Review Article

Diagnostic modalities of Tuberculosis- Then and Now

Abstract

Tuberculosis is one of the most ancient diseases known to affect humans and according to WHO remains major cause of death after HIV/AIDS. It remains a global public health problem due to increasing number of undiagnosed and drug resistant cases. Early diagnosis and immediate initiation of treatment is crucial to prevent the extensive spread of this deadly disease. Nowadays advancements in molecular based test imparted better and rapid diagnosis of TB. Several commonly used methods to screen and diagnose TB are clinical, immunological, microscopy, radiography, and bacterial culture. Molecular diagnostic methods including loop-mediated isothermal amplification (LAMP), line probe assays (LPA), GeneXpert, whole genome sequencing (WGS) etc have been employed to diagnose and characterize TB. Here we reviewed use of these currently available and other future promising diagnostic methods along with their sensitivity, specificity, advantage and disadvantage of each method. Although new diagnostic methods have been developed, they are still uncertain and require extensive amount of studies to validate their confirmatory role in the diagnosis of TB. There is increased requirement for biomarker discovery, validation and translation into clinical tools. High-burden countries will need to improve their efficiency of health care delivery and ensure better uptake of new technologies.

Key Words: Mtb, Biomarker, NAATs, Pulmonary

Introduction

Tuberculosis (TB) is one of the major leading infectious disease causing morbidity and mortality worldwide. *Mycobacterium Tuberculosis (Mtb)* is the pulmonary (primarily) pathogen responsible for this deadly disease[1]. Mtb can exist either in metabolically active or inactive (latent) disease state[2]. Sputum smear microscopy and culture of Mtb are the most commonly used diagnostic tests for TB[3]. Despite the efforts of vaccination and newer drugs being developed for this disease, it remains a great concern due to emergence of drug resistance and significant number of undiagnosed TB cases[4]. In accordance with World Health Organization (WHO) TB report 2021, there was a big global drop in new TB case notification in 2020 as compared to 2019. The number of new TB case diagnosed and notified in 2020 was 5.8 million only which was 7.1 million in 2019 and 5.7-5.8 million observed earlier in 2009-2012. Hence, these numbers indicate reversal of previous progress in 2019 to the level of 2012. Therefore, this fall in trend of new TB case notifications necessitates urgent need of early diagnosis[5].

Over the years several new TB diagnostics have been developed including rapid molecular test, radiological, biochemical and immunological assays. Pulmonary TB can be diagnosed by symptoms, chest radiographs, sputum smear microscopy and culture of Mtb[6]. Recent advances in the field of molecular biology and better understanding of molecular mechanism for drug resistance have contributed in rapid diagnosis[7]. WHO 2020 update recommended molecular assays as the initial test to diagnose TB instead of sputum smear microscopy due to high diagnostic accuracy of these assays[8]. Molecular diagnostic tests provide timely results for high quality patient care, low contamination risk and ease of performance and speed. For the purpose of early and easily accessible diagnostics, there is a need of simpler point of care tests. Current point-of-care (POC) or near-to-point of care tests are smear microscopy, GeneXpert (in well-resourced settings), and chest X-ray. Future POC tests might include LAM (Mtb pathogen) and IP-10 (host) biomarker assay, modified NAATs based on isothermal cycling or paper based lateral flow assay[9]. Hence, there are several approaches that are being developed for the diagnosis and have been included in the TB detection program. In the present review, authors have tried to include all the potential markers and their feasible implementation have been discussed in brief.

<u>Early techniques ('Gold Standard')for the TB diagnosis</u>

1. **Sputum smear Microscopy**

Sputum smear Microscopy (SSM) is the primary method for diagnosis of pulmonary TB in the resource-limited settings[10]. Expectorated sputum is stained using varied methods including Ziehl-Neelson, Kinyoun and Auramine staining (fluorochrome). The major disadvantage of SSM is its low sensitivity (22%-80%) depending on Bacilli count and false-positive results. *Fluorescence microscopy* and *front-loaded smear microscopy* (spot-spot microscopy) further facilitated increased sensitivity to sputum smear[11]. Whilst these methods could not efficiently detect TB in children and HIV or immunocompromised patients due to low Mtb count.

2. **Culture of Mtb**

Culture of Mtb is the gold standard for the diagnosis of active TB. Conventional method for culture relies on solid media such as Lowenstein-Jensen medium and Middlebrook agar[12]. Growth in sputum culture usually takes several weeks after incubation, hence it is laborious and time consuming (3-8 weeks) to obtain the results.

Current test for TB diagnosis

1. Culture of Mtb- The liquid medium such as BACTEC radiometric systems and MGIT (Mycobacteria Growth Indicator Tube, fluorescence-based detection system) allowed detection of growth in 9.7 and 20.2 days respectively with additional benefit of automated detection system. Therefore, combination of BACTEC and MGIT proves beneficial as it reduces the growth detection time to 9.9 days[13]. Briefly, the principle of mechanism of these two advancements has been discussed below:
    (a) *BACTEC radiometric system*: It has been used for several for isolation of bacteria. It is based on the measurement of $^{14}CO_2$ produced by the bacteria when it metabolizes ^{14}C labeled palmitic acid present in the liquid media. Several reports have demonstrated higher yield and rapid isolation of Mtb by BACTEC system[14].
    (b) *Mycobacterial growth indicator tubes*: MGIT has Middlebrook7H9 liquid medium along with oxygen quenched fluorochrome embedded in silicone at the bottom of tube. After successful cultivation of Mtb, oxygen is depleted due to which fluorochrome is not inhibited, resulting in fluorescence within MGIT tube when visualized under UV lamp[15]. It is faster than conventional culture methods and provide high degree of sensitivity and specificity

The cost of radiometric system and use of radioisotopes excluded its usage for routine purpose. Various other culture media or systems being developed for the cultivation of Mtb with different sensitivity and time of detection includes:

(i) Bactec MGIT 960: The BACTEC MGIT 960 system is a noninvasive, nonradiometric system that works similar to manual MGIT and the BACTEC 9000 MB system. A ruthenium pentahydrate oxygen sensor embedded in silicon at the bottom of a tube containing 8 ml of modified Middlebrook 7H9 broth fluoresces following the oxygen reduction induced by aerobically metabolizing bacteria within the medium[16].

(ii) Bactec 460 TB: This system is recognized as a reference method for detection of mycobacteria, combining the advantages of liquid media (Bactec 12B) with semi-automation. However, this system uses a radiometric method for the detection of mycobacterial growth[17].

(iii) MB/BacT: It is a well-automated system for the detection of Mtb in clinical specimens without using radioactive reagents. It utilizes a colorimetric sensor and reflected light to continuously monitor the CO2 concentration in the culture medium[18].

(iv) MB Redox: It is a culture system combining a liquid medium and a redox indicator which enables an easy macroscopic colorimetric vision of growth. MB Redox tubes contain an invisible tetrazolium salt which changes from red-to-violet particles when reduced by the growth of Mtb[19].

(v) Thin layer agar (TLA): It allows initial identification of Mtb based on colony morphology being visualized by microscope and with the help of *para*-nitrobenzoic acid in the medium[20].

(vi) Bactec 9000: It is a fully automated nonradiometric method which uses oxygen-quenched fluorescence indicator for the rapid detection of Mtb growth[21].

(vii) VersaTREK: It is based on the detection of pressure changes in the culture medium of a sealed vial during mycobacterial growth[22].

(viii) Microscopic-Observation Drug-Susceptibility (MODS): It relies on faster growth of Mtb in liquid media which can be observed earlier than solid media and also drugs can be incorporated in the same media to study for drug sensitivity[23].

(ix) Bio-FM: This system uses enriched Middlebrook 7H9 medium supplemented with vancomycin, colistin and amphotericin to enhance rapid and selective growth of Mtb. It contains a colored indicator which turns into dark blue color to violet upon positive cultures[24].

The culture of Mtb can also be used to study drug susceptibility for the patients. In 2011, WHO recommended use of non-commercial method for cultivation and ***Drug Sensitivity Test (DST)*** either directly by microscopic examination of growth in media with and without drug or indirectly by *Colorimetric Redox indicators* (Nitrate reduction) [25].

2. **Molecular Test:**

1.1 Nucleic Acid Amplification Tests (NAATs)

i. **Gene XPERT MTB/RIF (CBNAAT):** It is semi-automated nested Real-time PCR for detection of Mtb complex and Rifampicin resistance simultaneously from unprocessed sputum sample. The assay identifies most of the clinically relevant RIF resistance inducing mutations in the RNA polymerase beta (rpoB) gene in the *Mtb* genome using fluorescent probes[26]. Despite several strengths it offers variable and low sensitivity in immunocompromised and smear negative patients respectively.

ii. **Loop-mediated isothermal amplification (TB-LAMP):** It is a manual assay and results are interpreted under UV light easily. It involves use of loop primers which have sequences complementary to single-stranded loop region on the 5'end of hairpin structure. Thus, increasing the number of starting points for DNA amplification. Previous metanalysis study reported that TB-LAMP perform better than sputum smear and thus can be used in replacement of later[27]. But it performs similar to GeneXPERT, so it can be used as additional tool along with it. Inefficiency to diagnose LTBI and contamination risk in molecular biology lab are the majorly the disadvantages of this technique.

iii. **Line Probe Assay (LPA):** LPA belongs to DNA Strip-based test family that determines drug resistance profile of Mtb. Different pattern of amplified DNA fragments binding to probes targeted to resistance associated mutated genes in comparison to wildtype DNA predicts drug resistance. WHO recommended LPA to be used as additional tool with conventional DST[28]. It can rapidly detect resistance for INH and RIF drugs but showed less sensitivity and specificity for smear negative patients.

1.2 Molecular typing

1.2.1 DNA Fingerprinting by PCR RFLP: It is the most commonly used method in study of epidemiology and pathogenesis of TB. It has been used to differentiate strains of Mtb, to define strain clusters within population, to study molecular evolution and delineate the pathogenesis of TB[29].

1.2.2 Spoligotyping: It is based on polymorphism on chromosome locus DR (Direct Repeats) containing short variable repeats interspersed with nonrepetitive spacers[30].

3. **Immunological Diagnosis of TB**

   a. **TB Skin Test (TST):** TST is based on delayed-type hypersensitivity reaction. Mixture of Mtb antigen purified protein derivative (PPD) is injected intradermally and observed cutaneous hypersensitivity to antigen reflects a delayed response to Mtb antigen[31].

   b. **IFN-γ Release Assay (IGRA):** IGRAs are *in vitro* blood tests of cell-mediated immune response which measure IFN-γ released by T-cell release, following stimulation by antigens specific to the *Mtb* complex. The antigens used for stimulation are early secreted antigenic target 6 (ESAT-6) and culture filtrate protein 10 (CFP-10). There are two commercial IGRAs available including QuantiFERON-TB Gold In-Tube (QFT) assay (ELISA) and the T-SPOT.TB assay (ELISPOT)[31]. In addition to IFN-γ, ***IP-10*** *(IFN-γ Inducible protein)* has been reported as biomarker for TB and thus can be used for Mtb identification[32]. Major drawback of IGRAs is its low sensitivity and specificity in HIV/immunocompromised or pediatric patients.

Diagnosis of Latent Tuberculosis Infection (LTBI) and discrimination from active TB infection

Diagnosis and treatment of LTBI is one of the strategies recommended by the WHO to control TB disease worldwide[33]. In most individuals, initially *Mtb* infection is sustained by host defenses, the infection either remains latent or cleared, so that individual is asymptomatic and noninfectious. However, latent infection has the potential to develop into symptomatic active disease at any time. Identification and treatment of LTBI can reduce the risk of development of this disease[34]. In 2004, WHO approved only three test for identification of LTBI which included (i) TST and two IGRA assays (ii) QuantiFERON TB gold and (iii) T-SPOT.TB. C-TB test is a new advance method developed which used ESAT6 and CFP-10 antigens instead of PPD. Previous study reported that this test performed better than TST in BCG vaccinated population and had high relativity with QuantiFERON TB-Gold assay[35]. Therefore early and specific diagnosis of LTBI is sill dependable on newer research for LTBI biomarker.

c. **Lipoarabinomannan (LAM) ELISA**: LAM test is the only available and approved test to be done in urine samples for TB patients. Mtb has a unique cell wall with multiple lipid-based molecules. LAM is the major component of this cell envelope and accounts for 15% bacterial mass. It is one of the non-invasive and rapidly detecting methods useful in immunocompromised/HIV patients who are seriously ill[36].

4. **Radiological Test**

Chest X-ray can be primary radiological tool to evaluate suspected or proven pulmonary TB. Radiological presentation of TB may be variable but in many cases is quite characteristic. Treatment management and follow-up of these patients is also performed by Chest X-Ray and is extremely valuable for monitoring complications. In addition

Chest CT (Computed Tomography) is required sometimes to study fine lesions and assess bacterial activity by observing branching opacities[37].

5. **Non-microbiological Test**

Adenosine Deaminase (ADA) is an important enzyme in purine catabolic pathway which increases in TB because of T-Cell activation by mycobacterial antigens. It has been reported to be widely present in body fluids and serving in diagnosis of TB when negative smear staining is obtained. Specifically ADA has been found to be increased in tuberculous pleural effusion (TPE)[38]. As diagnosis of TPE is difficult due to low sensitivity of direct microscopy and culture, ADA proves to be a promising marker.

Future promising techniques for TB diagnosis

1. **Digital droplet PCR (ddPCR):** It is a third generation PCR which enables absolute gene quantification (exact nucleic acid targets) rather than relative one. This method is capable of detecting single copy of DNA[39]. It offers a higher sensitivity than qPCR and can be used to identify Mtb in sputum[40] and blood samples[41]. Thus ddPCR might be used as additional tool for the diagnosis of Mtb from pathological samples. The major drawback for ddPCR is prohibitively expensive and will require uninterrupted power supply.
2. **CRISPR-MTB**: The diagnostic power of CRISPR has been specified in the detection of viral infections. In a study, authors have developed a rapid CRISPR based assay for identification of Mtb and evaluated it in various clinical samples of patients who were part of a retrospective cohort study. This research highlighted the significance of CRISPR for both pulmonary and extrapulmonary TB[42]. Future extensive multi-centric research is necessary to confirm its utilization for clinical diagnosis. It is a culture free,

highly sensitive and specific method with rapid turnaround of less than 1.5 hour[43]. In spite of these advantages, non-specific targeting is feasible and efficacy for HIV or pediatric samples has not yet been established.

3. **Next-Generation Sequencing (NGS) Techniques**: In comparison to phenotypic testing, NGS provides detailed nucleotide sequence of multiple gene regions or whole genomes of interest. Sequencing information allows screening of these genomes for resistance conferring mutations. *Drug susceptibility testing (DST)* can be attained either via targeted NGS (tNGS) or whole genomic-sequencing (WGS)[44]. Currently targeted NGS approaches such as Deeplex-Myc TB assay are mainly focused due to their reliability and availability. It is a culture free multiplexed technique identifying large number of Mtb strains and provides drug resistance for 15 drug profile. But longer turnaround time and special molecular set up with expensive sequencing equipment make it difficult for limited-resources setting.

4. **MicroRNA (miRNA) detection**: miRNAs are small non-coding RNAs known to regulate the expression of genes post-transcriptionally involved in shaping immune responses. Also recent studies have established that innate immune response against Mtb is regulated by various miRNAs. The differential expression of miRNAs in TB can indicate about disease progression and further distinguish between latent and active TB infection. The different miRNAs upregulated in TB disease progression including miR-26-5p, miR-2-5p, miR-33, miR-155-5p etc function as inhibitor for innate immunity, inflammation and apoptosis thus evading host immune response[45]. miRNAs are easily detected in blood samples[46], therefore pointing towards its utilization in pediatric and

HIV patients (hard-to-diagnose). This technique might contribute variability in results and effective miRNA for TB diagnosis is yet to be proved as biomarker.

5. **Volatile organic compounds (VOCs) breathing test (Biosensor)**: In spite of well established highly sensitive diagnosis methods, there is a need of point-of-care and hand held approaches such as breath test using an automated device. There are limited studies reporting the use of electronic nose in screening of TB utilizing VOCs, hence this approach require adequate validation. This method is non invasive and highly portable with very less turnaround time of 10 mins but its sensitivity and specificity is still questionable[47].

6. **Raman spectroscopy**: It is a non-destructive technique which does not require Mtb cultivation. It detects the unique molecular fingerprints of bacteria when excited with certain wavelength. Conclusive research in larger sample size is required to implement this technique in diagnosis of TB[48].

7. **Artificial Intelligence (AI)**: AI is the area of computer science that helps in development of tools that can mimic human like thought processing, reasoning and self - correction abilities. A computer-aided detection (CAD) system is the need of the day for screening and diagnosis of TB and other lung diseases using chest x-rays[49]. It will help to diminish the human error in result interpretation as well as workload on pathologists. Sputum smear image database has been developed. This database can be used to generate algorithms and thus can assist in developing methods for automated microscopy[50]. Despite of several advancements, this technology requires study on large sample size and also it has been shown vary widely depending on the population being

used. Hence AI is being developed by several companies in the world and under consideration by WHO as tool that can help combat TB.

The sensitivity and specificity parameters of all the diagnostic methods have been discussed in table 1. Many studies have been performed for developing a rapid and sensitive method for TB Diagnosis and several are in queue for approval. The methods are needed to be designed in such manner that TB is diagnosed at earliest and at initial presentation of symptoms so that patients do not fall under category of ‘diagnostic defaulters’.

Conclusion

Present review discussed several well-established as well as under-debate diagnostic approaches for TB. The sputum smear microscopy and Mtb culture remain the gold standard for TB cases identification. As these approaches are time-consuming and cannot be used for pediatric and immunocompromised patient samples, newer molecular methods have been added in TB diagnostics strategy. For new method being developed commercial or in-house, our final concern should be that they are being evaluated in well designed clinical trials and tested in high-endemic, limited-resources system, where the implementation is critically essential for the improvement of tuberculosis control.

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Table 1: Accuracy of Various Diagnostic Tools for Tuberculosis

S No.	Diagnostic Method	Sensitivity	Specificity	WHO Approval
1.	Sputum Smear Microscopy	34-80%	97-98%	√
2.	Mtb Culture	80-93%	98%	√
3.	Tuberculin Skin Test (TST)	48-78%	57-81%	√
4.	Radiological Methods	92%	63%	√
5.	Nucleic Acid Amplification Test (NAAT)	80%	98-99%	√
6.	Gene XPERT	82-88%	96-98%	√
7.	Line Probe Assay (LPA)	95.6-97.5%	98.7-99.5%	√
8.	TB-LAMP (Loop-Mediated Isothermal Amplification)	85.6-92.6%	91-96%	√
9.	IFN-γ Release Assay (IGRA)	61-86%	57-81	√
10.	TB-LAM (Lipoarabinomannan) ELISA	13-93%	87-99%	√
11.	Adenosine Deaminase (ADA)Test	83.3%	66.6%	√
12.	Digital droplet PCR (ddPCR)	61.5%	98%	Future-Promising
13.	CRISPR/Cas (Clustered Regularly Interspaced Short Palindromic Repeats Cas system) Based Test	79%	98%	Future-Promising
14.	Volatile Organic Compounds in Breath Test	93%	93%	Future-Promising
15.	Whole Genome Sequencing	>95%	>95%	Future-Promising
16.	Raman Spectroscopy	84-86%	65-89%	Future-Promising
17.	MicroRNA (miRNA) detection	24.7-39.9%	>90%	Future-Promising
18.	Artificial Intelligence (AI)Processing	68-96%	72-85%	Future-Promising