study, a stochastic decision analysis model was used to analyze the cost effectiveness of tNGS in DR-TB detection in LMIC like Georgia, South Africa and India. In all three countries, bacteriological confirmation is done by Xpert MTB/RIF and once RIF detected, Xpert XDR and pDST is used in South Africa and Georgia while LPA and pDST is used in India. The cost of tNGS over existing DST practices proved cost effective in South Africa but not Georgia. In India, tNGS proved cost effective over pDST providing greater health impact at a lower cost. A one-year project cost of tNGS was US$ 57 130 727 as compared to a total US$ 57 719 097 for LPA and pDST.[35–37] In LMIC setting, the number of labs testing for pDST of Group A drugs like BDQ and LZD would be limited thus missing out patients for DR-TB testing. Besides, considering the two-step process of detecting additional drug resistance with associated delays, there is a fair chance of Loss-to-follow-up (LTFU) cases.

In the current scenario, a simple workflow for testing RR-TB patients with tNGS that could be cost effective (Fig. 2b). In this workflow, a second sample for a patient tested RR-TB in Tier1 lab can be sent to a Tier2 lab for additional drug resistance testing by LPA, tNGS and pDST. At the Tier 2 lab, sample should be decontaminated, DNA extracted and tested using FL/SL-LPA and tNGS while pDST can be performed from MGIT culture. With introduction of a newer technique, there are technical constraints in initial set up and avoidance of false positives/negatives and contaminants. In Tier2 labs with a LPA facility in place, the master mix and PCR hood can be extended for use in tNGS initially. Surface decontamination included in daily maintenance, dedicated pipettes and unidirectional flow within the facility can avoid cross contamination and reduce false positives. Swab testing can be done as part of monthly maintenance and if false positives or contaminants are detected, extensive decontamination of pipettes, work surfaces and floor with sodium hypochlorite is recommended. Once tNGS is established in a pilot scale, programmatic intervention and policy decision on diagnostic algorithm to include tNGS could be effectively implemented as a stand-alone follow-on test to prove more cost effective.

5. Conclusion

The studies on tNGS in direct detection of mycobacterial drug resistance shows remarkable advancement with different sample types

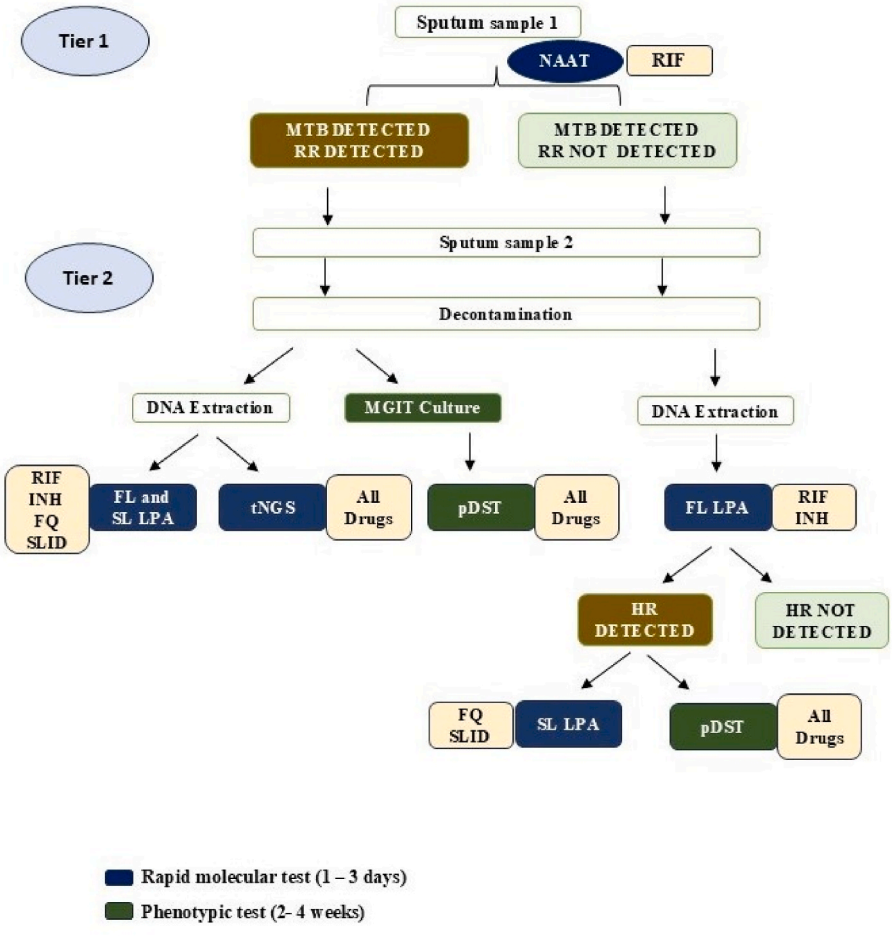

Fig. 2b. Workflow for the proposed inclusion of tNGS for RR-TB patients.
The image describes the workflow for the proposed inclusion of tNGS for RR-TB patients to perform comprehensive DST testing at Tier 2 laboratory from direct sample.
Genotypic tests are indicated in green while phenotypic tests are indicated in blue
LPA-Line probe assay; FL – First line; SL – Second line; RR – Rifampicin resistance; HR – Isoniazid resistance; NAAT- Nucleic acid amplification technology; MGIT-mycobacterial growth indicator tube; pDST – phenotypic drug susceptibility testing; tNGS – targeted next generation sequencing; RIF -Rifampicin; INH – Isoniazid; FQ – Fluoroquinolones; SLID – Second line injectable drugs.

including stool and across standard methods of DNA extraction. The identification of a user friendly DNA extraction method in peripheral setting combined with a simple portable sequencing platform and transferring resources/samples to existing laboratory set up can also help in reducing costs per test/TAT in a low-resource TB burden setting. As for bioinformatics tools, the persistent growth of user-friendly, open-source bioinformatics tools and pipelines can make tNGS data analysis more accessible to a broader range of researchers and clinicians. With this, tNGS can be integrated into a broader diagnostic algorithm, including other methods like culture, microscopy, PCR and pDST, to improve diagnostic accuracy. Besides, tNGS can recreate an essential role in public health efforts, assisting in understanding TB epidemiology, tracking outbreaks, and monitoring drug resistance trends.

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Declaration of competing interest

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