CHAPTER

22

Molecular Diagnosis of Tuberculosis

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INTRODUCTION

Tuberculosis (TB) is an age old disease. In 1882, Sir Robert Koch discovered Mycobacterium tuberculosis (M-TB), but even today the diagnosis of TB remains elusive. The symptoms of pulmonary TB (PTB) like cough, fever, hemoptysis, weight loss, night sweat etc. are nonspecific, and those may occur in any chronic lung infection. Similarly signs of PTB may vary from total absence to gross abnormalities depending on the site of disease, severity of disease and associated complications. Symptoms and signs of extra-pulmonary TB (EP-TB) are nonspecific and depend on the site of lesion. Chest X-ray and other radiological imaging techniques may be suggestive of TB but are non-specific, and have unacceptably high inter and intra personal variation even among radiologist. Positive tuberculin test suggests infection not disease, and may be negative in active TB. Biochemical tests are nonspecific and ADA has more negative prediction value than positive prediction value.

The best method of diagnosing an infectious disease is to demonstrate the causative organism in representative samples of tissue or fluid by either staining and or by culture. Sputum microscopy is accepted world-wide as the first line test as it is rapid, inexpensive and can be done in field condition. But it is less sensitive as it requires at least 10^4 bacilli per ml of sputum to be positive and it may be falsely positive in many conditions including environmental mycobacterial infection. Culture in solid media is the reference standard but it takes long time (4-6 weeks). Rapid culture in liquid media (BACTEC- MGIT) takes at least 10-14 days.

So there is an urgent necessity for tests that can quickly diagnose TB. Hence the most promising approach was to demonstrate remnants of the TB bacilli in representative tissue / fluid samples. Detecting even small amounts of bacterial DNA was feasible due to the development of various molecular diagnostic tests for TB.

DNA PROBE

DNA probes are single stranded complementary DNA (cDNA) / RNA directed against target DNA / RNA of TB bacilli. The labeled probe is first denatured (by heating or under alkaline conditions such as exposure to sodium hydroxide) into single stranded DNA (ssDNA) and then hybridized to the target ssDNA. Previously radio-active iodine (^{125}I) labeled DNA probes were used. Now that has been replaced by acridinium-ester labeled DNA probes. Probe-target hybrid produces light (chemo-iluminecent assay) that can be detected by a laminometer. The DNA probe for culture confirmation had sensitivity and a specificity of 100%. However, DNA probes are available only against limited number of species of mycobacteria, on the other hand M. terrae may cause false positive result. The main limitation of DNA probe is that it requires at least 10^5-10^6 DNA fragments to be positive. DNA fragments have to be amplified by nucleic acid amplification test (NAAT) before applying them to DNA probe.

NAAT

NAAT is a molecular technique used to detect a virus or a bacterium. The term includes any test that directly detects the genetic material of the infecting organism. As few as 100 organisms/ mL may give a positive result with NAAT.[1] NAAT results must be interpreted in the context of clinical and epidemiologic factors. Contamination and laboratory errors can cause false-positive results. In addition, NAAT can detect nucleic acid from dead and live organisms, so testing can remain positive even after appropriate therapy. Therefore, this method is only appropriate for initial diagnostic purposes.

Various types of NAAT are available and they are:

1. Polymerase chain reaction (PCR).
2. Reverse transcriptase PCR (RT-PCR) is used for HIV and other RNA viruses.
3. Branched DNA (quantiplex bDNA) tests use a molecule that links to the specific genetic material.
4. Ligase chain reaction.
5. Transcription mediated amplification (TMA).
6. Nucleic acid sequence-based amplification (NASBA).

The commonly used NAAT in TB is PCR.

PCR

Kary Mullis got Novel Prize in 1993 for developing PCR. Basic principle of the test are as follow (Figure 1): heat is applied to denature double stranded DNA to separate the strands, then they are allowed to cool down, then specific primer is added to produce template - primer complex, then nucleotides and heat stable DNA (taq) polymerase are added to produce first filial generation. First filial generation serve as template for further DNA synthesis. The process is repeated to produce millions of copies of DNA fragments (amplicons). Then the amplicons can be identified with the help of DNA probe.

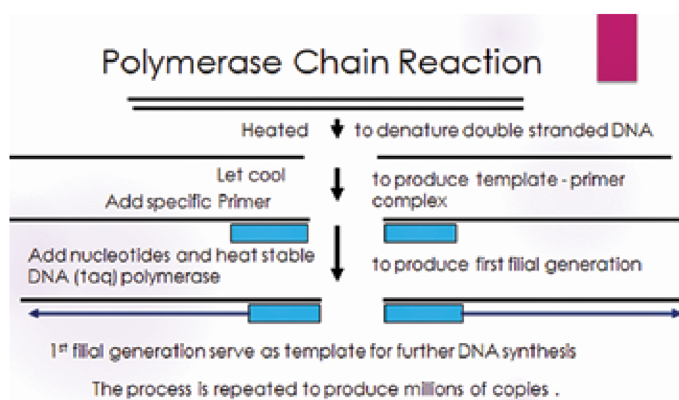

Fig. 1: Steps of polymerase chain reaction

During the latter part of the last century the advantages of conventional PCR tests for TB were widely publicized. However despite the excitement of the initial results it was found that PCR lacks sensitivity in smear negative and culture positive cases. PCR has a sensitivity of 95% in smear positive TB and 50-60% in smear negative TB. The specificity of PCR is around 98%. PCR is more sensitive than smear but less sensitive than culture.[2] PCR for rRNA indicates multiplying bacilli hence infection. Another important problem with PCR is that PCR may be positive intermittently and it is less accurate if patient is on anti-TB treatment.

The effectiveness of PCR in TB is related to experience and accuracy of the personnel conducting the assay. Thus TB-PCR results widely vary from laboratory to laboratory, and false positive results were found in 0 to 20% cases though all laboratories targeted IS6110 for amplification.[3] Due to the complexity of performing the test and the expenses involved, PCR tests lacked the ability to be used as point of care tests (POCT) of any reliability. POCT is defined as medical diagnostic testing performed outside the clinical laboratory in close proximity to where the patient is receiving care and is typically performed by non-laboratory personnel and the results are used for clinical decision making.

RESTRICTED FRAGMENT LENGTH POLYMORPHISM (RFLP)

In this process, DNA is extracted from cultured of M-TB, then a restricted endonuclease cleaves the elements in base pairs, IS6110 base pair is used as it is specific for M-TB, then DNA fragments are separated by electrophoresis in agar gel, then DNA fragments are transferred to a membrane (Southern blotting), DNA fragments are detected with DNA probe and DNA fragments are depicted as bands on a X-ray film to create a unique strain specific DNA finger printing. RFLP is used for research and epidemiological purposes. But the test is not suitable for use in clinical purpose.

CARTRIDGE BASED NAAT (CBNAAT)

Even in best laboratory of the world, 1-2% cross contamination in NAAT has been reported. To avoid cross contamination, CBNAAT, where separate cartridge is used for each test, is introduced. CBNAAT is a totally automated test and can be used as POCT. CBNAAT may be used for rapid diagnosis of TB where results are obtained within 1 -2 days. In AFB smear positive respiratory specimens, the sensitivity and specificity are 95 and 98% respectively whereas they are about 75 to 88 percent and 95 percent in smear negative specimens respectively.[3] In smear positive setting NAAT can help in distinguishing TB from non-tubercular mycobacterium (>95 percent). CBNAAT can rapidly establish the presence of tuberculosis in 50 to 80% of AFB smear-negative specimens (which would eventually be culture positive).[4]

Sensitivity of CBNAAT for TB diagnosis, when compared to liquid culture as gold standard, is high in FNAC / biopsy specimen from lymph nodes, biopsy specimen from other tissue and cerebro-spinal fluid, but lower in pericardia, ascetic and synovial fluid samples and still lower in pleural fluid. A positive CBNAAT provides useful confirmation but a negative test does not always rule out TB. CBNAAT does not replace the roles of routine AFB smear and culture. Tissue to be tested by CBNAAT should be collected without formalin. Tissue samples should only be processed at laboratories with appropriate biosafety requirements.

XPERT MTB/RIF ASSAY (GENE XPERT)

The Xpert MTB/RIF assay is an automated CBNATT that can simultaneously *identify* M. tuberculosis and rifampicin (RIF) resistance within two hours. The Xpert MTB/RIF assay has been approved by the WHO in 2013 for the diagnosis of TB in patients on therapy for less than seven days.[5] The assay is simple to perform with minimal training, is not prone to cross-contamination, and requires minimal biosafety facilities. This test has the potential to dramatically reduce the time to diagnosis and the time to initiation of effective therapy. But it requires a reliable power supply and operating temperatures below 30°C. Sputum should be of good quality, and it should be concentrated by usual laboratory methods.

In a systemic review and meta-analysis including 18 studies, the sensitivity and specificity of Xpert MTB/RIF assay (compared with culture) were 83 and 94 percent respectively in lymph nodes; 81 and 98 percent respectively in cerebrospinal fluid; and 46 and 99 percent respectively in pleural fluid.[6] Pleural fluid is a suboptimal sample. A pleural biopsy is the preferred sample. The Xpert MTB/RIF can be done in all EP-TB specimens except stool, urine and blood.

Xpert MTB/RIF is designed to identify RIF-resistance mutations in an 81-bp region (codons 426 to 452) of the rpo-β gene known as the RIF-resistance–determining region (RRDR). The accuracy for identification of RIF-resistance was 98 percent. However, a study done in Swaziland demonstrated that the assay may not be able to detect wild type mutations for RIF-resistance outside the rpo-β I491F domain.[7]

THE WHO RECOMMENDATIONS FOR XPERT MTB/RIF ASSAY ARE AS FOLLOWS[8]

For diagnosis of Pulmonary TB

Table 1: Difference between line probe assay and Gene Xpert

Line probe assay	Gene xpert
Can be done only in smear positive cases	Can be done in both smear positive and negative cases
Difficult to be done in crude clinical specimens	Can be done on crude clinical specimens
Results obtained in 2 days	Results obtained within 2 hours
Can detect both rifampicin and isoniazid resistance	Can detect only rifampicin resistance
Has less value in HIV and extra-pulmonary TB	Has more value in HIV and extra-pulmonary TB
Can only be used in national and regional level	Can be used in district and sub-district level
Technically less robust	Technically more robust
Less automated	More automated

- Initial diagnostic test in adults with HIV or suspected of having MDRTB
- Initial diagnostic test in children with HIV or suspected of having MDRTB
- May be used over smear and culture in adults and in children suspected of having TB (conditional recommendation considering resource constraints, low quality evidence)

For diagnosis of extra-pulmonary TB

- Initial diagnostic test of choice over microscopy and culture, for CSF specimens from patients suspected of having TB meningitis (strong recommendation given the urgency of rapid diagnosis, very low-quality evidence).
- Replacement test for usual practice for testing specific non-respiratory specimens (lymph nodes and other tissues) from patients suspected of having EP-TB (conditional recommendation, very low-quality evidence).

Line probe assay (LPA); MDRTB plus — The assay MTBDR plus (Hain's Test) is a molecular probe capable of detecting RIF and isoniazid (INH) resistance mutations within 2 days (rpo-β gene for RIF resistance and katG & inhA genes for INH resistance). In a study done in South Africa on 536 smear-positive specimens from patients at risk for MDR-TB, the test was ≥ 99% sensitive and specific for detecting MDR-TB compared with standard DST results.[9] The test can reduce time to initiation of therapy for MDR-TB. The difference between LPA and CBNAAT has been depicted in Table 1.

MDRTB sl: The assay detects resistance to second-line fluoroquinolones and second-line injectable drugs, and it may be used as an initial test for second-line drug resistance. A positive result is reliable for detection of drug-resistant TB but a negative result may not always rule out the presence of drug-resistant TB, and that should be confirmed by conventional culture and drug sensitivity test (DST).[10]

For drug resistant TB: Genotypic testing is much faster than phenotypic methods, as these are not growth based tests. Drug sensitivity test results by solid LJ media has a turnaround time of up to 84 days, liquid culture (MGIT) up to 42 days, LPA up to 72 hours and CBNAAT by 2 hours.

MOLECULAR TEST IN RNTCP[11]

In our National TB Control Program (RNTCP), both CBNAAT and LPA are approved. The recommendations for CBNAAT in RNTCP are as follows:

i. TB suspects in all key population like PLHIV (persons living with HIV).

ii. All smear positive patients with risk for drug resistance*.

iii. All smear negative cases where chest x-ray is suggestive of TB.

iv. In all presumptive cases of EP-TB appropriate specimens from the presumed sites of involvement must be obtained and CBNAAT is preferred over other tests.

v. In pediatric TB, sputum examination should be carried out among children who are able to give good quality specimen and CBNAAT is the preferable investigation of choice. Where CBNAAT is doable, smear examination may not be done.

*In smear positive cases without risk for drug resistance, at present CBNAAT is not advocated by RNTCP and those people are to be categorized as microbiologically confirmed TB.

Whenever indicated, alternative specimens like gastric aspirates, induced sputum, broncho-alveolar lavage should be subjected to CBNAAT.

For CBNAAT, a single specimen is required for testing. For "RIF indeterminate result", an additional CBNAAT should be done to get a valid result. If the second result is also indeterminate, then an additional specimen should be sent to nearest intermediate reference laboratory for LPA or liquid culture & DST. The need for a second specimen for CBNAAT arises in case the result is "invalid" or "RIF indeterminate". For "Error", "No results" the test can be repeated on the same specimen after appropriate trouble shooting.

Two specimens should be collected (spot-early morning or spot-spot) for examination by LPA which can be performed directly on sputum specimen which are positive on microscopy or on culture isolates of specimen which were negative on microscopy.

Conventional microscopy and culture remain essential for monitoring therapy and for performing DST for anti-TB agents other than RIF. In EPTB, a single negative result of Xpert MTB/RIF should undergo further diagnostic

testing, and those with high clinical suspicion for TB (especially children) may be treated with anti-TB drugs.

CONCLUSION

Molecular techniques have revolutionized the diagnosis of TB (both PTB and EP-TB), as well as MDR-TB. CBNAAT (Xpert MTB/RIF) can be used in field condition, sub-district level, where 24 hour electricity is available as it requires minimum training and biosafety. RIF resistance is considered as surrogate marker of MDR-TB as practically all RIF resistant bacilli are resistant to INH. However, it should be remembered that a positive result suggest but a negative result do not exclude TB as well as MDR-TB. At present CBNAAT has not totally replaced the traditional smear and culture for TB.

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