Disease of consumption to extensively drug resistance tuberculosis: Race of techniques to enhance diagnosis

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Abstract. Tuberculosis is one of the most ancient infectious diseases known to mankind. Detection of the causative organism, *Mycobacterium tuberculosis*, has experienced a series of advances from the late 19th century through early 21st century. The hierarchy of diagnosis ranges from smear microscopy with traditional Ziehl–Neelsen staining to advanced light-emitting diode–fluorescence microscopy staining to both solid and liquid culture, and more recently, to genotypic methods like nucleic acid amplification. Each procedure has its own merits and drawbacks in terms of specificity, sensitivity, minimum detectable number of bacilli, and most crucially, turnover time. The latest diagnostic tools, like geneXpert and line probe assays, have gone a step further by detecting drug resistance. Both early and accurate diagnosis based on the drug susceptibility test implies precise and prompt initiation of treatment, thereby curtailing not only the agony of patient but also the burden tuberculosis places on the health system.

Keywords: *Mycobacterium tuberculosis*, microscopy, culture, rifampicin, drug resistance

INTRODUCTION

Tuberculosis (TB) has existed for millennia and remains a major public health concern globally. The number of deaths caused by TB, which was known as the consumption, phthisis and white plague in the beginning of 20th century is unclear, but seems likely to be high. Tuberculosis has been prevalent in India since at least 1500 BCE, when its symptoms of wounds, hunger and fatigue were noted (Barberis *et al.*, 2017). Historically, the fatality rate of the disease has been very high, as there was no diagnostic or treatment modality available.

Despite many decades of development of both methods for diagnosis and treatment, TB

remains one of the top 10 causes of deaths globally, ranking even above HIV/AIDS. Globally, there are around 10 million cases of tuberculosis and 1.2 million deaths have been reported. India ranks first in terms of absolute number of TB cases, contributing 27% of the global burden, while 35% of global deaths due to TB occur in that country (India TB report, 2019).

The diagnosis of TB was mostly symptom-based until Robert Koch's 1882 discovery of *Mycobacterium tuberculosis*, its causative organism, and development of a technique for staining the bacilli (Barberis *et al.*, 2017). After finding the TB bacteria, researchers were reluctant to grow and

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study its characteristics. Although animal models were promising, at that time, direct study of the causative microbe required a pure tubercle bacilli population.(Herzog, 1998).This gave rise to a search for an appropriate medium to grow TB bacilli.

The first medium used to successfully grow the TB bacilli was a solid medium suggested by Dorset in 1902. In 1915, Petroff modified the medium by adding gentian violet, which provided a contrast to the tubercle colonies and the white medium.(WHO, 2009). He also introduced a sodium hydroxide treatment for decontamination and homogenization (Van Pinxteren *et al.*, 2000).Eventually, Petroff's methods replaced all previous methods used to isolate tuberculosis. Even though the growth of TB bacilli remained slow and time-consuming, with maintenance of a pure and contamination-free culture quite challenging, Petroff's methods replaced the need to use guinea pigs as the primary means to isolate TB.

In the 1990's, Middlebrook introduced an advanced, liquid medium method of culture for research (Singh *et al.*, 2019). Although all the diagnostic modalities were promising, there was a need of fast and accurate diagnosis. This led researchers to brainstorm and produce techniques for diagnosis at point of care, including nucleic acid amplification and real-time polymerase chain reaction (PCR) assays. In this review, we seek to offer an overview of past and presently evolving technologies in diagnosis of TB and its resistance to first- and second-line drugs. (WHO, 2009).

1. Light smear microscopy

Smear microscopy is a technique initially developed over a century ago after Koch's discovery of tubercle bacilli and iteratively refined since. Because it utilizes cheap equipment and materials, most developing countries uses sputum microscopy as the basic tool for diagnosis (Parsons *et al.*, 2011). Microscopy is highly specific for *M. tuberculosis*; it appears curved, long and beaded; however, this method does require expertise, and its accuracy is observation dependent. The sensitivity of microscopy is 60% with a detection threshold 10^4 – 10^5 bacilli/ml under optimal conditions (Singhal *et al.*, 2015) However, with an increase in testing burden;

microscopy meets with technical and operational constraints.

Therefore, light microscopy using traditional Ziehl–Neelsen (ZN) staining for Acid fast bacilli AFB has been replaced by fluorescent staining in high-throughput setups. LED microscopes were recently approved by the World Health Organization (WHO) for countries dealing with a high burden of comorbid HIV and TB(Reza *et al.*, 2013). Though the microscopy technique is very much dependent on the technical and operational subjects, it can be enhanced by modifying the factors influencing microscopy, like sample collection, staining techniques and microscope quality. As a manual process, it is subjected to human error (Chakravorty *et al.*, 2005). Sample collection and site must be correct to get the best results from the technique. For example, to diagnose pulmonary tuberculosis, the collected sample must originate from the respiratory tract using induced sputum, Bronchial Alveolar Lavage (BAL) or a lung biopsy. The specimen required for extra-pulmonary TB is particularly site dependent. The most common sites of infection are biopsies, aspirates, pus, urine and sterile body fluids including cerebrospinal, synovial, pleural, pericardial or peritoneal fluids(Cuevas *et al.*, 2011) However, the sensitivity of microscopy is a concern because of the paucibacillary nature of extra-pulmonary samples.

2. Culture medium-based techniques

The standardized procedures are conventional methods using Lowenstein–Jensen medium which took 8–12 weeks to grow *M. tuberculosis*. These methods were also used for drug susceptibility testing (DST) to test the laboratory strains for resistance. This technique is widely adapted as it was relatively inexpensive. But it requires a minimum 6–8-week period, which causes challenges of diagnosis and treatment due to the delay in results, increasing morbidity and mortality among patients.

A research media by Middlebrook in the form of liquid culture came as a boon to the field in the 1990s decreasing testing turnaround times to 2–4 weeks. The most common technique used a BACTEC460 (Tuberculosis) radiometric system, which was a major breakthrough and was later replaced by *Mycobacterium* growth indicator tube (MGIT) system for growth (Ardito *et al.*, 2001).

This also proved to be an excellent technique to perform first- and second-line DST for drugs. A comparison of three techniques for growth, two in liquid and one in solid media, revealed that the semiautomated BACTEC 460 radioactive and fully automated BACTEC 960 could provide reports in 4–12 days, while solid media took 3–4 weeks (Tortoli *et al.*, 1999), this represented a clear breakthrough. These techniques require a minimum of 10–100 *M. tuberculosis* to grow a culture (Heifets *et al.*, 2000). The sensitivity of these techniques ranges from 80 to 95%, with specificity as high as 98%. (Kenyon *et al.*, 2002)

Limitations

Smear microscopy and culture are the oldest and most established techniques for TB diagnosis but have some limitations. Sensitivity in microscopy is dependent on the skill of the technician as well as the bacterial load, which typically ranges from 10^3 to 10^5 bacilli per ml of sputum. False negatives cannot be completely avoided, especially in paucibacillary sample (Odubanjo *et al.*, 2011) In respect to culture, microscopy has low sensitivity, with no ability to distinguish between species of *M. tuberculosis* (Das *et al.*, 2019).

Though culture is the gold standard for diagnosing tuberculosis, with high sensitivity of 10 bacilli per ml, it also has limitations. The wait of 6–8 weeks for test results causes delays in diagnosis and treatment. (Asmar *et al.*, 2015). The establishment of TB culture labs in peripheral areas, or even in every district is not possible in low-income and developing countries; these techniques require good infrastructure with facilities such as BSL II or BSLIII containment labs with highly trained technical staff (Nienhaus *et al.*, 2011).

3. Rapid detection of drug resistance

Although the techniques discussed earlier are capable of TB detection, the sensitivity (in sputum microscopy) or the duration to arrive at diagnosis (in culture) is often less than ideal. Moreover, those techniques cannot address the increase in drug resistant TB. Therefore, development of a method which not only reduces the turnover time but can address drug resistance was a dire need. The rapid techniques followed the molecular changes in the genes targeted by the two most

important anti-TB drugs, rifampicin and isoniazid.

Rifampicin inhibits the DNA-dependent RNA polymerase *rpoB* gene. In almost 96% of MDR cases of *M. tuberculosis* isolates there are mutations in the 81bp region coding 507–533 of the *rpoB* gene, famously called hot spot region. (Ninan *et al.*, 2016) Meanwhile, for isoniazid resistance that targets both the *katG* and *inhA* genes; the highest level of resistance and the most prevalent mutations are found in the region of S315T of the *katG* (Bollela *et al.*, 2016). Whereas in *inhA*, low levels of resistance to isoniazid is seen at -15C/T (Vilhèze *et al.*, 2014) The detection of mutations for Rif in *rpoB* serves as a surrogate for MDR resistance (Mani *et al.*, 2001). Many techniques to detect genetic abnormalities have been developed; they including InnoLipa, Rif TB Inno Lipa Mycobacteria V2 and Genotype MTDR plus (Kristen *et al.*, 2019). These techniques have since advanced into more sensitive and faster techniques, like molecular beacon tests and nucleic acid amplification assays.

4. Probe-based assays

The assays are rapid and probe-based, involving multiplex PCR with sophisticated reverse hybridization with specific probes tested on nitrocellulose strips. The first few assays that used these techniques are INNO-LiPA®Rif.TB and INNO-LiPA MYCOBACTERIA v2 developed by Innogenetics (Tortoli *et al.*, 2003). More advanced assays, such as GenoType®MTBDRplus (Hillemann *et al.*, 2007), are being used to detect both *M. tuberculosis* and resistance to rifampicin and isoniazid. Though these techniques cannot replace the gold standard, they can reduce the time gap between the diagnosis and treatment. Probe-based assays offer sensitivity of up to 92.2% and specificity of 99.3%. The original Genotype®MTBDRsl assay ver.1 (HainLifescience, Nehren, Germany), is able to detect resistance to second line drugs by detecting mutations in *gyrA*, *rrs* and *embB* genes. Whereas, the advance version of the assay (i.e., MTBDRsl v2.0) detects *eis* promoter region and *gyrB* mutations in addition to the mutations detected in version 1, which improves the performance of the assay (Lacoma *et al.*, 2012). Then there is advance version of the technique

incorporated *gyrA*, *gyrB*, *rrs* and *eis* genes to detect resistance to secondline drugs fluoroquinolones and second line injectables (Tagliani *et al.*, 2015). The sensitivity and specificity (95% CI) were 100% and 90% respectively. According to the WHO's recommendations, the GenoType®MTBDR plus and GenoType®MTBDRsl ver.2 cannot replace the conventional culture methods used to detect MTB but can be used in the screening of MDR and extensively drug resistance XDR (Migliori *et al.*, 2008).

Limitations

Probe-based assay are gene-based, and thus cannot differentiate between live and dead bacteria. Though they are fast to give a sensitive result, many countries and the WHO guidelines do not allow their use on a smear-negative sample, which means these methods do not necessarily address the challenge of quick diagnosis and immediate treatment (Chopra *et al.*, 2020). In addition, the sensitivity of this method for extrapulmonary samples is very low. The rate of false positives for resistance cannot be overlooked, as silent mutations cannot be checked. The techniques are not so develop to test all the drugs used as antituberculosis and many drugs must be tested on liquid culture media for sensitivity.

5. Molecular beacons and nucleic acid amplification-based tests

The researchers were heading towards faster techniques to diagnose tuberculosis. Molecular beacons are like nucleic acid hybridization probes. These probes, attached to turn-on fluorescent beacons, target a specific region of DNA and bind to the required sequence. The phenomenon is so precise that a single nucleotide change prevents the beacon from binding and fluorescing. These assays allow the detection of mutation in less than 3 h without a loss of sensitivity and specificity (Nyendak *et al.*, 2009). The specificity and sensitivity of these beacon tests is greater than 97.5% and 100%, respectively, for rifampicin. Though it cannot replace smear microscopy and culture, nucleic acid amplification assays are currently the most popular diagnostic procedures in multiple settings. The turnover time is around

2 h, requiring less than 10 bacilli per ml, and offering high specificity and sensitivity.

The Xpert®MTB/RIF (Cepheid) assay, one example of this type, is a powerful tool for tuberculosis control. It identifies portions of the rifampicin resistance determining region (RRDR), part of the *rpoB* gene in *M. tuberculosis* complex (Mboowa *et al.*, 2014). All five overlapping molecular probes, A to E covers the whole 81 bp core region of the *rpoB* gene. Some of the techniques based on nucleic acid amplification tests (NAAT) and molecular beacons (Sharma *et al.*, 2018) along with their ability to detect resistance to drugs, are listed in Table 1 (Alemu *et al.*, 2020). Amplified *Mycobacterium Tuberculosis* Direct Test (Boehme *et al.*, 2010; Boehme *et al.*, 2011; Kenyon *et al.*, 2002; Sollai *et al.*, 2014). The use of Xpert®MTB/RIF (Cepheid) has revolutionized TB treatment and companies are striving to expand the detection limits and sensitivity for a wider panel of drugs. These and other advances are discussed further in the section-New Era of diagnostics.

Limitations

The technology used in NAAT is again gene-based, and whether the detection is of a live or a dead bacterium cannot be differentiated. Techniques such as Xpert®MTB/RIF (Cepheid) are quick and can be used at the point of care, but are expensive, which make it difficult for low income and developing countries to afford. The sensitivity of the technique is low in extrapulmonary samples (Cuevas *et al.*, 2011).

7. Non-microbiological diagnostic techniques for latent tuberculosis infection

The diagnosis of TB used to be based on clinical investigation before the rise of technology. The diagnostic techniques and their advances have given a microbiological response to the clinician, thus increasing specificity for case detection and true disease treatment. However, diagnosis of latent TB infection (LTBI) and the test to detect the presence of *M. tuberculosis* in the patient was based on the hypersensitive reactions thus associated. LTBI is defined as a state of persistent immune response to stimulation by *M. tuberculosis* antigens with no clinical evidence of active TB infection (Kasambira *et al.*, 2011). Since, there is no “gold standard” test for LTBI, the burden is

not known with certainty. However up to one-third of the world's population is estimated to be infected with *M. tuberculosis* (Kenyon *et al.*, 2002; Bokhari *et al.*, 1987) and the vast majority have no signs or symptoms of TB disease. Though they are not infectious, still they are at risk for active TB disease and for becoming infectious (Kasambira *et al.*, 2011). With one-fourth of the global burden of TB, 40 percent of the population of India is infected with MTB. Several studies have shown that, on average 5–10% of those infected will develop active TB disease over the course of their lives, usually within the first 5 years after initial infection (Pai, *et al.*, 2015). So, in order to achieve End-TB targets within the stipulated time period, it is essential to address this part of the population.

There is no gold standard method for diagnosing LTBI. A tuberculin skin test (TST) and interferon gamma release assay (IGRA) require a competent immune response in order to identify people infected with TB, and these are not perfect tests for measuring progression to active disease.

Current guidelines recommend using only two of the commercially available IGRAs (QuantiFERON®-TB Gold In-Tube and T-SPOT®.TB) (Sollai *et al.*, 2014). The Guideline Development Group (GDG) concluded that comparison of TST and IGRA in the same population does not provide strong evidence that one test should be preferred over the other for predicting progression from LTBI to active TB disease (Biraro *et al.*, 2016). TST may require significantly fewer resources than IGRA and thus may be more practical in resource-constrained settings.

The GDG also noted that equity and access may affect the choice and type of test used. The preferences of clients and programmes are, however, affected by several factors, such as the requirement for sophisticated laboratory infrastructure (for IGRA) and possible additional costs incurred by clients for travelling and by organizations for facilities and testing. The GDG recommended the two tests as equivalent options, with relatively similar advantages and disadvantages. The less accurate performance of these tests can lead to false-negative results, particularly for young children and immune-compromised individuals, such as people living with HIV. Hence, LTBI testing should not be a

requirement for initiating TB-preventive treatment for people living with HIV and child household contacts aged less than 5 years, particularly in countries with a high TB incidence, given that the benefits outweigh the risks (Klausner *et al.*, 1993).

The availability and affordability of the tests could determine which LTBI test is used. Other considerations include the structure of the health system, feasibility of implementation and infrastructure requirements. Bacille Calmette-Guérin (BCG) vaccination plays a decisive role in reducing the specificity of TST, suggesting a wider choice of IGRA-only strategies. The GDG noted, however, that the impact of BCG vaccination on the specificity of TST depends on the strain of vaccine used, the age at which the vaccine is given, and the number of doses administered. When BCG is given at birth, as is the case in most parts of the world, it has a variable, limited impact on TST specificity (Zwerling *et al.*, 2011). Therefore, the GDG agreed that a history of BCG vaccination has a limited effect on interpretation of TST results later in life; hence, BCG vaccination should not be a determining factor in selecting a test.

Operational difficulties should be considered in deciding which test to use. IGRAs are more costly and more technically complex to perform than the TST. For example, IGRA requires a phlebotomy, which can be difficult, particularly in very young children, laboratory infrastructure, technical expertise and expensive equipment. However, only a single visit is required to obtain a result, although patients may have to make a second visit to get the result. TST is less costly and can be performed in the field, but it requires a cold chain for storage (2–8°C) two health care visits, and training in intradermal injection, reading and interpretation.

Blood tests and skin tests are sensitive enough to support the presence of latent TB, but active disease cannot be ruled out—less than half of all cases are likely to be the result of latent TB reactivation. There are many tests to detect interferon gamma in whole blood. Commercially available assays include QuantiFERON In-Tube® (Cellestis Australia), the T.SPOT.TB® (Oxford Immunotech) (Islam *et al.*, 2013). A meta-analysis of different studies compared the specificity of IGRA to TST, finding 85% to 95% and 45% to

60% respectively. The WHO has even issued a guideline to stop using serological tests for diagnosis in countries with high TB burdens.

8. New Era of Diagnostic

Advances in the diagnosis of TB have been incremental, in step with advances in research and technology. New techniques have been developed, with some approved by the WHO and others by the US FDA.

COBAS TaqMan MTB is a real-time PCR technique developed by Roche Diagnostics and

approved by the FDA. The technique detects 16sRNA of *M. tuberculosis* in a smear-positive sample with a turnaround time of 2.5 h (Yang *et al.*, 2011). Studies have shown that the sensitivity of the assay is 96.9–98% in smear-positive samples, and 34.9–79.5% in smear-negative samples. It is an easy technique to implement, one which does not require an advanced laboratory, but its limitations cannot be ignored. Notably, it cannot detect drug resistance, a major consideration for point-of-care testing.

Table 1. Techniques in modern era used for detection of tuberculosis and their ability to detect resistance.

Technology	Amplification reaction	Test detects
EasyNAT	Cross priming, Amplification	<i>M. tuberculosis</i>
Xpert TB/RIF	RT-PCR	Resistance to rifampicin
Xpert XDR	RT-PCR	Resistance to fluoroquinolone, aminoglycosides and isoniazid
RPA	Recombinase polymerase amplification	<i>M. tuberculosis</i>
Truenat	PCR based Semi	Resistance to rifampicin
Genedrive	PCR	<i>M. tuberculosis</i>
Amplified MTD	Transcription-mediated, Amplification	<i>M. tuberculosis</i>
COBAS TaqMan W MTB Test	RT-PCR	<i>M. tuberculosis</i>
LoopampW Tuberculosis Complex Detection Reagent Kit	LAMP	<i>M. tuberculosis</i>
Genotype MTBDR Plus & MTBDRsl	Multiplex PCR and reverse hybridization	Resistance to rifampicin, isoniazid, fluoroquinolones and aminoglycosides

Loop-mediated isothermal amplification, or LAMP, is a technique developed by Eiken Chemical Co. Ltd. of Japan. LAMP is based on an isothermal nucleic acid amplification technique, which amplifies nucleic acid at a constant temperature without the need for a thermal cycler. This method amplifies even low quantities of DNA with high specificity and a turnaround time of less than 1 h (Mitarai *et al.*, 2011). Many studies have shown that the technique is simple and easy, though it provides higher sensitivity for smear-positive samples (92.1%–100%) than for smear-negative samples (52.1%–90.3%). Notably, aerosol contamination due to mishandling and improper techniques may result in false positive results, and the technique does not detect any markers of drug resistance.

EasyNAT TB is an isothermal nucleic acid amplification technique from Ustar Biotechnologies Co. of China. The technique, which offers a turnaround time of 90 min, has been approved by the FDA. However, EasyNAT does not allow detection of mutations in drug resistance markers.

The gendrive is a technique developed by Epistem (Manchester, UK) for the diagnosis of tuberculosis from samples such as sputum and urine. It is a benchtop test that uses real-time detection by PCR amplification to detect TB with a sensitivity >90–95%, and specificity >95% with the POC of <45 minutes (Amplified *Mycobacterium Tuberculosis* Direct Test Package Insert). Truenat is a technique developed by Molbio Diagnostics Private Ltd, Goa India. The company has developed POC technique which is economical

semi-automated and quantitative which can detect tuberculosis in the peripheral health centers in developing and low-income countries (Ruth *et al.*, 2015).

True NAAT MTB is a chip-based NAAT technique approved by the WHO. Its turnaround time is <60 min. This assay can also detect mutations in the *rpoB* gene to check for resistance to rifampicin. (Ling *et al.*, 2008). This molecular technique give a see through in the shortest time in all the techniques being endorsed in public care. While liquid culture technique remains the gold standard in TB, diagnosing gene sequencing appears to be more sensitive in diagnosing and detecting resistance. The advancement in the field of geneXpert technology developed by Cepheid has been rapid. GeneXpert Omni is a single cartridge based for mobile diagnosing, not evaluated by WHO. XpertMtb Rif/Ultra and Xpert XDR is a next-generation cartridge-based system for the diagnosis of TB, resistance to rifampicin, isoniazid and second-line drugs. The technique was evaluated by the WHO, which issued guidance for its use in 2017. The test has a turnaround time of <2 h. The Xpert MTB/RIF Ultra amplifies two targets IS6110 and IS1081. It covers 25 different RRDR region of *rpoB* gene. The Ultra technique has been made more sensitive, reducing the detection limit from 112.6 cfu/ml of sputum to 15.6 cfu/ml (Horne *et al.*, 2019). A study performed by Foundation for Innovative New Diagnostics (FIND) revealed that Ultra was 5% more sensitive than geneXpert MTB/Rif. The higher sensitivity raises the issue of non-specificity, which is a substantial concern in patients previously treated for TB. However, the assay is still under development and the WHO has not evaluated the test (Horne *et al.*, 2019).

GeneXpert Omni is a new method; it is a single cartridge-based technology which detects tuberculosis and resistance to rifampicin. The machine is battery operative and portable with a shelf life of 4 h.

In 2017, Genomics England, the National Institute for Health Research (NIHR) and the Wellcome Trust worked in partnership to develop whole genome sequencing (WGS) (Stark *et al.*, 2019). WGS has proven to be an accurate diagnosing tool for the detection of TB and drug resistance. It also allows the detection of mutations in the regions other than the regions

covered in different diagnostic techniques. The technique provides a quick and complete view of the genotype of *M. tuberculosis*. Many studies reveal that WGS is 99% in concordance to DST of drugs in culture based medium for drugs rifampicin, isoniazid, pyrazinamide, fluoroquinolones, kanamycin, ethambutol, streptomycin and ethionamide. Despite its benefits, this technique is being used for diagnosis only in developed countries and those with low disease burden. Moreover, the complexity of data interpretation and result assessment makes the technique tough and mandates the requirement of specialist in bioinformatics and medical microbiology.

CONCLUSION

We have covered the methods to diagnose of tuberculosis, from recognition of symptoms to sputum microscopy, and from culture to nucleic acid amplification tests. The target we have set for ourselves, as sustainable development goals (SDGs) or through END TB, sound unrealistic given the current progress, but advancements in diagnosis will definitely be one of the key components necessary to achieve them.

Accurate, fast diagnosis is the first step to move toward the elimination of TB. Undoubtedly, TB notification has shown an upward trend in the last decade, thanks to newer diagnostic tools with higher sensitivity. Similarly, the number of TB deaths is showing a significant reduction due to early diagnosis and prompt initiation of treatment. Newer diagnostics have improved the overall sensitivity of TB detection in pediatric patients, and in patients with HIV co-infection or extra-pulmonary disease. The availability of these newer techniques has left serological tests far behind. However, there is still a long way to go because of the rise in the number of drug-resistant cases, especially in developing and poorer nations. It is possible to bridge the gap between disease and drug resistance detection with the new advances made in the field. We must simultaneously address social determinants of health like poverty, malnutrition, overcrowding, alcohol, tobacco and diabetes if we are to achieve our targets.

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