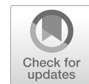

13 Advancement in the Molecular Diagnosis of Tuberculosis

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13.1 Introduction

Mycobacterium tuberculosis (MTB) is an airborne infectious disease that causes tuberculosis (TB), which affects mainly the lungs. Eighty percent of all cases of tuberculosis are pulmonary (PTB). The extrapulmonary form of tuberculosis (EPTB) can affect the colon, meninges, lymph nodes, bones, joints, kidneys, and skin. Depending on the response of the host immune system, exposure to MTB bacilli results in eradication or persistence of the pathogen [1]. TB is the most prevalent infectious cause of mortality in adults' worldwide [2]. According to WHO India represents one-fifth of the global burden of TB. Early and accurate diagnosis is crucial for disease management and improved patient outcomes. With the advent of more recent microscopy, culture, and molecular techniques, diagnostic modalities for tuberculosis have improved significantly over the past few decades (Fig. 13.1). For the purpose of determining a diagnosis, monitoring treatment and stopping the spread of tuberculosis, improved laboratory techniques are essential. In contrast to traditional culture systems, which take several weeks to diagnose TB, molecular detection offers quicker and more affordable ways to identify and confirm resistance to treatment in TB cases. New developments in the molecular diagnosis of tuberculosis, such as the quicker and easier nucleic acid amplification test (NAAT) and whole-genome sequencing (WGS), have sped up the diagnosis and, consequently, TB treatments [3]. There are new methods for improving culture medium, such as culture system improvement, early growth detection, strict oxygen tension control, and MALDI-TOF-MS (matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry) for identification [4]. Most molecular tests have focused on identifying specific nucleic acids to MTB, both in DNA and RNA, using amplification techniques such as polymerase chain reaction (PCR) and identifying gene mutations associated with drug resistance through sequencing or nucleic acid hybridisation [5].

Molecular methods for the detection of tuberculosis (TB) are based on the amplification and detection of specific DNA sequences from the *Mycobacterium tuberculosis* complex, the causative agent of TB. These methods include polymerase chain reaction (PCR) techniques such as real-time PCR (qPCR) and conventional PCR, as well as newer techniques such as loop-mediated isothermal amplification (LAMP) and nucleic acid sequence-based amplification (NASBA) [6, 7].

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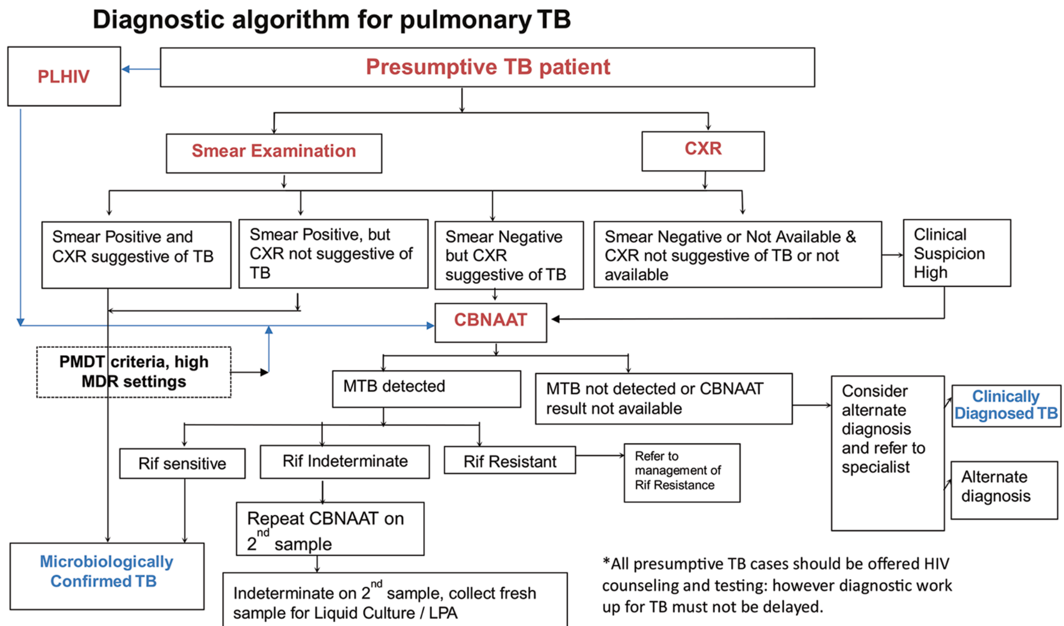

Fig. 13.1 Diagnostic algorithm recommended by Central TB division, India. (Adopted from National TB Elimination Program (NTEP), India)

These molecular methods offer several advantages over traditional TB diagnostic methods including:

1. Speed: molecular methods can provide results within a few hours, whereas sputum culture can take several days or even weeks.
2. Sensitivity: molecular methods can detect TB in patients with low bacterial loads, including those with extrapulmonary TB and those who are HIV positive.
3. Specificity: molecular methods are highly specific for *M. tuberculosis*, reducing the risk of false-positive results.
4. Reduced need for skilled technicians: some molecular methods, such as LAMP, do not require specialised training or equipment and can be performed in low-resource settings.

Therefore, the rapidity provided by the molecular methods has placed them in the first level of screening of the bacillary forms. But the classical phenotype-based method of diagnosing the tubercle bacilli cannot be written-off as it is still the confirmatory testing method and offers an array of advantages which are discussed in following lines. Moreover these are the gold standard methods used for validating the newer molecular methods in most scenarios apart from the use of WGS.

13.2 Direct Method

Direct methods for detecting tuberculosis include acid-fast bacilli (AFB) staining, culture methods, histopathology, and ultrasound imaging [8].

13.2.1 Smear Microscopy

The most popular technique for identifying pulmonary TB is direct sputum smear microscopy, which is available in the majority of primary healthcare labs. This method involves the collection of sputum sample followed by smear

preparation and observing the smear under microscope after staining. At least two sputum specimens are required and cannot be collected at ease from the children makes sputum microscopy less favourable. Fortunately, the majority (95–98%) of smear-positive TB patients can be identified by high-quality microscopy. This uses the special capability present among the entire family of mycobacteria, the property of the cell wall which is acid fast and so are resistant to acid treatment after staining with a primary stain. But the degree of fastness varies between species. MTB is resistant to 25% acid and techniques are available for light and fluorescent microscopy. The older method of using strong carbol fuchsin, followed by 25% acid treatment and counter staining with methylene blue is called as the Ziehl-Neelsen method in which the smears can be read using a light microscope but requires heating step during the carbol fuchsin treatment to make the fatty acid layers permeable to the stain in use. Moreover the smears are read using the oil immersion objective. In the Auramine Phenol (AP) method which is a fluorescent based technique where in the heat fixed smears are stained using AP, followed by the decolorising step using 1% acid alcohol and counter staining by 0.1% potassium permanganate. Auramine-O dye or auramine-rhodamine dye are the two main dyes used in the fluorochrome process. When observed under a fluorescence microscope, the hydrochloride dye auramine-O causes stained AFB to exhibit fluorescence (green or yellow). Heating is not necessary, in contrast to the Ziehl-Neelsen procedure, for the stain to penetrate the germs. However, for the acid-fast organisms to adequately pick up the stain, there must be a minimum of 20 min of stain to smear contact time. This method has several advantages; they are more sensitive [9] and the smears can be read using the high-power objective lenses and hence more filed can be scanned in lesser time. But in general, smear microscopy is less sensitive as it requires a minimum of 10,000 bacilli per ml of the specimen for detection.

13.2.2 Culture Methods—Solid Egg-Based Methods

Culture-based methods are more sensitive and can also be used for the detection of drug resistance. Egg-based solid media (Lownstein Jenson, Ogawa) are the most favourite ones where the morphology of MTB results in a typical rough, tough and buff-coloured colonies are characteristics of MTB. The carbon source is glycerol, L-asparagine serves as the nitrogen source and malachite green is used as a contaminant inhibitor. LJ is less expensive than agar-based medium, simpler to prepare, and less prone to contamination during preparation because it is inspissated after being poured in bottles. Mycobacteria toxic materials are neutralised by the medium's high buffer capacity. It has a long shelf life if refrigerated (several weeks). It allows for direct visual recognition of *M. tuberculosis* colonies and contamination growth, just like other solid media. Using liquid media instead of solid media reduces the cultivation time from 12 weeks to 3 or 7 weeks and then they can be stored for longer periods at room temperature. The slow-growing nature of the bacilli makes the method less favourite and the liquid-based systems such as MGIT960, BacTAlert are automated liquid based closed systems where the time to detection is much more reduced than solid based cultures but are very expensive. These techniques involve fluorescent based dyes in the culture medium which offers to detect the growth of the tubercle bacilli in a system that is built with an agitator and incubator.

13.2.2.1 Agar-Based Medium

Middlebrook media: Middlebrook 7H11 and 7H10 medium consists agar as a solidifying agent. Compared to LJ medium, Agar-based media can detect growth more quickly by developing visible small early colonies. On one hand, transparent media can be easier to identify colonial morphology. These media are supplemented with oleic acid, albumin, dextrose, and catalase (OADC) to promote MTB growth and are also

suitable for quantitative colony counts, which have been used in phase 2 early bactericidal activity (EBA) trials of TB drugs and drug combinations [10].

13.2.2.2 Nitrate Reduction Test (NRA)

Bacteria utilise nitrate (NO_3^-) as a terminal electron acceptor or as a source of oxygen during the anaerobic respiration process. In either of these processes, nitrate is first converted to nitrite (NO_2^-) and then to other by products such as molecular nitrogen gas (N_2), ammonia (NH_3), hydroxylamine, etc., or it may not be further converted depending on the metabolism of the bacterium or the presence of specific enzymes. These microorganisms are known as nitrate-reducing bacteria (denitrifying bacteria). These bacteria produce the enzymes needed to convert nitrate to nitrite, nitrate reductase. The basis of NRA is the ability of *Mycobacterium tuberculosis* (MTB) to convert nitrate present in the medium to nitrite, which may be identified by a change in colour when Griess reagent is added. The fastest and most accurate way to identify drug resistance in tuberculosis is direct NRA using middle brook 7H11 agar. The nitrate reductase assay (NRA) is one of the methods for rapid detection of resistance. WHO recommends that the NRA be used as a direct test on smear-positive sputum sample or as an indirect test on Mycobacterium tuberculosis isolates grown from conventional solid cultures such as LJ media. Additionally, 1 mg/ml KNO_3 was added to the LJ medium as a nitrate source [11].

13.2.2.3 Liquid Culture Systems

The BACTEC MGIT 960 instrument is a fully automated system that detects mycobacteria growth in culture by utilising oxygen. Oxygen molecules act as a quencher to the fluorescence indicator. Following the oxygen reduction induced by aerobically metabolising bacteria within the medium, the ruthenium pentahydrate oxygen sensor embedded in silicon at the bottom of a tube containing 8 ml of modified Middlebrook 7H9 broth fluoresces [12]. It is highly reliable, delivering consistent and reproducible results. It is equipped with advanced sensors and algorithms that monitor the growth of mycobacteria, ensuring accurate and timely detection. The MGIT960 system offers high sensitivity and specificity in TB detection. It can detect as few as 100 mycobacteria per millilitre of the sample, making it highly effective even in cases with a low bacterial burden. Its ability to differentiate between *Mycobacterium tuberculosis* and other non-tuberculous mycobacteria is crucial for accurate diagnosis. When compared to LJ culture, MGIT 960 was found to be a more rapid method of mycobacteria detection and to be highly sensitive (100%), specific (93.3%), and accurate (93.6%) [13]. It can provide results within 2–3 weeks [14]. The automated nature of MGIT960 eliminates subjective interpretation and human error, leading to enhanced diagnostic accuracy. The MGIT960 system is equipped with a built-in susceptibility testing module, enabling the detection of drug resistance in TB bacteria. This feature assists healthcare providers in choosing appropriate treatment regimens, thereby preventing the spread of drug-resistant strains [15, 16]. These systems require power backup round the clock and the use of PANTA to control the normal flora and contaminants present in the specimen. The chances of contamination are higher than those of the solid-based cultures but are quite rapid. The initial investment and maintenance costs associated with the MGIT960 system can be substantial, especially for resource-limited settings. This can restrict its widespread implementation, particularly in regions with limited healthcare resources. The MGIT960 system requires a dedicated laboratory space and access to a stable power supply. Establishing and maintaining the necessary infrastructure can pose challenges, particularly in remote areas or regions with inadequate healthcare facilities. Although the MGIT960 system offers faster results compared to conventional methods, the 2–3 week turnaround time may still be a limitation in urgent cases requiring immediate diagnosis and treatment initiation.

13.2.2.4 MODS Assay

Microscopic Observation Drug Susceptibility assay (MODS) is an approved technique by WHO which does not require any radioactive isotope or florescent indicator like other phenotypic method and this technique is reliable, rapid, and suitable for developing country laboratories [17]. Several research has proved the safety, effective DST for screening of MDR-TB of MODS assay [18]. Moreover first and second line drugs can be used simultaneously in MODS assay in 24 well plates. MODS assays are less expensive in terms of the reagents used when compared with MGIT960, because 0.9 ml of enriched 7H9 medium is used per well in the MODS assay, therefore around 3.6 ml of enriched 7H9 medium is used to determine the first-line drug resistance per sample. For MGIT, more than 7 ml of enriched 7H9 medium are used for primary isolation and there after this much volume is required for each of the drugs for DST.

13.2.2.5 Skin Test: Tuberculin Skin Test

To evaluate if a person has been exposed to MTB, either from a prior TB vaccination or environmental exposure, the tuberculin skin test (TST) is employed. TST is based on the skin reactivation of tuberculin PPD in patients with delayed-type hypersensitivity (DTH). A combination of protein precipitated from modified mycobacterial culture filtrate is known as tuberculin PPD. An immune competent person who has been exposed to MTB experiences an active immune response that leads to the generation of antibodies and memory B cells that will recognise MTB antigens upon reinfection and create MTB antibodies to fend against future TB infections. TST comprises the injection of a small amount of tuberculin fluid containing MTB protein antigen, purified protein derivative, PPD, into the forearm of a patient. Within 48–72 h after injection, PPD reacts with pre-existing antibodies, causing inflammation that results in apparent tissue swelling. The risk that someone has been exposed to MTB increases with the size of the skin oedema. TST can't tell the difference between latent and active TB, though. In addition, those who have received a BCG vaccination to prevent TB will test positive on the TST.

13.3 Indirect Methods

In these methods, the samples collected are incubated for a period of time with appropriate composition of growth supplements that promote the proliferation of TB. Simultaneously, drug susceptibility is checked to select a suitable drug for treatment. A cellular immune response to mycobacterial antigens is required for the diagnosis of long-term bacterial infection (LTBI), which is an indirect method. Indirect tests for diagnosis generally includes immunological and serological tests. Latent tuberculosis infection (LTBI) is described as a condition in which there is a persistent immunological response to stimulation by *M. tuberculosis* antigens but no sign of clinically manifested active TB. Those with LTBI who have negative results from bacteriological tests can opt for the presumptive diagnosis of LTBI which is based on a positive result of a skin (tuberculin skin test, TST) or blood (interferon-gamma release assay, IGRA) test indicating an immunological response to *M. tuberculosis* infection [20].

13.3.1 Interferon-Gamma Release Assays (Igras)

Since IGRAs monitor a patient's immunological response to MTB, they are thought to be more accurate than TSTs Blood samples from people who are thought to have MTB infection are exposed to MTB antigens, if the person is TB-positive, their peripheral blood lymphocytes respond by generating IFN-γ. A single test can provide a definitive diagnosis of TB, and these assays are believed to be extremely sensitive. Two more IGRAs have received FDA approval: the QuantiFERON® TB Gold In-Tube test (QFT-GIT) and the T- SPOT® TB test (T-SPOT). The T-SPOT test counts the number of cells actively producing IFN- γ, whereas the QFT-GIT test measures the concentration of IFN- γ.

13.3.2 QFT-GIT

Whole blood is taken as the test sample to test for single peptides such as the early secretory antigenic target 6 (ESAT-6) as MTB antigens which measures the IFN-ɣ concentration in enzyme linked immuno sorbent assay within the processing time of 24 h and the possible results generated can be positive, negative or intermediate.

13.3.3 T-SPOT

Peripheral blood mononuclear cells (PBMC) blood is taken as a test sample to test for one or both peptides (ESAT-6 and CFP-10) as MTB antigens which measures the number of cells that produce IFN-ɣ in the aptamer-linked DNA immuno sorbent assay within the processing time of 24 h and the possible results generated can be positive, negative or intermediate [19].

13.3.4 Limitations to Phenotypic Methods

The major limitations of liquid and solid DST methods are accuracy, availability, and turnaround time. The DST results may take even more than a month sometimes in culture-based phenotypic assays and this may lead to delay and inaccurate treatment which may increase the resistance proposition. The infrastructure required to carry out phenotypic methods are not available in resource-limited set-ups in many countries. Drugs such as streptomycin, pyrazinamide, and ethambutol suffers from sub-optimal reproducibility. Phenotypic DST is not at all suitable for mixed cultures. There is a fair chance of contamination during the setup of phenotypic DST and also if non-tuberculous mycobacteria is present. This may lead to false-positive results.

13.3.5 Genetic Markers to Identify MTB

1. IS6110: The insertion sequence IS6110 is a widely used genetic marker for MTB. It is a repetitive DNA sequence that is present in multiple copies within the genome of MTB but absent in most other mycobacterial species. The IS6110 element can be detected using polymerase chain reaction (PCR) or other nucleic acid amplification techniques.
2. hsp65: Another frequently used genetic marker for MTB identification is the gene encoding the heat shock protein 65 (hsp65). It encodes a protein that mycobacteria greatly conserve. PCR-based techniques, such as the widely used GenoType MTBDR plus assay, can be used to amplify and detect the hsp65 gene.
3. rpoB: As a genetic marker for MTB detection and medication resistance testing, the RNA polymerase beta subunit (rpoB) gene has been employed. One of the main drugs used to treat tuberculosis, rifampicin, has been linked to certain mutations within the rpoB gene that cause resistance to it. Both species identification and rifampicin resistance data can be obtained using rpoB gene amplification and sequencing.
4. 16S rRNA: The 16S ribosomal RNA (16S rRNA) gene is a conserved region found in the bacterial genome. Specific regions of the 16S rRNA gene can be targeted for amplification and sequencing to identify MTB and differentiate it from other bacteria.
5. Region of Difference (RD) markers: Areas of difference (RD) are genomic regions that are unique to MTB and are absent or markedly different in other mycobacterial species. For PCR-based tests to identify MTB, these RD areas make good targets. RD9, RD12, RD702, and RD750 are a few examples of RD markers.

13.3.6 In-House PCR

PCR is a molecular biology technique that amplifies small amounts of DNA in a sample, making it possible to detect even trace amounts of the target DNA. In-house PCR refers to the use of PCR technology in a laboratory that is part of the same healthcare facility where the sample is collected. This setup allows for faster and more convenient testing, as well as increased cost savings compared to sending samples to an external laboratory.

In the case of tuberculosis (TB), internal PCR is used to detect the DNA of *Mycobacterium tuberculosis*, the bacterium that causes TB [21]. This method is highly sensitive and specific, making it a valuable tool for early diagnosis and prompt treatment of TB.

One of the main benefits of in-house PCR for TB diagnosis is the speed of results. Compared to traditional TB diagnostic methods, such as sputum culture or X-ray, internal PCR can provide results in hours, rather than days or weeks. This fast turnaround time is especially important in areas endemic to tuberculosis, where prompt diagnosis and treatment are crucial to prevent the spread of the disease.

In addition, internal PCR is less prone to contamination and false negative results, compared to sputum culture, and is more accurate than X-ray in detecting TB in people with extrapulmonary TB, such as TB of the lymph nodes, bones, or organs [22, 23].

Despite its many benefits, internal PCR for TB diagnosis is not without limitations. For example, the cost of equipment and reagents can be prohibitive for some healthcare facilities, especially in low-income countries. Additionally, the need for trained personnel to operate and interpret the results of the test can also pose a challenge in resource-limited settings.

13.3.7 Random Fragment Length Polymorphism (RFLP)-PCR for Diagnosis

Random Fragment Length Polymorphism (RFLP)-Polymerase Chain Reaction (PCR) is a diagnostic tool used for the identification of tuberculosis (TB) caused by *Mycobacterium tuberculosis* in human samples. The method is based on the detection of specific genetic regions in the bacterium, which are amplified and fragmented, then analysed to determine the specific strain of TB.

Conventional methods of diagnosis of tuberculosis, such as sputum smear microscopy and culture, are time-consuming and have limitations in terms of sensitivity and specificity. RFLP-PCR, on the other hand, is a highly sensitive and specific diagnostic method that can detect the presence of *M. tuberculosis* in clinical specimens within a few hours. The procedure involves the amplification of specific genetic regions of *M. tuberculosis* using PCR, followed by digestion of restriction enzymes and analysis of the resulting fragments. The pattern of fragments produced can be used to distinguish between different strains of *M. tuberculosis.*

One of the main advantages of RFLP-PCR is its ability to detect TB in a variety of clinical samples, including sputum, bronchoalveolar lavage fluid, and tissues. This makes it a valuable tool in the diagnosis of extrapulmonary TB, which often goes undiagnosed using conventional methods.

Another advantage of RFLP-PCR is its ability to detect TB in immunosuppressed individuals, who may not show typical symptoms of the disease. This is particularly important for patients with HIV, who are at increased risk of developing TB. RFLP-PCR can also be used to monitor the response to treatment and to detect the emergence of drug-resistant strains of *M. tuberculosis*.

However, there are some limitations to the use of RFLP-PCR for the diagnosis of TB. The method requires specialised equipment and expertise and is more expensive than conventional diagnostic methods. In addition, false-positive results can occur if the specimens are contaminated with other species of Mycobacteria.

In conclusion, RFLP-PCR is a highly accurate and precise diagnostic technique for the diagnosis of TB brought on by *M. tuberculosis*. It is an effective tool in the fight against TB since it can find TB in a range of clinical specimens and in immunosuppressed people. However, the process is more expensive than customary diagnostic techniques and calls for specialised equipment and knowledge. For widespread usage in the global TB control effort, more research is required to increase the RFLP-price PCR's and accuracy.

13.3.8 Nucleic Acid Amplification Test (NAAT)

NAAT assays identify the MTB complex organisms by detecting the mycobacterial insertion element IS6110 [24]. Acid-fast bacilli (AFB) smear-positive and AFB smear-negative sputum samples can both be used to find DNA from the *Mycobacterium tuberculosis* complex (MTBC) (deoxyribonucleic acid) in sputum or other respiratory samples. NAA testing comprises a procedure that amplifies (or replicates) the genetic material because the amount of DNA in a sample is so tiny. A typical NAAT method used in laboratory diagnosis is polymerase chain reaction (PCR). The GeneXpert® MTB/RIF test is a PCR that simultaneously identifies MTBC and the genetic mutation that results in resistance to rifampin (RIF). Due to its high sensitivity and specificity, nucleic acid amplification testing (NAAT) has become the preferred method as a potential substitute for the traditional bacteriological diagnosis of tuberculosis (TB), which has a number of drawbacks. A NAAT can detect MTBC genetic material even when very small amounts are present in the tested sample. NAAT results are typically available in 24–48 h. Rapid results enable an earlier diagnosis of tuberculosis, earlier initiation of treatment, a reduced period of infectiousness, and improved patient outcomes.

The nucleic acid amplification test (NAAT) is a highly sensitive and specific diagnostic test that has become an essential tool in the diagnosis of tuberculosis (TB). It is a highly infectious disease that can spread quickly, especially in populations with weakened immune systems, and early and accurate diagnosis is critical to controlling its spread and improving patient outcomes.

NAATs are able to detect very small amounts of *M. tuberculosis* DNA in a patient's sample, making them ideal for the rapid diagnosis of TB, even in patients with early-stage or asymptomatic infections. This is a significant advantage over traditional TB diagnostic tests, such as the sputum smear microscopy test, which can miss up to 50% of cases of TB and can be affected by the presence of concurrent infections or other factors that can interfere with the results [25].

Another advantage of NAAT in the diagnosis of tuberculosis is its specificity, which reduces the risk of false-positive results. This is particularly important in TB-endemic areas where misdiagnosis can lead to inappropriate treatment, further spread of disease, and potential development of drug-resistant strains. NAATs are able to identify the specific subtype of *M. tuberculosis*, providing crucial information for effective treatment and tracking the spread of the disease.

Several commercially available NAATs exist for TB diagnosis, including the polymerase chain reaction (PCR) test. These tests can provide results in just a few hours, allowing for rapid diagnosis and initiation of treatment. Additionally, NAATs can be performed on a variety of sample types (such as urine, blood, and sputum), making them useful for a wide range of diagnostic applications.

13.3.9 XPERT MTB/RIF Assay

The XPERT MTB/RIF assay is a molecular diagnostic test used to detect tuberculosis (TB) and resistance to rifampin, a crucial first-line TB drug. A cartridge based on the GeneXpert Instrument System is used in the nucleic acid amplification-based XpertMTB / RIF assay [26]. This test offers several advantages over traditional TB diagnostic methods, including improved speed, accuracy and versatility.

One of the key benefits of the XPERT MTB/RIF assay is its speed. Results can be obtained in as little as 2 h, making it much faster than traditional TB culture methods which can take several weeks to produce results. This quick turnaround time can help ensure that patients receive timely and effective treatment for their condition.

The XPERT MTB/RIF assay is also highly accurate. Studies have shown that the test has a high level of sensitivity and specificity for detecting TB and rifampin resistance, meaning that it is able to accurately identify TB cases and distinguish between TB and other similar conditions. This helps to reduce the risk of misdiagnosis and ensure that patients receive the correct treatment for their condition. The test is also versatile as it can be performed on a variety of sample types, including sputum, broncho alveolar lavage fluid (BAL), and pleural fluid. This makes it suitable for use in a range of clinical settings, including

resource-limited settings where access to laboratory facilities may be limited.

The 81 bp core region of the bacterial RNA polymerase (rpoB) gene, which encodes the enzyme's active site, is the target DNA sequence. Mutations are located in this region in more than 95% of all strains resistant to rifampicin. Furthermore, *M. tuberculosis* specific DNA sequences flank the rpoB core region. Consequently, it is possible to test both *M. tuberculosis* and resistance to rifampicin at the same time [27].

In addition to these benefits, the XPERT MTB/RIF assay is relatively easy to use and requires minimal technical training. This makes it a suitable option for use in a variety of settings, including hospitals, primary care clinics, and low- and middle-income countries. Overall, the XPERT MTB/RIF assay is a highly effective and convenient diagnostic tool for detecting TB and rifampin resistance. Its fast turnaround time, accuracy, versatility, and ease of use make it a valuable tool for improving TB diagnosis and treatment globally.

Steingart et al. [28] has done a meta-analysis and reported that in the low- and middle-income countries GeneXpert MTB/RIF has 89% sensitivity and 99% specificity. The sensitivity is higher in smear-positive cases (98%) than in smear-negative cases (67%). The main limitations of this method are it cannot distinguish between living and dead and so treatment progression cannot be assessed. The sensitivity is lower in case of extrapulmonary and paucibacillary. There is a chance of getting false-positive results in certain silent mutations [29]. The high cost is also one of the limitations. The GeneXpert MTB/RIF Ultra assay is a next generation assay that has higher sensitivity than the previous version.

GeneXpert 10-colour instrument is one of the recent additions by Cepheid wherein the instrument can detect drug resistance to Rifampicin, amikacin, ethionamide, fluoroquinolones, and isoniazid. WHO has recommended the use of this instrument and stated that this is comparable to the existing Xpertsytem in detecting rifampicin resistance (Use of Xpert MTB/RIF and Xpert MTB/RIF Ultra on GeneXpert 10-colour instruments: WHO policy statement. Geneva: World Health Organization; 2021. Licence: CC BY-NC-SA 3.0 IGO).

13.3.10 Line Probe Assays

The line probe assay (LPA) is a molecular diagnostic test that is used to detect the presence of specific genetic mutations in a patient's DNA. The test works by amplifying and then hybridising the target DNA to a set of probes that are attached to a solid support (such as a strip). It is based on the reverse hybridisation theory [30]. It is based on reverse hybridisation theory. If a mutation is present in the target DNA, it will bind to the corresponding probe, causing a visible line to appear on the strip. The presence of *M. tuberculosis* can be seen by looking at the coloured band that appears on the strip.

Advantages of LPA include its high sensitivity and specificity, rapid turnaround time (results are typically available within a few hours), and its ability to detect multiple mutations in a single reaction. This makes it a useful tool for the diagnosis of tuberculosis and human immunodeficiency virus [HIV]), cancers (such as lung and colon cancer), and inherited disorders (such as cystic fibrosis).

In addition to its diagnostic applications, LPA has also been used to monitor the effectiveness of treatments for certain diseases, such as tuberculosis and HIV and for monitoring the development of drug resistance in patients.

There are several different commercial LPA kits available, each with its own specific set of probes designed to detect specific mutations. It is important to choose the correct LPA kit for the intended application, as using the wrong kit may result in false negative or false-positive results.

13.3.11 MTBDR*plus* and MTBDR*sl*

MTBDR*plus* and MTBDR*sl* are line probe assays for rapid screening and DST of tuberculosis. MTBDR*plus* detects isoniazid (katG and inhA) and rifampin (rpoB) resistance, whereas MTBDR*sl* detects ethambutol (embB), aminoglycosides, and cyclic peptides (rrs) and fluoroqinolones (gyrA). The time taken for these two assays are approximately 5 h. Bai et al. in 2016 [31] has done a meta-

analysis and reported that the sensitivity and specificity to detect isoniazid resistance is 91% and 99%, respectively. For rifampicin resistance, the sensitivity and specificity is 96% and 98%, respectively, and for MDR, it is 91% sensitivity and 98% specificity. In case of direct smear-negative sputum samples, the early detection percentage for MDR-TB is low [32–34]. GenoTypeMTBDR*sl* 2.0 version is the updated version with higher detection capability of mutations [35–37]. The sensitivity and specificity in detecting fluoroquinolone resistance for direct testing of smear-positive samples are 97% and 98%, respectively, and for smear-negative samples it is 80% and 100%, respectively. In case of second line injectable agents, the sensitivity and specificity for smear-positive samples are 89% and 90%, respectively, whereas for smear-negative, it is 80% and 100%, respectively. Further evaluation should be done for susceptible smear-negative specimens.

13.3.12 Nipro NTM+MDRTB

The Nipro NTM+MDRTB is one of the line probe assays that can identify four species of Mycobacterium, namely *M. tuberculosis*, *M. kansasii*, *M. intracellularle,* and *M. avium*. It also detects resistance to isoniazid (inhA and KatG) and rifampin (rpoB) [38]. The sensitivity and specificity of direct test was reported as 96.5% and 98.5%, respectively, whereas for resistance, the sensitivity and specificity was reported as 94.9% and 97.6%, respectively. Nipro has also designed a test called NiproGenoscholar PZA-TBII line probe assay to detect resistance for pyrazinamide in pncA gene [39].

13.3.13 Anyplex Plus MTB/NTM/MDR-TB and Anyplex II MTB/MDR/XDR Kit

Anyplex plus system is one of the multiplex real-time PCR systems that is used to detect *M. tuberculosis* complex and non-tuberculous mycobacterial species. Resistance to rifampicin (rpoB) and isoniazid (katG and inhA) is also detected in case of *M. tuberculosis*. This assay can be completed in 3 h time. The sensitivity for the detection of Mtb complex is 86.4% and the specificity is 99% [40]. Lower sensitivity is reported in extrapulmonary cases. Anyplex II is a newer version that can identify resistance in rifampicin (rpoB), isoniazid (katG and inhA), aminoglycoside (rrs and eis), and fluoroquinolone (gyrA). When compared with phenotypic DST, this test has lower sensitivity [41].

13.3.14 Truenat MTB, Truenat MTB Plus, and Truenat MTB-RIF Dx

Truenat system is a chip based real-time micro-PCR assay developed in India by Molbio Diagnostics Pvt. Ltd. Truenat MTB and Truenat MTB Plus are diagnostic assays for TB. If the assay is positive, then Truenat MTB-RIF Dx is used to detect resistance for rifampicin. The sensitivity and specificity of Truenat MTB are 88% and 97.2%, respectively, whereas the sensitivity and specificity of Truenat MTB plus are 91.7% and 97.2%, respectively [42]. This assay only requires 0.5 ml of the sputum sample. This can be used as a point of care test as this is portable and battery operated. The total time taken for Truenat MTB Plus followed by MTB-RIF Dx is approximately 2 h. This has an advantage over Xpert system, wherein there are two different chips for Mtb detection and Rifampicin detection and hence this will be more cost effective when compared to Xpert.

13.3.15 Loop-Mediated Isothermal Amplification Technique

LAMP (Loop-mediated isothermal amplification) is a molecular diagnostic technique used to detect the presence of *Mycobacterium tuberculosis*, the causative agent of tuberculosis (TB). It is a rapid, sensitive, and specific method for diagnosing TB, especially in resource-limited settings. LAMP uses specific primers to amplify DNA under isothermal conditions, eliminating

the need for thermal cycling and making it a low-cost alternative to traditional PCR-based methods. The results of [43] LAMP can be read visually or by using a fluorescence reader, providing results in as little as 60 min.

13.3.16 Whole-Genome Sequencing (WGS)

Whole-genome sequencing (WGS) is a powerful tool for the diagnosis of *Mycobacterium tuberculosis* (MTB), the causative agent of tuberculosis (TB). It has the potential to revolutionise the diagnosis and management of TB, particularly in resource-limited settings. WGS provides a comprehensive view of the complete genomic makeup of MTB, allowing the detection of genetic variations that are not discernible by traditional diagnostic methods. This information can be used to differentiate between closely related strains, track the spread of tuberculosis, and monitor the emergence of drug-resistant strains [44]. One of the key advantages of WGS is its high resolution, allowing for the identification of mutations that are associated with drug resistance. This is particularly important in the context of TB, where drug resistance is a major challenge to effective treatment. By detecting resistance early, WGS can inform the selection of appropriate treatment regimens, reducing the risk of further spread of drug-resistant tuberculosis. Because MTB must be cultured for several weeks before a substantial amount of DNA can be recovered, WGS has not been used as a standard diagnostic method for TB. Workflow to extract DNA from frozen isolates without re-culturing is significant since slow-growing MTB must be cultured before DNA extraction, which takes a lot of time in WGS [45, 46]. Despite the many benefits of WGS for the diagnosis of MTB, there are also some challenges associated with its implementation. One of the main challenges is the cost, which can be a barrier to widespread adoption, especially in resource-limited settings. Additionally, WGS requires specialised equipment and skilled personnel, which may not be readily available in some regions. In conclusion, WGS has the potential to significantly improve the diagnosis and management of MTB, particularly with regard to the detection of drug-resistant strains and the differentiation between active and latent infections. However, cost and access to equipment and personnel remain barriers to widespread implementation. Further research is needed to address these challenges and make WGS a more accessible and practical diagnostic tool for TB. WGS is becoming more accessible in diagnostic microbiology laboratories as costs decrease and technology advances. Pathogens can be thoroughly characterised at the molecular level to research whole-genome evolution (such as the compensating mechanisms offsetting the fitness cost of resistance mutations), population structure, and phylogeny, as well as for epidemiological applications [47].

13.3.17 Mycobacterial Load Detection Assay

Mycobacterial load detection assays are in-house methodologies that are critical in the management of tuberculosis (TB) and other mycobacterial infections. The assay is used to measure the number of mycobacterial cells in a patient's sample, which is an important predictor of disease progression and response to treatment. The assay can be performed using a variety of methods including culture-based assays, molecular assays, and antibody-based assays. Culture-based assays, such as the Lowenstein–Jensen (LJ) culture method, have been the gold standard for mycobacterial load detection for several decades. However, these assays have significant limitations, including a long turnaround time, low sensitivity, and a requirement for specialised laboratory equipment and personnel. Molecular assays, such as real-time PCR, have emerged as a promising alternative to culture-based methods. These assays are highly sensitive, rapid, and can be performed with relatively simple and inexpensive equipment. However, their accuracy may be influenced by the presence of inhibitors in the patient's sample and the need for accurate and standardised calibration. Antibody-based assays,

such as the ELISpot assay, are designed to detect the presence of specific mycobacterial antigens in the patient's sample. These assays are rapid and simple to perform, but their sensitivity and specificity may be limited compared to culture-based and other molecular assays.

13.3.18 Other Advanced Molecular Diagnostic Techniques for TB

1. RFLP: The restriction fragment length polymorphism (RFLP) is a widely used technique for identifying MTB. This is based on the analysis of the differences in the restriction patterns of the MTB genome produced by different restriction enzymes. RFLP is a powerful tool for differentiating between different strains of MTB and has been widely used for epidemiological studies of TB.
2. Spoligotyping: This is another widely used technique that utilises genetic markers for identifying MTB. It is beneficial for laboratories studying population genetics, evolution, molecular epidemiology, and clinical care [48]. It is based on the analysis of the spacers between the direct repeats in the MTB genome. Spoligotyping is a powerful tool for differentiating between different MTB strains and has been widely used for epidemiological studies of TB.
3. MIRU-VNTR: The mycobacterial inter spersed number of tandem repeats in repetitive units (MIRU-VNTR) is a relatively new genetic marker that is used to identify MTB [49]. This marker is based on the analysis of the number of copies of a specific sequence of DNA in the MTB genome. MIRU-VNTR is a powerful tool for differentiating between different MTB strains and has been widely used for epidemiological studies of TB.

13.4 Conclusion

So far, there were several advances with respect to molecular diagnostics of drug resistance in *Mycobacterium tuberculosis*. Yet there are some limitations. The major disadvantage of molecular diagnostics of DST apart from not being a good prognostic indicator is the lack of studies in underling mechanisms of genetics involved in drug resistance, especially for drugs other than rifampicin. But the rapidity offered by the molecular methods are contributing so much that the drastic decrease in the time to detection is very much valuable especially in limiting the spread of the disease and transmission. The prominence in the placement of the molecular methods in the diagnostic algorithm reflects this statement. Generally, mutations that are specific to particular type of resistance are identified and then the molecular mechanism of resistance is studied using sophisticated tools. But in some cases resistance is present due to a cumulative effect of many mutations that act together. Bioinformatics approaches can be used to identify such patterns irrespective of point mutations specified for a particular resistance. For example, machine languages are used to create AI software that are involved in chest radiograph for patients having TB suspicion [50]. Likewise, bioinformatic tools can be used to filter the large data that are generated from whole-genome sequencing. Thus, software that are user friendly and highly streamlined which process automatically the data from whole-genome sequencing and gives us the report of drug resistance that are clinically relevant are needed. The other major limitation is lack of laboratories that have all the expensive instrumentation required for molecular diagnosis of drug-resistant TB. Establishment of many such central facilities will avoid the time taken for transportation of specimen from primary clinic to central facility. Closed cartridge systems are highly recommended over open tube systems for easy procedure with no cross contamination. Finally, as newer molecules having antiTB activity are being discovered now and then, the molecular genotypic determinants of resistance should be analysed and evaluated and should be included in the molecular tests. The resistant strains can be a part of the molecular diagnostic tool in the near future.Contri butionAB, SK and SAS contributed equally for the preparation of this chapter, AD was responsible for the entire flow and critical reviewing of the chapter.

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