Next Generation Sequencing : A Paradigm shift in the elimination of Tuberculosis.

Siddhant Shetty,
Fellow, Department of Infectious Diseases
PD Hinduja Hospital and Medical Research Centre,
Mumbai, India
drsiddhantshetty@gmail.com

Priti Kambli,
PhD student, Department of Microbiology,
PD Hinduja Hospital and Medical Research Centre,
Mumbai, India
priti_kambli88@rediffmail.com

Camilla Rodrigues,
Head, Department of Microbiology,
PD Hinduja Hospital and Medical Research Centre,
Mumbai, India
dr_crodrigues@hindujahospital.com

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I. TB : PROBLEM STATEMENT

Tuberculosis (TB) is still a significant community health problem and is far from being eradicated. Globally, in 2020, approximately 10 million people were infected with TB including 1.1 million children and a total of 1.5 million people died from TB (including 214,000 people having HIV co-infection). Globally, it is the 13th commonest cause of death and, after COVID-19, is the second leading cause of death from an infection (surpassing HIV). India is the epicentre of TB in the world, accounting for over 25% of the world's cases. The COVID-19 pandemic has made matters worse due to neglect and interruption of previously well established programs for the control and elimination of treatable diseases like HIV and TB.

II. DRUG RESISTANCE AND CURRENT CHALLENGES IN THE ELIMINATION OF TB

Even though the infrastructure for detecting cases of TB and starting the patients on treatment, and their monitoring has been put in place through the RTNCP (Revised National Tuberculosis Control Program), our current challenge is of drug resistance. This problem has stemmed through rampant empiric use of TB drugs, under-dosing patients, non-compliance and interruption of treatment. Rapid urbanisation and people living in close proximity has also made the spread of these resistant strains much easier in the last few decades.

WHO recommends that all tuberculosis patients should undergo testing for resistance to Rifampicin and Isoniazid routinely, while resistance to fluoroquinolones (FQs) must ideally be tested in cases where Rifampicin and Isoniazid resistance has been ascertained [4]. The mechanisms of resistance to Isoniazid and FQs are well researched and understood, and we have commercially available molecular tools for their detection. However, they come with the limitation that these genotypic drug-susceptibility testing (DST) assays have a lower sensitivity than those for resistance to RIF, and additional phenotypic testing is required to detect mechanisms of resistance that are not picked up on genotypic DST.

Survey data shows that incidence of primary drug resistant tuberculosis (DR-TB) is quite high in urban areas where people live in close contact, such as Mumbai, up to 20% in certain areas as per some reports. Sequential selection of drug resistant mutants during inadequate therapy breeds Multi-drug resistant (MDR) and Extensive drug resistant (XDR) infections in previously treated patients. However for new patients, which are on the rise of late, such resistance is due to the transmission of a resistant strain. This is compounded by problems associated with currently available large scale diagnostic techniques. MDR-TB treatment differs from susceptible TB by means of longer treatment duration (6-9 months vs 18-24 months), use of daily injectables, higher cost and worse drug toxicities, many of which are irreversible. Subjecting a patient to drug toxicity without strong evidence of drug susceptibility in today's era is unjustified.

In our cohort, more than 5 months typically pass from diagnosis to MDR-TB treatment. Most MDR-TB in our cohort is resistant to quinolones (Pre-XDR-TB) or to 2 or more of the 3 group A drugs namely Bedaquiline, Linezolid and quinolones (XDR-TB). Sole reliance on newer molecular tests like Xpert MTB/RIF ("GeneXpert") and Xpert

MTB/RIF Ultra (“GeneXpert Ultra”) in our setting would provide most MDR-TB patients with potentially inadequate treatment. When patients with incomplete DST receive ineffective treatment, they suffer unnecessary toxicity and may develop additional resistance. In addition to the personal impact, this prolongs community transmission. One study at our centre reported that pre-XDR and XDR-TB patients took longer to convert their sputum cultures than those with MDR-TB, indicating longer transmission in homes, offices, and communities.

TB diagnostics used currently can be broadly classified into phenotypic methods and genotypic methods. The phenotypic method consists of culture techniques like TB MGIT (Mycobacterial growth Inoculation tube) and subsequent drug susceptibility testing (DST). Genotypic methods consist of the Xpert MTB/RIF, Xpert MTB/RIF Ultra, Line Probe Assays (LPA) and Sequencing techniques.

III. CURRENT TB DIAGNOSTICS (AND THEIR LIMITATIONS) : PHENOTYPIC

For decades, culture-based drug susceptibility testing (DST) has been the World Health Organization (WHO) standard for determining resistance to anti-tubercular drugs in MTB isolates. Universal access to accurate DST on the culture growth is essential for effectively diagnosing and treating MDR-TB/XDR-TB. Traditionally, DST for MTB depends on growth on culture and sensitivity testing in liquid or solid media. These samples are processed in biosafety level 2/3 laboratories, which are not widely available. Typically, it takes around 3-6 weeks for a growth, followed by another 2-3 weeks for a DST to get phenotypic results, which is very crucial time for a TB patient to be started on directed treatment.

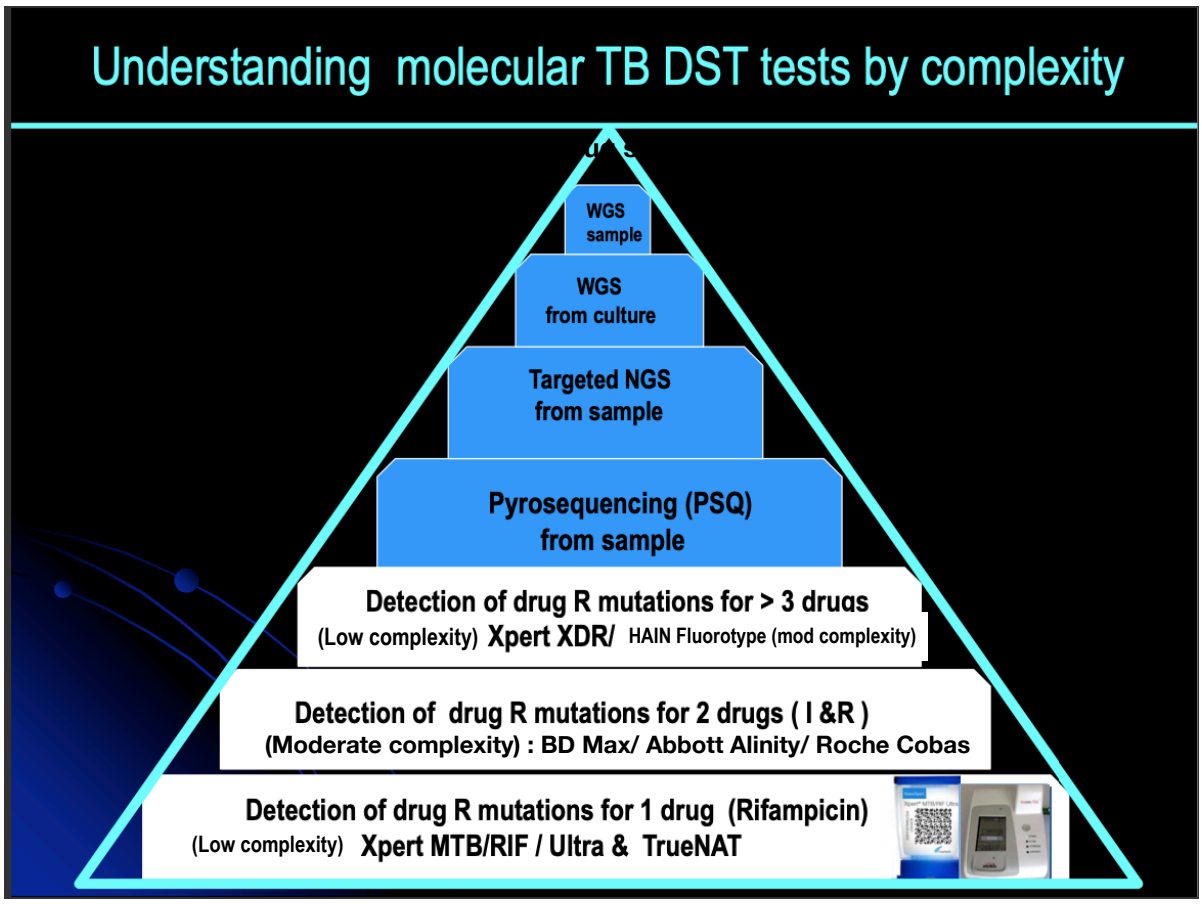
Several studies show that drug resistance in MTB is rather heterogenous and not a homogeneous entity. More specifically, phenotypic drug resistance at different levels (low, moderate and high-level) are found in MTB and these are caused by very different and distinct genetic mechanisms. Generally, there is a good correlation between the phenotypic resistance and genetic resistance mechanism. Clinical resistance is determined on the breakpoints of therapeutic drug failure during treatment. However, the DST ignores low-level resistance mechanisms where the MIC increases without reaching or crossing the breakpoint, therefore indicating evolution towards high resistance in the future. This clearly has important biological implications. Low levels of drug resistance may not be consistent with resistance clinically. On the contrary, high levels of resistance will render the drug useless. The implications of moderate levels of resistance lack clarity, and should be better explored in future studies considering PK/PD (pharmacokinetic/pharmacodynamic) parameters. Drug susceptibility testing methods need to be modified to acknowledge and solve these issues. Lastly and most importantly, protocols for quantitative testing of drug susceptibility to first and second line drugs should be standardised and be a prerequisite for prospective studies which will address the impact of resistance heterogeneity on clinical outcomes, by correlating the data from quantitative resistance studies. Also, phenotypic susceptibility testing for drugs such as pyrazinamide is limited by low precision and lack of reproducibility. Drugs available for the treatment of XDR-TB are quite limited, hence it is important to utilize potential drugs for MDR-TB to treat the majority of cases. TB culture is less sensitive than molecular techniques, but is very specific since it does not detect inactive bacteria like molecular techniques do.

IV. CURRENT TB DIAGNOSTICS (AND THEIR LIMITATIONS) : GENOTYPIC

Some genotypic tests have been recommended by the WHO for the diagnosis of MDR and XDR-TB, including cartridge-based nucleic acid amplification tests (CB-NAAT) and line probe assays (LPA) which can be utilised in peripheral TB laboratories. In recent years, rapid molecular testing of drug resistance in MTB, primarily by GeneXpert MTB/RIF, has contributed significantly to the detection of DR-TB. A sensitivity of 98% for smear positives and 70% for smear negatives has been reported for the test. The Xpert MTB/RIF detects TB bacillus and Rifampicin resistance but does not detect resistance to other drugs, and certain mutant loci detected on the rpoB gene for Rifampicin resistance have questionable clinical significance. The Xpert MTB/RIF Ultra has better sensitivity (detects 2 loci on the TB bacillus instead of 1 on its predecessor) which comes at the cost of specificity, detecting some false positives, especially in samples from unsterile sites. The earlier concept of isolated rifampicin resistance representing MDR-TB (Multi-drug resistant TB - defined as resistance to both Isoniazid and Rifampicin) is now becoming irrelevant with the recent rise in isolated isoniazid resistance leading to treatment failures in “drug susceptible” TB.

This drawback of the GeneXpert detecting only Rifampicin resistance (thus missing out on certain Isoniazid mono-resistant strains) has been partially overcome by introducing the 1st line LPA which detects Isoniazid resistance along with Rifampicin resistance, however with limited availability.

These resistance interpretations in molecular tests are established on the basis of rapid detection of genetic mutations. However, they measure resistance indirectly and consider only a few of the available genetic targets, thus rendering the DST results incomplete.



V. SEQUENCING FOR DIAGNOSING DRUG RESISTANT TB

The molecular tools employed at present in TB diagnosis target the "most important" regions of a limited number of genes to detect resistance to a limited number of drugs. The exact mutation upon which phenotypic resistance is predicted is not always reported. An all-in-one solution is required, at least at the reference laboratory level, to help individual clinical decisions for treating patients harbouring the most complex resistant strains.

Sequencing is the latest molecular technique which has the ability to promptly diagnose DR-TB directly from a given sample. Developed in the 1970s, Sanger DNA sequencing provided the first genomic sequences, but its application has remained limited due to the complex nature and high cost of this technology, when it is applied to larger genomic regions. The advent of pyrosequencing dramatically increased sequencing capacity, followed by NGS methods, after costs incurred reduced, in the mid-2000s.

Sequencing Workflows have the following steps:
- DNA Extraction
- Library Preparation
- Cluster Generation
- Sequencing
- Data Analysis

The MTB genome is quite suitable for sequencing due to the lack of integrative vectors and low mutation rates. The only technical challenge in sequencing the MTB genome is the presence of repetitive and difficult-to-sequence regions which contain a high GC content. Higher accuracy in sequencing requires sufficient genome-wide sequencing depth, thus impacting costs.

Pyrosequencing (PSQ) is the sequencing technology currently used in TB molecular diagnosis, currently validated for liquid samples, which can detect the TB bacillus and also resistance to 5 drugs including first line and second line drugs. Its disadvantages are that it is slightly less sensitive than the GeneXpert Ultra and is less cost effective, and hence not easily available. Currently, there is just one centre doing the test in the entire city of Mumbai. Even though the LPA and PSQ provide rapid, reliable results, their application remains limited to the genes associated with drugs resistant in XDR-TB.

Next Generation Sequencing (NGS) techniques consist of Targeted NGS (tNGS) and Whole Genome Sequencing (WGS). WGS is currently performed on culture isolates only, since direct testing of samples by WGS results in sequencing all the genetic material, including huge amounts of contaminating human DNA other commensals [4]. In contrast, tNGS can be performed directly on samples. This is in keeping with the current molecular testing where

only the genetic targets of interest are amplified. The amplified fragments are sequenced at reasonable depth, providing nucleotide-level detail and detecting minor variants in heterogenic populations, instead of using probes to detect variants. [4].

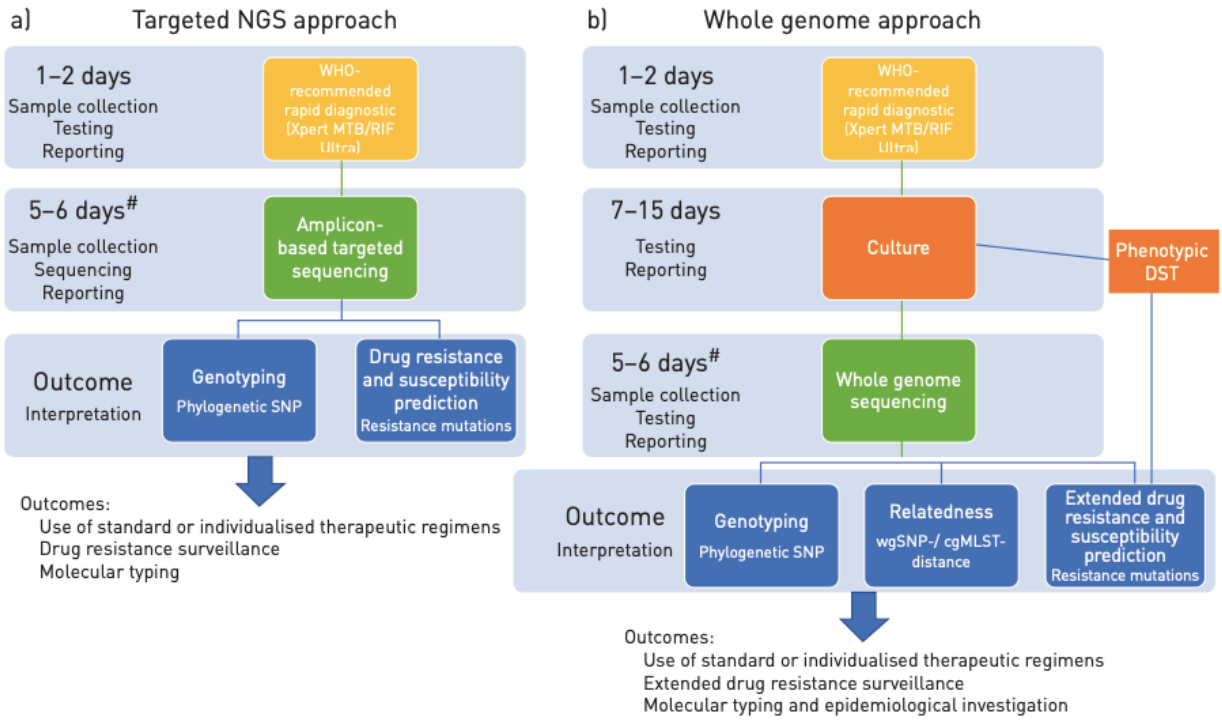


Figure 2 : targeted Next Generation Sequencing vs Whole Genome Sequencing [6]
Courtesy: Cabibbe et al, European Respiratory Journal (Jan 2018)

Unlike Whole Genome Sequencing (WGS) that is currently best performed on TB isolates, Targeted, amplicon-based deep sequencing has the ability to promptly diagnose drug resistant TB directly from uncultured sputum samples. tNGS can provide rapid sequence information for a much larger number of loci than the existing molecular tests, and the bioinformatics can be performed on a simple automated web based pipeline. One of the tNGS platforms is Deeplex Myc-TB (Genoscreen, Lille, France) that detects targets in 18 genes associated with drug resistance in MTB, namely *rpoB* (Rifampicin); *katG, fabG1, ahpC, inhA* (Isoniazid/Ethionamide); *pncA* (Pyrazinamide); *embB* (Ethambutol); *gidB, rpsL, rrs, tlyA, eis* (Aminoglycosides); *gyrA, gyrB* (Fluoroquinolones); *ethA* (Ethionamide); *rplC, rrl* (Linezolid); *rv0678* (Bedaquiline/Clofazimine). It also enables simultaneous species identification and detection of hetero-resistance. Newer platforms such as Oxford Nanopore Technologies (ONT) and Bacteriochek TB (ABL) offer 16 gene and 13 gene targets respectively. With a turnaround time of 2-3 days, tNGS solutions currently seems to be one of the most attractive, affordable and accessible methodologies for DST.

Whole Genome sequencing (WGS) provides us with information of the entire genome but is performed currently only on MTB isolated on cultures. WGS on sputum is still evolving, and needs relatively high quantity and good quality DNA to generate adequate depth. Currently, primary culture takes weeks to provide viable results, thus delaying DST informed treatment decisions. While WGS performed on uncultured sputum is still evolving, it remains less cost effective and requires complex bioinformatics infrastructure and setup, limiting its use and availability to just a few high-income countries., whereas the need for it is primarily in the lower and middle income countries. WGS offers excellent resolution for genotyping, investigating outbreaks and determining known sequence variants which are associated with antimicrobial resistance, which with the help of tNGS, can undergo further deep sequencing of selected genomic regions. WGS-based approaches have been proposed for surveillance of bacteria included in the World Health Organization (WHO) “priority list”. Currently, WGS has been validated on culture samples of *Klebsiella pneumoniae, Escherichia coli, Pseudomonas aeruginosa, Acinetobacter spp., Staphylococcus aureus, Streptococcus pneumoniae, Clostridium difficile, Salmonella spp.,* and *Neisseria gonorrhoeae* in addition to the global priority group of *Mycobacterium spp.* (including *Mycobacterium tuberculosis*).

VI. NGS : NEED OF THE HOUR

Molecular diagnostics such as LPA and GeneXpert that are based on rapid detection of genomic mutations associated with resistance has shown to be alternative to phenotypic DST. The reduction in time to diagnosis of MDR-TB using Xpert MTB/RIF has been seen, but such tests do not allow clinicians to select optimal drug treatment (and have not been associated with mortality reductions). If therapy were determined by Xpert MTB/RIF alone, rifampin-resistant patients in Mumbai would receive drugs to which 67-96% of patients are resistant, leaving an important role for NGS. Similarly, other current rapid tests (line probe assays and pyrosequencing) evaluate genes associated with isoniazid, quinolone, and injectable drugs, but do not fully evaluate treatment options for such patients. Ideally, such rapid tests should be screening tools to rule out resistance, with positive results triggering NGS to evaluate alternative drug options.

NGS can be potentially used for the rapid diagnosis of drug resistant tuberculosis (DR-TB) in various clinical reference laboratories worldwide. NGS has made it possible to overcome the challenges associated with conventional phenotypic testing and the limitations of other molecular tests, by providing rapid and detailed sequence information for multiple gene regions or whole genomes of interest. However, NGS has not yet been accepted for the diagnosis of DR-TB due to cost concerns, their addition into existing laboratory workflows, technical training and skills required for the application of the technology, and the need for expert guidance for managing and clinically interpreting the data derived from sequencing.

Targeted New Generation Sequencing (tNGS) can be performed using cloud-based analysis platforms and provides sequence information quickly for a much larger number of loci than detected by currently used molecular tests. This method is highly accurate, easy to perform and has a simplified analytic pipeline that does not require significant bioinformatics expertise when implemented at a local level. This technology can be adapted to incorporate our constantly evolving knowledge of specific mutations associated with resistance to new drugs, as they present. The currently used rapid molecular assays like Xpert MTB/RIF and Line Probe Assays screen few genomic loci which are routinely involved in drug resistance, thus neglecting many other relevant drug resistance-associated loci which can be potentially involved. WGS will potentially screen the loci included in the rapid molecular tests used at present, as well as other known resistance-associated loci which are not screened by them, thus enabling us in identifying new mutations associated with drug resistance which are not explained by currently available diagnostics, and that too at a much faster speed.

A. Whole Genome Sequencing (WGS) for Mycobacteria

WGS of MTB has two main corresponding uses in clinical microbiology and public health:

1. To predict the phenotype of drug-resistant strains.
2. The determine genetic similarity, hence categorising transmission chains in a potential outbreak.

Both of these can be of benefit to the patient.

In TB diagnostics, WGS results can be interpreted much earlier giving it a clear advantage over conventional DST. Also, in the future, it is going to get much more faster and cost effective. Until we can routinely do WGS on sputum, results will be delayed by 1-2 weeks compared to the current molecular tests. The immensely increased progress in WGS results means improved accuracy and better prediction of drug resistance than is currently possible.

B. WGS in Drug Resistance

WGS works well to differentiate between persistent infection and re-infection in isolates taken from the same patient at different times. WGS was used to demonstrate that reinfection and persistent infection in MDR-TB based on the number of Single Nucleotide Polymorphisms (SNPs), i.e. 2 SNPs within 8 months for persistent infection vs >60 SNPs within 2 years for re-infection.

C. Direct DNA from Sputum and WGS

All the above studies describing the WGS and their clinical applications utilise the DNA from the cultured isolates. However, if we can eliminate the culture step, a further 1-2 weeks could be saved. It is difficult to apply WGS directly to sputum (Fig. 3) as it depends on the load of organisms and it also contains human and non-mycobacterial DNA which are contaminants. There are 2 recent studies that have attempted to address this problem, where sputum was being tried as the direct source for WGS.

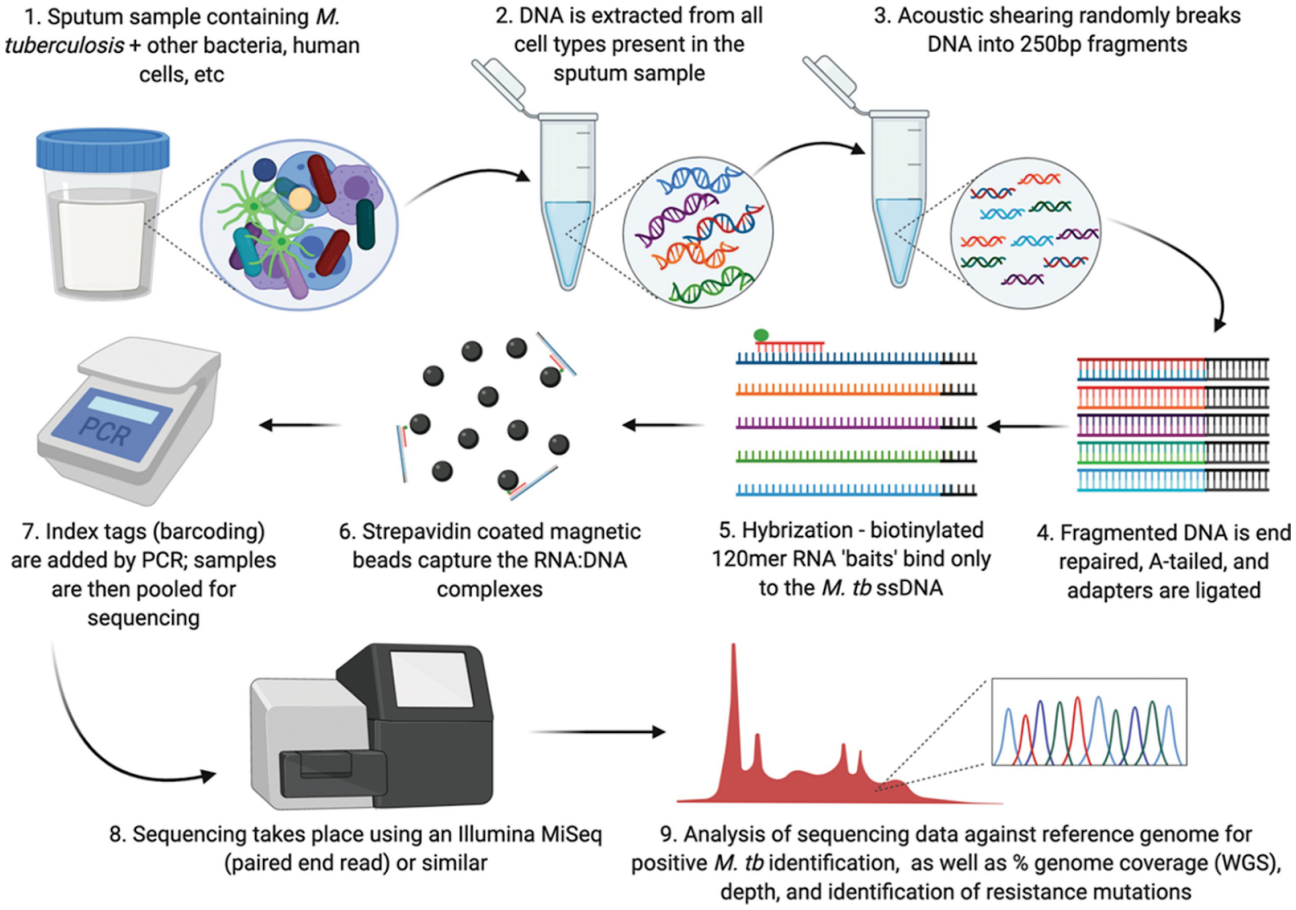


Figure 3 : Whole Genome Sequencing from sputum [7] sample
Courtesy : Brown, A.C, Methods in Molecular Biology (2021)

The need for culture based testing poses a challenge to NGS in its complete implementation and as an effective alternative to conventional molecular methods, especially in situations with limited resources. Hence we are trying to develop protocols to perform WGS directly on clinical specimens, to include differential lysis steps, enrichment of the TB bacilli and automated DNA purification. It is expensive at present, with the low starting material of *M. tuberculosis* and the material being contaminated with human and commensal flora genetic material posing a great challenge to their utilisation. A possible alternative to direct sequencing could be a targeted approach taking advantage of the selective amplification of phylogenetic and drug-resistance related regions.

We need to validate and certify these methods to completely implement this technology into our routine workflows. We must assess and evaluate the reproducibility, accuracy, performance, quality control steps, quality thresholds (e.g. on the depth/breadth of genome coverage), clinical management, impact on turnaround times, and use of standards and development of standard operating procedures, of these techniques. External proficiency testing programmes which are already in use for molecular testing in TB will be made mandatory for the microbiology laboratories planning to introduce these technologies. Clinicians are helped by simple yet comprehensive clinical reports to arrive at best decisions while managing TB. NGS generates large quantities of data, however our knowledge in completely interpreting it is lacking, and we are constantly learning of how best to utilise this data. A report must inform us on the quality of sequencing and in identifying mutations to infer genotyping and drug resistance profiles, and give details on the exact mutations and standardised prediction of resistance levels (ideally after analysing data of minimum inhibitory concentration). WGS and tNGS will eventually replace the standard for DST and epidemiological investigation in TB, and for other high-priority bacterial pathogens in the future. However, we need more studies to address the feasibility of WGS directly from clinical specimens, and also for standardising and automating the laboratory procedures and post-sequencing analyses, and for implementing the NGS platforms in resource limited settings, especially with high burden of disease.

VII. NEXT GENERATION SEQUENCING : REAL WORLD EXPERIENCE

In a study[2] conducted at Hinduja Hospital, Mumbai in 2019-2020, results from tNGS performed directly on sputum samples was compared to Xpert MTB/RIF, LPA, PSQ, and phenotypic DST. tNGS done on uncultured sputum samples yielded results quickly and achieved very good concordance with phenotypic drug testing for isoniazid and

rifampin, and second-line injectable drugs, and good concordance with phenotypic drug testing for fluoroquinolones, leading to a sensitivity of 83.5%, similar to that of LPAs. Inspite of using very little quantity of sputum, this technique was successful in predicting the same drug resistance profiles for all drugs that were tested with an overall sensitivity and specificity of 83.5% and 100%, respectively compared to phenotypic DST. It correctly identified MTB and predicted drug resistance successfully in 97.5% of the smear and Xpert MTB/RIF positive samples which were tested. Also, lineage of the strains and resistance to bedaquiline, clofazimine, linezolid, and pyrazinamide were correctly predicted by tNGS done directly on sputum samples, thus establishing its supremacy over currently used molecular tests and being consistent with updated MDR-TB treatment guidelines. In addition to this, tNGS was able to pick up resistance mechanisms which were not picked up by other rapid molecular tests, due to its depth of sequencing. tNGS from cultured and uncultured sputum samples yielded the same results overall for all samples.

Another study [1] from India has used WGS on enhanced sputum samples and have concluded that this technique had a sensitivity and specificity of 90.9% and 95.0% respectively in predicting drug resistance, when compared to phenotypic DST using minimal sputum sample. A few studies [3,5,6] conducted in Europe have reported high efficacy of the tNGS in detecting TB and drug resistance associated genes.

In 2011, Gardy *et al* [8] utilised WGS to study the dynamics of TB transmission in an outbreak from 32 MTB isolates obtained in the Canadian province of British Columbia over a three year period and was the first study to do so. WGS was able to show two distinct lineages in contrast to contact tracing, which had suggested a single outbreak. Super-spreaders (individuals likely to spread the disease to others) could also be identified. Walker *et al* [9] showed the microevolution of strains within community outbreaks in their study following up on the findings of Gardy *et al*, which could not be evaluated by conventional genotyping tools. They interpreted the direction of transmission of the disease by setting an upper threshold of 5 SNPs, identifying super-spreaders and estimated the mutation rate at 0.5 SNPs/ genome/year. Local or international control measures can be optimised by using NGS as shown by some other recent studies which have shown the superiority of WGS while investigating outbreaks in the community over the prevalent conventional methods.

WGS at the frontline has the potential to minimise turn-around time, empower hospital-based microbiology, and have a positive impact on local activities like infection control initiatives. Our understanding of the complex nature of antibiotic resistance is better because of the tremendous amount of data derived from Whole Genome Sequencing. To add to that we are now more knowledgable about the possible mechanisms contributing to drug resistance, by identifying mutations within genes or intergenic regions that are evolving which could give a selective advantage in presence of drugs. Another recent study by Coll *et al* [10] applied genome-wide association study (GWAS) approach and analysed 6,465 MTB clinical isolates from more than 30 countries. They revealed new epistasis relationships and identified additional genes associated with resistance, along with new mutations to drugs such as ethionamide, cycloserine, and para-aminoslicyclic acid. These provide new targets for molecular diagnostics and for developing new therapies against resistant TB strains. Using specific patho-biological characteristics, WGS has provided a system to differentiate clinical isolates into major lineages and sub-lineages, by using a nomenclature framework, thus paving the way for further investigations into lineage or sub-lineage. Strain type may also affect the transmission, variation in vaccine efficacy clinical presentation, disease outcome, and emergence of drug resistance, hence it is important to understand lineage for improving tuberculosis control efforts.

Laboratories can choose to test a given specimen or isolate from a vast variety of methods available to them, which may lead to certain discrepancies between methods. Such discrepancies may not be significant in certain scenarios, such as resistance to Streptomycin in places that use primarily Isoniazid, Rifampicin, Pyrazinamide, and Ethambutol, but they may have huge implications for treating drug resistant TB in areas where the number of drugs available are quite limited. The discordance between genotypic and phenotypic assays is becoming troublesome for microbiologists and clinicians alike and are inevitable. They are only likely to increase in frequency in the future as new technologies and techniques are adopted, yet it has been slightly neglected. The causes of such discordances are multifold ranging from pre, post and analytic errors to co-existence of non-tuberculous mycobacteria, silent mutations, mutations outside the resistance-determining region, non-canonical mutations conferring increased MICs below the Critical Concentration (CC) in some phenotypic DSTs, and hetero-resistance (either due to mixed infections with two or more MTB strains or to the endogenous development of two populations following inadequate treatment) . Borderline resistance or resistance due to the different CCs used for DST setting can also lead to such discordance. Individual drug MIC values help in resolving such disagreements. The imperfect agreement between genotypic and phenotypic DST results impairs clinical decision making to a large extent, necessitating the need for a tiebreaker, unfortunately with limited choices.

VIII. NGS : THE FUTURE??

Therefore, the future beholds a culture-free WGS method for identifying MTB and predicting its resistance pattern. Rapid molecular testing for detecting drug resistance in MTB currently is predominantly done by Xpert MTB/ RIF, which has proved to be revolutionary for the diagnosis of drug resistant TB. However, despite its speed and convenience, this assay does not detect resistance to drugs other than rifampicin, which is insufficient to determine and give appropriate treatment to patients in regions like Mumbai with high prevalence of drug resistant TB. LPA too (like

Xpert MTB/RIF) also provides quick and reliable results, however it remains limited to the genes conferring resistance to a limited number of drugs.

WGS technique was found to be equivalent to phenotypic DST when predicting drug resistance profiles with good sensitivity and specificity, inspite of using very little pus DNA. When performed on uncultured pus samples and compared to each of the existing methods, WGS was found to have good specificity and sensitivity for the XDR-TB defining drugs, when it was assessed directly from pus samples compared to phenotype. In addition, WGS directly from pus samples detected lineage and predicted linezolid and pyrazinamide resistance, being more beneficial than any of the existing molecular tests currently being used and consistent with updated MDR-TB treatment guidelines. Sequencing uncultured pus samples is time effective by reducing the processing time from 6 to 9 weeks for phenotypic testing to 3 days. Also, this technique has high accuracy, is simpler to perform and possesses a simplified analytic pipeline that requiring minimal bioinformatics expertise in the field, and can be adapted easily to include new loci as we get more information pertaining to newly detected mutations associated with resistance to new drugs. Additionally, WGS can identify resistance mechanisms not picked up by other rapid molecular tests due to its high sequencing depth. Conventional molecular tests may fail to detect heterogenous resistance. Discordance between WGS and other molecular tests are also observed which has been addressed earlier.

Injectable drugs are now being less commonly used in the treatment for DR-TB and the treatment is now slowly transitioning towards the use of linezolid and newer oral drugs like bedaquiline and delamanid, and it will only increase the relevance of tNGS and could potentially decrease the need for phenotypic drug susceptibility testing for those drugs.

A standardised, comprehensive catalogue of mutations is lacking thus limiting the utilisation of sequencing technologies and of next-generation molecular diagnostics for comprehensive genotypic DST. Also there is a lack of data of their association with individual drug resistance[4]. The uptake and the clinical relevance of these tests has a been limited by the technical uncertainty about the identity, number and clinical interpretation of genomic resistance-determining regions, especially for new and repurposed drugs. There is a need for a good quality, comprehensive catalogue of genetic markers of phenotypic resistance, along with their confidence grading, to distinguish clinically significant resistant strains from those not associated with clinically significant resistance or those already with sufficient data.

The World Health Organisation (WHO) is currently cataloguing the data of mutations associated with drug resistance from all over the world. It is thus creating an index which will connote the level of confidence of the mutation in causing a particular drug resistance, thereby dictating if a particular drug can be used to treat a particular patient or not. They are currently classifying the mutations in the catalogue into 5 categories.
Group 1: Associated with resistance
Group 2: Associated with resistance – interim
Group 3: Uncertain significance
Group 4: Not associated with resistance – Interim
Group 5: Not associated with resistance

Thus, when we have large scale data available in the future, a sample will be run on the system after enrichment, its mutations assessed and compared to the database available and its interpretation as to the drugs which can be used, and not, will be provided by the software with a turnaround time of 3 days, and probably lesser in the future. Hence we are heading towards a world of individualised TB treatment compared to the currently used algorithm based mass scale treatment protocols. The drawbacks will be scaling the availability and cost of this technology.

For WGS to truly replace phenotypic testing in the future, it needs to incorporate additional loci of resistance such as *atpE, pepQ*, and the multiple loci associated with delamanid and pretomanid resistance. In summary, WGS directly from uncultured pus samples is invaluable in diagnosis of TB and drug resistance, and potentially in the future it may replace phenotypic DST for detecting drug resistance. WGS directly from uncultured pus samples provides results in a short time, achieved good concordance with phenotypic susceptibility testing for isoniazid and rifampicin, second-line injectables, and fluoroquinolones. Compared to the conventional molecular assays, it has a faster turnaround time, adaptable to newly identified loci associated with resistance and can detect mixed infection.

WGS currently takes weeks to culture sufficient MTB DNA. To shorten time to results, WGS needs to move beyond this limitation. WGS evaluates all DNA in a sample, so 75% of sputum sequencing reads align to human DNA. Recent attempts to overcome this limitation have tried shotgun sequencing, lysis of human DNA, hybridized precipitation using RNA baits, amplicon sequencing and single molecule overlapping reads. Amplicon sequencing is promising due to speed, reliably, and high depth, but it can only evaluate so many targets at a time. Attempts at lysis of human DNA and enrichment of MTB DNA have also been promising, but are not yet consistent enough for DST. Studies of WGS directly from sputum have not been applied yet to large numbers of samples, to smear negative samples, in clinical cohorts with outcome data, or at sufficient depths to evaluate 2nd line drug resistance in the majority of samples tested.

IX. CONCLUDING NOTE:

For accurate diagnosis and treatment of TB, detection of MTB and testing for drug resistance is of paramount importance. Cheap test cost is a very important factor such that available treatment reaches the common people at the earliest. Therefore, it is essential to advance alternative cheaper technology for the detection of MTB to ensure that low and middle income countries (LMICs) also benefit from them.

WGS and tNGS directly from uncultured pus samples is a potential alternative to phenotypic DST, seems feasible in a high-incidence setting, and has a potential to completely replace the phenotypic DST in the future. They have important advantages over conventional molecular assays like shorter turnaround time, adaptability to newly identified resistance-associated loci, coverage of comprehensive resistance targets and the ability to detect mixed infections. WGS directly from pus samples will be useful for moving NGS from research setting into the clinical setting. Transforming this new technology from a research setting to a clinical setting in a high TB burden country like ours is a challenge for the next few decades to come.

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