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Laboratory Diagnosis of Tuberculosis: Conventional and Newer Methods

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Abstract: ***Tuberculosis is the main cause for loss of life from a single infectious agent known as Mycobacterium tuberculosis (M.tb). Tuberculin skin test (TST) and interferon-gamma release assays (IGRAs) are two approaches available for testing of latent TB infection but none of these can accurately differentiate between active TB disease and TB infection. Emergence of drug resistant tuberculosis particularly multidrug-resistant (MDR) and extensively drug-resistant (XDR) tuberculosis is a challenge to eliminate tuberculosis worldwide. The acid-fast bacillus (AFB) smear microscopy remains the foremost frequently used microbiological test for detection of TB, however it can neither differentiate between live and dead bacilli, nor give any information regarding drug susceptibility testing (DST). Phenotypic culture remains the gold standard but is time consuming and requires good infrastructure. Therefore, significant efforts have been made in last few years for advancement in molecular/genotypic technologies like line probe assay (LPA), cartridge based nucleic acid amplification test (CBNAAT), Truenat MTB test, loop-mediated amplification (LAMP) test, whole genome sequencing etc. Some of these methods have been endorsed by the WHO and have proven to improve case detection and management of TB patients. Presently there is no single test which can be used for detection of tuberculosis in all patients. We have to depend upon combination of different tests and see how to place them in diagnostic algorithm so as to appropriately use all the available diagnostic modalities taking into account factors such as the capacity of different levels and requirement of infrastructure/man power.***

Keywords: Mycobacterium tuberculosis, Molecular techniques, Drug resistance

1. Introduction

Tuberculosis (TB) is the main cause for loss of life from a single infectious agent known as *Mycobacterium tuberculosis (M.tb)*, which is spread when an individual sick with TB illness expels microorganism into the air by coughing, sneezing. The microorganism generally infects the lungs (pulmonary TB) but can even have an effect on different sites of body (extrapulmonary TB). If not handled promptly, this infectious illness can be fatal. In 2018, an estimated 10 million individual's fell ill with tuberculosis [1], and 87% of the cases occurred in 30 high burden nations together with India (27%) and China (9%). The emergence of drug resistant tuberculosis (DR-TB), particularly multidrug-resistant (MDR) and extensively drug-resistant (XDR) tuberculosis is a challenge to eliminate tuberculosis worldwide. Globally, 3.4 % of new TB cases and 18% previously treated cases are estimated to have MDR-TB. There have been roughly half a million new cases of rifampicin-resistant TB (RR-TB) of which 78% had MDR-TB. India, China and Russian Federation share the biggest number of DR-TB. The average proportion of MDR-TB cases with XDR-TB is 6.2%.

Early detection of the pathogen and its drug susceptibility testing (DST) is extremely crucial for End TB Strategy, because it helps in prompt initiation of treatment and thereby curtail the chain of transmission of the disease. [2]. The acid-fast bacillus (AFB) smear microscopy remains the foremost frequently used microbiological test for detection of TB, however it does not differentiates between live and dead bacilli and thence it's not very helpful for follow up patient. It can neither produce DST patterns, nor predict the presence of non-tuberculous mycobacteria (NTM) [3]. Another test called culture is thought-about as the most sensitive methodology, and remains the gold standard for identification of TB. Additionally, it can provide the DST patterns of various anti-TB drugs. Yet, because of slow growth of mycobacteria, results can take week to months. Conventional culture with egg based solid media referred to as Lowenstein-Jensen (LJ) media, might take upto eight weeks for mycobacterial growth and additional 6 weeks for DST results. Now, liquid medium based rapid culture method is available but it also takes 1-3 weeks for mycobacterial growth [4]. Therefore, significant efforts have been made in last few years for advancement in molecular/genotypic technologies (based on nucleic acid amplification test, DNA hybridization, etc) for reducing the time to few days. This review is intended to highlight the diagnostic performance of conventional and newer TB diagnostic techniques.

2. Conventional Method

Microscopy

AFB smear microscopy is fast, less expensive method for detecting TB in developing countries with high incidence of TB [5]. In this method, the sputum specimen are smeared directly on the cleaned glass slides and subjected to Ziehl-Neelsen (ZN) staining. Although it is most practical, simple and furnish fast results, it detects roughly 50% of all active cases of TB. The higher load of bacilli (10^4 to 10^5 bacilli per ml) that needs to be present leads to a varying sensitivity from 20% to 60% relying upon many factors such as quality of specimen and the training of laboratory person. As per current National TB Elimination Programme (NTEP) of India, two samples (spot and early morning) should be collected for

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diagnosis. However, one sample is sufficient for testing of follow-up patient.

Light-emitting diode fluorescent microscopy (LED-FM)

Light-emitting diodes (LED) microscopy is novel diagnostic tool which is considered superior over ZN microscopy. The technique is more robust, less expensive, user friendly and required less maintenance, thus allow resource constraints parts of the world access to the benefits of FM [6]. The diodes are very durable and do not produce ultraviolet (UV) light, do not require dark room and significantly decrease the instrument's power consumption, allowing long lasting battery life. LED-FM increases the sensitivity of microscopy and it allows a much longer larger area of the smear to be seen, resulting in morefaster examination of the specimen. As compared to ZN microscopy, the LED-FM is slightly more sensitive while specificity is comparable of both methods. Being WHO endorsed in 2009, the NTEP has adopted LED-FM to replace ZN method in high load designated microscopy centres (DMCs) across India in phase manner.

Rapid phenotypic culture methods

BACTEC MGIT 960 system

In the recent years a non-radiometric, fully automated Mycobacterial Growth Indicator Tube (BACTEC MGIT 960 – Becton Dickinson USA) has been introduced for detection of *M.tb* and DST and suitable with all types of clinical specimens except blood. The culture tube contains 7 ml Middle brook 7H9 broth base, to which an enrichment supplement containing oleic acid, albumin, dextrose, catalase (BBL MGIT OADC) is added at the time of inoculation. In addition, an antibiotic mixture of polymixin B, amphotericine B, nalidixic acid, trimethoprim and azlocillin (BBL MGIT PANTA) is added to the culture tube at the time of inoculation of decontaminated samples. The culture tube contains a fluorescent sensor that detects the concentration of oxygen in the culture medium. The level of fluorescence corresponds to the amount of oxygen consumed by the organisms in the inoculated specimens. This, in turn, is proportional to the bacillary load. When the certain level of fluorescence is reached, the instrument indicates that the tube is positive. The test is considered as superior over conventional egg based solid culture method in terms of low turn around time (TAT) and more mycobacterial recovery, however, contamination rate is more (6-8%) as compared to conventional method (4-5%). Sensitivity for detection of mycobcteria with this method is 88 to 97.2% in culture confirmed specimens. The average TAT for the recovery of *M.tb* complex is 10 to 14 days and additional average time 6-10 days for DST results [7],[8]. The test is recommended for DST of both first and second line anti-TB drugs [9]. In addition, it is also suitable to detect mycobacteria other than tuberculosis (MOTT). Every culture obtained by this techniques needs to be subjected for AFB microscopy (ZN staining) and commercially available immunochromatographic assay for identification of *M.tb* complex.

BacT/ALERT 3D system

The BacT/ALERT system (bioMerieux , Durham, NC, USA) is fully automated, nonradiometric system with a revised antibiotic supplement kit designed for the recovery of mycobacteria from clinical specimens and their DST patterns. It is based on the detection of CO_2 released by actively proliferating mycobacteria into the liquid media [10]. Sensitivity for detection of *M.tb* is between 78 to 99%. Digestion and decontamination of clinical samples from nonsterile body sites is required for optimal recovery of *M.tb*. The mean of time detection of growth for *M.tb* and NTM were found as 16.4 and 10.9 respectively [11]

Microscopic observation drug susceptibility assay

It is a liquid culture based diagnostic method in which incorporation of drugs permits rapid and direct DST concomitantly with mycobacterial growth detection [12]. Characteristic cord formation can be seen microscopically at an early stage. Various studies have shown it accurate and sensitive test that is promising for use in high burden countries for early diagnosis of MDR-TB [12], [13],[14]. The only equipments needed to perform the MODS assay are an inverted microscope, tissue culture plate, biological safety cabinet and incubator. This method is now endorsed by WHO for use in the resource-constraint settings as an interim solution to increase detection of TB [15].

Genotypic method

Nucleic Acid Amplification Test (NAAT)

The nucleic acid amplification test (NAAT) is based on polymerase chain reaction (PCR) which provide early identification of *M.tb* and DST results simultaneously [16],[17]. This is advantageous over culture based method in terms of fast results, less chances of contamination and requirement of fewer infrastructures [18]. Because of low TAT it is very useful in early treatment initiation, thereby, reducing the spread of drug resistant strains of *M.tb* [2],[19]. A positive AFB smear microscopy results with a positive NAAT would indicate presence of *M.tb* whereas a positive AFB smear results with negative NAAT would suggests the possibility of NTM [19]. Liquid culture system is more sensitive than NAAT and hence samples with NAAT negative can be tested for culture additionally. Various type of NAAT are endorsed by WHO like Xpert MTB/RIF assay, line probe assay etc.

XPERT MTB/RIF assay

Cepheid GeneXpert MTB/RIF assay, can detect MTB complex and associated rifampicin (RIF) susceptibility status directly from clinical samples using ultrasensitive hemi-nested PCR (by amplifying 81-bp hot spot region of *rpoB* gene of *M.tb*) and molecular beacon technology. It is also called cartridge based nucleic acid amplification test (CBNAAT). It requires minimum handling and training. The analytical limit is 131cfu/ml and yields results within 2 hours. The assay was shown to be 90.3 to 90.4% sensitive (98.3 to 98.7% in smear-positive specimens, 75.0 to 76.9% in smear-negative specimens) and 98.4 to 99% specific in HIV negative culture confirmed pulmonary tuberculosis [20],[21]. Co-infection with

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HIV substantially decreases the sensitivity of microscopy, but did not significantly affect Xpert MTB/RIF performance. Rif – resistant PTB was diagnosed with around 94.1% for sensitivity and 97.0% for specificity. In addition to pulmonary samples, the test is applicable for extra-pulmonary samples as well, however, national program of TB do not recommended to use with urine, stool and blood samples. The performance with samples from extra pulmonary TB patients depends on type of samples. However, overall pooled sensitivity with extrapulmonary samples is 80.4% and specificity was 86.1%. Recently, a new version called Xpert MTB/RIF Ultra has been developed which is considered superior over present version in greater *M.tb* detection rates. Ultra cartridge incorporates two additional molecular target for *M.tb* detection and therefore its performance has improved to detect 16 bacilli per ml sputum sample. The increased sensititivity is not only with cases of paucibacillary TB, such as those of HIV-TB co-infection, but also in pediatric TB, EPTB, and AFB smear-negative TB [22]. Now, NTEP has revised the diagnostic algorithm to allow the CBNAAT in place of microscopy in phased manner especially for diagnosis of presumptive DR-TB cases [23]. Limitation of assay includes requirement of uninterrupted and stable electrical power supply and annual validation of the system, which may pose a problem in rural / remote settings. Storage of cartridges in high-volume settings is a concern given lack of adequate space [24]. Temperature of environment ideally should not be exceeding more than 30 degree Celsius.

Line Probe Assays

Line Probe Assays are DNA strip-based tests that detects *M.tb* complex and provide DST profile through the pattern of binding of amplicons (amplified DNA products) to probes targeting the most common resistance associated mutations to first- and second line anti-TB drugs and to probes targeting the corresponding wild-type DNA sequence [25]. The post hybridization process leads to the development of coloured bands on the test strips detecting probe binding. The tests can be used directly on AFB smear-positive sputum specimens (direct testing) or on culture isolates (Indirect testing). The tests involve three steps: DNA extraction, multiplex PCR amplification, and reverse hybridization. The contamination chances are less associated as compared to phenotypic DST and results can be obtained within 2 to 3 days. In 2008, the WHO endorsed the use of first line LPA, the GenoType MTBDRPlus assay (version1) for rapid detection of MDR-TB [26]. Subsequently the improved version of LPA (referred to as GenoType MTBDRPlus ver.2) has been developed since 2011 with aim to enhance its sensitivity to detect *M.tb* complex, and simultaneous detection of resistant patterns of rifampicin and isoniazid by detecting mutations in the *rpoB*, *katG* and *inhA* gene [25], [27]. Another version to detect resistance to second-line anti TB drugs is also available which detects the mutations associated with fluoroquinolones, and second-line injectable (SLI) anti-TB dugs in RR/MDR-TB. Although, LPA can detect mutations that occurred most frequently in identified resistant strains of *M.tb*, some mutations that confer resistance are outside the regions covered by the test and hence resistance cannot be completely excluded. Therefore, in some cases additional phenotypic DST may be required to provide full assessment. The test has limitations like it cannot be used with specimens of extra-pulmonary tuberculosis and require sophisticated infrastructure.

GenoType MTBDR*Plus* Assay (Ver.2)

It is referred to as first line LPA (FL LPA). The assay identifies mutations in the *rpoB* gene for rifampicin resistance whereas *katG* and *inhA* gene for isoniazid resistance. The test has good performance for detection of RIF resistance in AFB smear-positive sputum samples. The sensitivity, specificity are around 97%, 98%, respectively for detecting rifampicin resistance while it is 94%, 100%, isoniazid resistance, and 95-97%, 99%, for detecting MDR-TB as compared to conventional method [28],[29]. The test can also be used with samples having positive CBNAAT results to know the status of INH susceptibility. Geographical variations in the prevalence of mutations associated with RIF and in particularly INH resistance may result in varying performance of LPA in different geographical settings. The comparatively low performance of detecting INH resistance indicates the presence of some unidentified mutations in other genomic regions (like *ahpC, kasA, furA* etc) which is not targeted by the assay.

GenoType MTBDR*sl*

The initial version of GenoType MTBDRsl can be processed from smear-positive pulmonary samples and culture isolates. The second version (GenoType MTBDR*sl* version 2.0) is even more sensitive and therefore can also be performed using smear-negative pulmonary samples. However, the yield of valid result is low with smear negative samples. The test detects mutations associated with *gyrA* (codon 85-97) and *gyrB* (codons 536-541) of quinolone-resistance determining region for fluroquinolone and resistance determining region of rrs gene for second line injectable drugs (SLID). This test also includes eis promoter region which is responsible for low level kanamycin resistance. The test is suitable for early detection of XDR-TB among patients previously diagnosed with MDR-TB. As compared to phenotypic method, its sensitivity and specificity estimates (95% CI) were 97 to 100% and 98 to 100% respectively for direct detection of resistance to fluoroquinolone; and these were 62% to 89.2% and 91 to 98.5% respectively for second line injectable drugs [30],[31]. Resistance to individual drugs in class of fluoroquinolone cannot be determined by this test. The performance of detecting XDR-TB is also good with high sensitivity (79-100%) and specificity (97-100%) with smear-positive sputum samples. The test could not eliminate the need of conventional phenotypic DST which is necessary to monitor the resistance to additional anti-TB drugs.

Truenat MTB test

This is recent example of the developments in the field of TB diagnostics (developed by Bigtec Laboratories, Bangalore, India). It is a battery-operated, portable chip based nucleic acid test [32], endorsed by WHO to identify *M.tb*. If sample is found to be *M.tb* positive, the Truenat MTB-Rif Dx chip may

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be used as a follow-on test for detection of resistance to rifampicin, using the previously extracted DNA. The results can be printed using a Trulab microprinter. However, analyzer has in-built connectivity allowing for transmission of results via simcard, bluetooth or wifi. It needs skilled and trained technician. The test involves three steps 1. Liquification and cells lysis (sample preparation) using Truprep AUTO MTB sample pretreatment pack, 2. DNA extraction and purification using Truprep AUTO universal cartriadge based sample prep kit and device and,3. Amplification and fluorescent probe-based detection using Truenat chip and Truelab^{TM} Dx real time quantitive micro PCR analyser [33]. The performance of Truenat assays showed comparable accuracy with Xpert MTB/RIF assay for detection of *M.tb* and RIF resistance. Its accuracy is also comparable with TB-LAMP assay as replacement tests for AFB smear microscopy. Considering with Composite Reference Standard (CRS), the sensitivity and specificity for detection of *M.tb* were reported as 91.1% and 100% by [34].

Loop-mediated amplification (LAMP)

It is a novel NAT platform manufactured by Eiken Chemical Co Limited (Tokyo, Japan). The speed of the reaction, no need of thermal cycler, and visual inspection of fluorescence or turbidity of the labeled probe makes LAMP a promising platform for the development of a simple and sensitive tool for the molecular detection of tuberculosis in developing countries. Lamp amplifies DNA with high efficiency under isothermal conditions using six sets of primers [35] targeting the six regions of the *gyrB* and 16S *rRNA* genes. Due to its high amplification efficiency, DNA ~~being~~ amplifies 109-1010 times in 15-60 min [3]. The LAMP detects *M.tb* complex within an hour and can be read with the naked eye under ultra violet light. The pooled sensitivity for detecting pulmonary TB in adult is found higher than smear microscopy ranging from 77.7% to 80.3% (with 95% confident interval), and pooled specificity ranging from 97% to 98% (95% CI). The pooled sensitivity among sputum AFB smear-positive patients at 95% CI ranging from at 95.2% to 96.6%, depending upon the varying reference standard, while its specificity ranged from 90 to 99% [36]. The test can be used as replacement test for conventional microscopy for diagnosis of presumptive pulmonary TB in adults. However, due to inability to provide DST, it cannot replace Xpert MTB/RIF assay. In places where Xpert MTB/RIF assay could not be implemented, it may be considered as an alternative test.

Diagnostic test of latent TB infection

Detection of latent TB infection (LTBI) is very crucial to overcome the probability of its progression to TB disease. Currently there are two methods available for testing of latent TB infection: Tuberculin skin test (TST) and Interferon-Gamma Release Assays (IGRAs). Both depend on the cell mediated immune system (memory T cell response), but none of these two can accurately differentiate between active TB disease and TB infection [1].

Tuberculin Skin Test (TST)

TST is based on delayed-type hypersensitivity reaction (type IV). The test is commonly performed by using mantoux technique by injecting two tuberculin unit (TU) of RT-23 or five TU of purified protein derivatives S (PPD-S) [1]. The report is obtained by measuring induration in transverse diameter. This technique requires trained health care worker and require two visit. Although the test is used widely, it has drawbacks. The specificity of TST decreases in population where BCG vaccination after infancy is widely used and where prevalence of NTM is high. The sensitivity of the test also reduced in case of immunodeficiency (people living with HIV) and malnutrition [37].

Interferon-Gamma Release Assays (IGRAs)

Interferon-Gamma Release Assays (IGRAs) is single visit test. Its specificity is not affected by prior BCG vaccination and certain NTM infection. Two types of IGRAs are available commercially, one is T-SPOT.TB blood test and other is QuantiFERON-TB Gold-in-Tube test (QFT-GIT). Both these test are based on principle that when T cells of infected person restimulated with *M.tb* antigens, will produce cytokine interferon-gamma (IFN-γ). T-SPOT.TB test uses separate mixtures of CFP-10 and ESAT-6 synthetic peptide as *M. tb* specific antigen and measures number of IFN-γ producing cells (peripheral mononuclear cells) through enzyme-linked immunospot (ELISPOT) assay, whereas, QFT-GIT test uses single mixture of CFP-10, ESAT-6 and TB7.7 synthetic peptides and measures IFN-γ concentrations in international unit (IU) per milliliter by taking whole blood.

Limitation of rapid diagnostic techniques

A number of rapid methods and assays have been recently evolved for the detection of *M.tb*, with or without detection of drug resistance. However, rapid methods are not a complete substitute of mycobacterial culture, most are not reliable when used with AFB smear-negative specimens. Therefore it is most tricky in areas where occurrence of smear negative HIV-infected TB patients is high. Confirmation of XDR-TB still sometimes needs culture and DST. Many rapid methods still require appropriate laboratory facilities, an adequate infrastructure, need for continuous electricity, supply chain systems and technical expertise.

Diagnostic strategy and NTEP

The Revised National TB Control Programme now called National Tuberculosis Elimination Programme (NTEP), based on the globally recommended Directly Observed Treatment Short-course (DOTS) strategy, was launched in 1997 and expanded entire country in a phased manner with the target of 85% cure rate and 70% case detection among new sputum positive (NSP) patients. Since then every five years National Strategic Plane (NSP)s have been made by government of India and many huge improvements have been made such as covering of complete geographical area, banning of sero-diagnostic tests, notification by private sector, active case finding, cash incentives for TB patients and nikshay portal development. A laboratory network of LPA and CBNAAT , Truenat, whole genome sequencing (WGS) laboratories, national reference laboratory (NRL), Intermediate reference

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laboratory (IRL), Culture & Drug Susceptibility Testing (C&DST) laboratory, and various district level laboratories have been established, and many are under development [38]. India has set up target with high priority to end TB by 2025, five years ahead of global target.

3. Conclusions

Building capacity of all IRLs and CDST labs under the program is necessary to achieve TB elimination goals. This paper has described several new and established methods as well as some of the issues associated with the techniques. Several of these methods have been endorsed by the WHO and have proven to improve case detection and management of TB patients. There is no single test which can be used for detection of tuberculosis in all patients. We have to depend upon combination of different tests and see how to place them in diagnostic algorithm so as to appropriately use all the available diagnostic modalities taking into account factors such as the capacity of different levels and requirement of infrastructure and man power

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