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, 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 2019 was 7.1 million which reduced to 5.8 million in 2020 comparable to cases (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 [5]. The observed decline in TB cases diagnosis might be attributed to COVID-19 pandemic situation worldwide (one of the plausible causes). During this period patients were not able to approach healthcare centers for disease diagnosis and following treatment. Other instances might include time-consuming and costly diagnostic tests available to the patients [6]. Therefore, this fall in trend of new TB case notifications necessitates urgent need of early diagnosis.

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 [7]. Recent advances in the field of molecular biology and better understanding of molecular mechanism for drug resistance have contributed in rapid diagnosis [8]. 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 [9]. Molecular diagnostic tests provide timely results for high quality patient care, low contamination risk and ease of performance and speed. In low-income and middle-income country settings (LMICs) use of molecular test is limited. Although GeneXpert labs in African countries increased from zero to a large number since its discovery in 2010 [10]. A report suggested cost of GeneXpert in routine healthcare is comparable to AFB staining if number of test performed through GeneXpert increases. Therefore scaling-up of NAATs in

LMICs is preferably required to increase lab-confirmed TB cases [11]. 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 [12]. Hence, there are several approaches that are being developed for the diagnosis and have been included in the TB detection program.

Detection of Pulmonary TB (PTB) and Extra-Pulmonary TB (EPTB) by any of diagnostic method requires appropriate clinical sample and detailed knowledge regarding compatibility, accuracy of sample with target diagnostic test to be used. Sputum sample collected either expectorated or induced is the foremost specimen used for PTB and body fluids are the preferrable sample for EPTB. The plausible specimens for both PTB and EPTB include body fluids i.e. pleural, pericardial, spinal, synovial, ascitic, bone marrow and pus; blood; tissue (lymph node, tissue biopsies); urine; gastric lavage; bronchial alveolar lavage etc. Most of the EPTB samples are paucibacillary in nature. Although these different clinical specimens can be used with diagnostic methods discussed in present review, their efficiency might be altered [13,14,15,16].

In the present review, authors have tried to include all the potential markers and their feasible implementation have been discussed in brief.

EARLY TECHNIQUES ('GOLD STANDARD') FOR THE TB DIAGNOSIS

1. SPUTUM SMEAR MICROSCOPY

Sputum smear Microscopy (SSM) is the primary method for diagnosis of pulmonary TB in the resource-limited settings [17]. Briefly, sputum sample is processed by mucolytic agent NALC (N-acetyl-L-cysteine) and NaOH for decontamination of bacteria other than mycobacteria [18]. Expectorated sputum is stained using varied methods including Ziehl-Neelson, Kinyoun and Auramine staining (fluorochrome). Both viable and non-viable acid-fast bacilli (AFB) are stained and counted in AFB examination using standard scoring system published by WHO/IUATLD and CDC-USA. A smear is considered positive with bacilli count of 10^4 per ml or greater. 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 [19]. Whilst these methods could not efficiently detect TB in children and HIV or immunocompromised patients due to low Mtb count.

2. CULTURE OF MTB

Bacteriological cultures can provide a definitive diagnostic of TB, hence it is the gold standard for the case finding of active TB. The primary advantage over SSM is its higher sensitivity allowing detection of very low number bacilli (☐10 bacilli/ml). Besides diagnosing at early stage, culture is also used to identify treatment failures, EPTB, species identification and drug susceptibility test. Conventional method for culture relies on solid media such as Lowenstein-Jensen medium and Middlebrook agar [20]. All cultures inoculated should be examined after 48-72 hours to detect gross contaminants. Thereafter these shall be monitored weekly upto 8 weeks. As growth in sputum culture usually takes several weeks after incubation, it is laborious and time consuming to obtain the results.

CURRENT TEST FOR TB DIAGNOSIS

1. ***Culture of Mtb***- WHO recommends expanded use of liquid culture systems even in resource-constrained settings. 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. Combination of BACTEC and MGIT proves beneficial as it reduces the growth detection time to 9.9 days [21]. Briefly, the principle of mechanism of these two advancements has been discussed below:

   (a) *BACTEC radiometric system*: It has been a commonly used method of bacteria isolation for several years. 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 of culture. Hence it detects the presence of mycobacteria based on metabolism rather than visible growth which provides an automated system. Several reports have demonstrated higher yield and rapid isolation of Mtb by BACTEC system [22].

(b) *Mycobacterial growth indicator tubes*: MGIT has Middlebrook7H9 liquid medium along with oxygen quenched fluorochrome embedded in silicone at the bottom of tube. The medium is enriched by addition of ODAC (Oleic acid, Dextrose, Albumin and Catalase) and PANTA (Polymyxin B, Amphotericin B, Nalidixic Acid, Trimethoprim and Azlocillin) used as antimicrobial mixture. After successful cultivation of Mtb in tubes, oxygen is depleted due to which fluorochrome is not inhibited, resulting in increased fluorescence within MGIT tube when visualized under UV lamp [23]. 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 [24]. Thus, growth of mycobacteria increases fluorescence in tube. Therefore, automated MGIT 960 has advantages of shorter-time and easy result interpretation with high-throughput.

(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. Specimens are cultured in liquid media containing ^{14}C labelled palmitic acid and PANTA antibiotic mixture Radioactive CO_2 released is measured by BACTEC system. However, this system also uses a radiometric method for the detection of mycobacterial growth [25].

(iii) *MB/BacT*: It is a well-automated system for the detection of Mtb in clinical specimens without using radioactive reagents. It utilizes specialized Liquid Emulsion Sensors (LES) present at the bottom of culture bottle which changes

color visibly. This change in color is due to decrease in pH after release of CO_2 produced by growth of mycobacteria. Increase in reflectance of light depicts CO2 concentration in the culture medium [26].

(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 is reduced by mycobacterial redox system to a pink-, red- and violet-colored formazan [27]. These formazan particles are accumulated on cell surface in a granular form and growing microcolonies appear as colored particles.

(v) *Thin layer agar (TLA)*: TLA is a non-commercial drug susceptibility test (DST) which allows initial identification of Mtb based on colony morphology being visualized by microscope and with the help of *para*-nitrobenzoic acid (PNB) in the medium (Middlebrook 7H11) [28]. Mtb can be differentiated from non-tuberculous mycobacteria (NTM) as PNB inhibits growth of Mtb.

(vi) *BACTEC 9000*: It is a fully automated, nonradiometric method with rapid and highly sensitive (92.8%) recovery of mycobacteria. It is a fluorescence- based method which uses modified Middlebrook broth supplemented with antibiotic mixture and nutrients. It utilizes oxygen-quenched fluorescence indicator for the rapid detection of Mtb growth [29].

(vii) *VersaTREK*: It is based on the detection of pressure changes in the culture medium of a sealed vial during mycobacterial growth [30]. This system uses glass bottles for culture which contain cellulose sponge to provide unique growth matrix, thus increasing the contact surface for Middlebrook 7H9 broth supplemented with nutrients and antibiotic mixture. The decrease in pressure due to O_2 consumption is measured by nanometer.

(viii) *Microscopic-Observation Drug-Susceptibility (MODS)*: MODS is a simple, rapid, low cost test for TB and MDR-TB. MODS assay is performed using Middlebrook broth supplemented with ODAC and PANTA. Mtb growth is easily detected in

liquid media and eliminates need of waiting for appearance of macroscopic colonies in media. In addition, drugs isoniazid and rifampicin can be incorporated in the same media to simultaneously detect MDR-TB [31].

(ix) *Bio-FM*: This system uses enriched Middlebrook 7H9 medium supplemented with vancomycin, colistin and amphotericin (VCA) to enhance rapid and selective growth of Mtb. It contains a chromogenic indicator which turns from dark blue color to violet upon positive cultures [32].

In 2011, WHO recommended use of non-commercial method for cultivation of Mtb and *Drug Sensitivity Test (DST)* is done either directly by microscopic examination of growth in media with or without drug as mentioned above. Another method used for analyzing culture for DST is a Biochemical test, Nitrate Reduction Assay (NRA) using *Colorimetric Redox indicators* [33]. It utilizes the ability of mycobacteria to reduce nitrate to nitrite. In NRA, inoculum of Mtb is incubated with broth containing nitrate which is reduced to nitrite by nitrate reductase produced by bacteria. The nitrite generated is detected by the presence of red precipitate formed after reaction of nitrite with sulfanilic acid and α-napthylamine.

WHO endorsed and recommended use of non-radiometric liquid culture for TB diagnosis in LMICs. Previous study carried out in Ethiopia laboratory services, showed improved recovery rate of MTB by MGIT system (liquid-media) as compared to solid LJ media [34]. The BACTEC MGIT 960 system has significant shorter turnaround time for both smear positive and negative sputum specimens, gives higher yield and has relatively high cost. Also, it requires measure to reduce the contamination rate and false positive results in high burden TB countries. Minimizing contamination risk could be a key to cost-effectiveness.

2. **MOLECULAR TEST:**

2.1 NUCLEIC ACID AMPLIFICATION TESTS (NAATS)

i. ***Gene XPERT MTB/RIF (CBNAAT)*:** It is semi-automated cartridge based nested Real-time PCR for rapid detection of Mtb complex and Rifampicin (RIF) resistance simultaneously from 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 [35]. This system integrates and automates sample processing, nucleic acid amplification and target sequence detection. It provides results within 2 hours. 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. This method depends on auto-cycling strand displacement DNA synthesis. 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 review reported that TB-LAMP perform better than sputum smear microscopy and thus can be used in replacement of later [36]. But its performance is similar to GeneXpert, so it can be used as an additional tool along with it. Inefficiency to diagnose LTBI and contamination risk in molecular biology lab are 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. It involves three basic steps of DNA extraction, PCR amplification and reverse hybridization. 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 [37]. It

can rapidly detect resistance for INH and RIF drugs but showed less sensitivity and specificity for smear negative patients.

2.2 MOLECULAR TYPING

2.2.1 *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, molecular evolution and delineate the pathogenesis of TB [38]. IS6110-based (Insertion-sequence) typing is widely applied genotyping method in molecular epidemiological studies *M. tuberculosis* [39].

***2.2.2 Spoligotyping*:** It is the most frequently used PCR-based approach for studying phylogeography of Mtb complex. It relies on polymorphism at chromosome locus DR (Direct Repeats) containing short variable repeats interspersed with nonrepetitive spacers [39].

The limited access and affordability to high-quality TB diagnostic tests in most LMICs illustrates major barrier to patients for quality and early treatment. GeneXpert (NAATs) is one such efficient diagnostic which has been endorsed in LMICs by WHO. Reliance on smear microscopy is replaced by Xpert MTB/RIF at local clinics of LMICs [40]. A survey conducted on the use of rapid test for TB in South Africa demonstrated more than 80% health care workers used Xpert MTB/RIF as a major tool for diagnosis both in public and private sectors. The Xpert MTB/RIF provides rapid and accurate diagnosis within 2 hours only [41]. The cost analysis of Xpert MTB/RIF in sub-Saharan African countries revealed that its cost is decreased with volume of test conducted and hence confirming it as cost-effective diagnostic tool [42]. In addition to this, GeneXpert has been recommended to be employed as point-of-care test even in LMICs [43]. Another type of NAATs available in limited-resources settings is TB-LAMP. In comparison to GeneXpert, TB-LAMP could be equipped in

peripheral laboratories in LMICs as it does not require specialized infrastructure and expertise training. Although TB-LAMP costs are considerably lower than GeneXpert, the lack of ability to detect drug-resistant TB (DR-TB) and ongoing scale up efforts for GeneXpert may limit cost-utility of TB-LAMP in particularly high prevalence DR-TB settings [44]. While sensitivity of TB-LAMP is lower than that recommended by WHO, it can perform better than conventional smear microscopy in diagnosis of MTB among presumptive TB patients. In high DR-TB prevalence countries, proper patient treatment is delayed due to slow process, financial, infrastructure, human resources requirements for conventional culture-based DST and its widespread implementation might be challenging in LMICs. Considering this, WHO had recommended use of LPA for rapid screening of MDR-TB in LMICs [45]. Multiplex PCR in LPA has the potential to substantially reduce the turnaround time of DST results. However, a study conducted in Uganda established LPA as an appropriate tool for rapid screening of MDR-TB in the reference laboratory settings [46].

3. IMMUNOLOGICAL DIAGNOSIS OF TB

i. ***TB Skin Test (TST) or Mantoux test*:** 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 [47] . Antigen is injected intradermally on ventral surface of forearm and interpreted after 48-72 hours of administration by trained health-care worker. This test is widely used around the world due to its low cost and straightforward implementation.

ii. ***IFN-γ Release Assay (IGRA)*:** IGRAs are *in vitro* blood tests of cell-mediated immune response which measure IFN-γ released by T-cell, following *in-vitro* stimulation by mycobacterial antigens. 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) [48]. QFT-GIT contains long peptide derived from ESAT-6

and CFP-10 to induce specific CD4 T-cell response and shorter peptides in another tube to induce IFN-γ production by CD4 and CD8 T-cells (specific for discriminating LTBI). In comparison to QFT-GIT, T-SPOT. TB assay require expensive reader, software and trained personnel to interpret T-SPOT.TB results. 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 [49]. 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 [50]. 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 [50]. 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 [51]. Therefore, early and specific diagnosis of LTBI is sill dependable on newer research for LTBI biomarker.

These two test TST and IGRA used for identification of latent TB patients are still under-utilized in LMICs due to several factors including costs, infrastructure, shortage of products such as PPD antigen and IGRA assay kits with consumables. Although TST is the commonly and preferably used method in limited-resource settings (Sub-Saharan Africa), a key barrier associated is follow-up-visit for result interpretation [52]. As per meta-analysis, studies revealed that pooled prevalence of positive TST and IGRA test results in LMICs and high-income countries were 61% and 25% respectively. In a survey analysis conducted in 2020, authors have reported varied

implementation of IGRA assay in high burden countries in which few reported its inclusion in NTP (National TB Program), whereas some countries incorporated in national policy but not NTP. Additionally, there are countries which do not recommend IGRA in epidemiological context [52]. Besides several benefits of IGRA assay including single visit of patient, no booster phenomenon, effective results in prior BCG vaccinated population, it is not completely implemented for LTBI screening in LMICs. The major hurdles confronted by health care facilities other than mentioned-above are insufficient capacity and quality-maintained (temperature sensitive) transport of specimen, and limited laboratory personnel in LMICs.

iii. ***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 lipopolysaccharide present in 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 [53]. Commercially available LAM test has been recommended by WHO since 2015 to assist in diagnosis of TB in people with HIV. Besides this, its uptake in high burden countries has been observed to be slow. Ease of LAM assay procedure, low cost, and reduced result time makes it a point-of-care test. In a survey conducted in high TB and HIV burden countries (2020) out of 31 countries, 46% adopted commercial LAM with 21% currently using the test and 61% were planning to implement in national policy in near future [54]. Hence scaling-up of LAM assay in high burden LMIC setting is crucial to control TB cases.

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 [55]. CT is a preferred imaging method for lymphadenopathy, bronchogenic spread and abdominal TB. CNS TB and tuberculous spondylitis are detected using magnetic resonance imaging (MRI) [56].

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. ADA has very limited diagnostic value in PTB and hence not recommended for its confirmation. On other hand, it is quite useful for EPTB along with other clinical correlation [57]. Specifically, ADA has been found to be increased in tuberculous pleural effusion (TPE) [58]. 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)*****:** ddPCR method is a third generation PCR which depends on water-oil emulsion droplet technology. The sample is fractionated into 20,000 droplets and PCR amplification occurs in individual droplets. Thus, it enables absolute gene quantification (exact nucleic acid targets) rather than relative one. This method can detect single copy of DNA [59]. It offers a higher sensitivity than qPCR and can be used to identify Mtb in sputum [60] and blood samples [61]. 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. A rapid CRISPR based assay was developed for identification of Mtb and evaluated in various clinical samples of patients who were part of a retrospective cohort study. This research indicated CRISPR-MTB as a highly sensitive, promising technology for *in-vitro* diagnosis of both pulmonary and extrapulmonary TB [62]. 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 [63]. 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) [64]. 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 include miR-26-5p, miR-2-5p, miR-33, miR-155-5p etc which function as inhibitor for innate immunity, inflammation and apoptosis thus evading host immune response [65]. miRNAs are easily detected in blood samples [66], 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)***: It is based on array of sensors diagnosing disease from the pattern of VOCs produced either by Mtb or host metabolism due to TB which would be different from standard conditions. Despite well-established highly sensitive diagnosis methods, there is a need of point-of-care and handheld 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 requires

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 [67].

6. ***Raman spectroscopy***: It is a non-destructive technique and optical method which does not require Mtb cultivation. It detects the unique molecular fingerprints of bacteria when excited with certain wavelength. They are ideally suitable for identification of microorganism at species and strain level. In a previous study, Raman spectroscopy was reported as promising, rapid and accurate technique for identification of clinically relevant Mycobacterium species [68]. Conclusive research in larger sample size is required to implement this technique in diagnosis of TB [69].
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 system is the need of day for screening and diagnosis of TB and other lung diseases using chest x-rays [70]. 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 [71]. Despite of several advancements, this technology requires study on large sample size and in multiple populations. Hence AI is being developed by several companies in the world and is under consideration by WHO as tool that can help combat TB.

The clinical research trials are still ongoing in Asian [72,73,74,75] and African [76,77,78,79] LMICs to study the sensitivity and specificity of these future-promising techniques with the purpose of including them into national diagnostic methods.

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.

CONFLICT OF INTEREST

Authors have no conflict of interest to declare. All co-authors have seen and agree with the contents of manuscript and there is no financial interest to this review.

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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