REVIEW

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Pulmonary Tuberculosis Diagnosis: Where We Are?

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In recent years, in spite of medical advancement, tuberculosis (TB) remains a worldwide health problem. Although many laboratory methods have been developed to expedite the diagnosis of TB, delays in diagnosis remain a major problem in the clinical practice. Because of the slow growth rate of the causative agent *Mycobacterium tuberculosis*, isolation, identification, and drug susceptibility testing of this organism and other clinically important mycobacteria can take several weeks or longer. During the past several years, many methods have been developed for direct detection, species identification, and drug susceptibility testing of TB. A good understanding of the effectiveness and practical limitations of these methods is important to improve diagnosis. This review summarizes the currently-used advances in non-molecular and molecular diagnostics.

Keywords: Tuberculosis; Diagnosis; Tuberculosis, Multidrug-Resistant

Introduction

Tuberculosis (TB), a major global health issue, is one of the leading infectious causes of death and remains to be a global health problem despite strenuous efforts to eradicate it[1]. In order to improve patient outcomes and decrease TB transmission, accurate and rapid detection of TB, including smear-negative TB and drug resistant–TB, have a critical role. As TB case detection is still dependent on the microscopic smear examination of Ziehl-Neelsen (ZN) or auramine stained specimens, specificity, and culture, early diagnosis is essential for optimal control, which generally provides results in at least 2–3 weeks[2].

Diagnostic methods for *Mycobacterium tuberculosis* (MTB) have witnessed major improvements over the past decades such as nucleic acid amplification test (NAAT) and other methods now make rapid and sensitive detection at the clinical level possible[3,4]. Rapid diagnosis results in reducing TB-related morbidity and mortality rates as well as risk of person-to-person transmission. The advent of molecular methods for the detection of *M. tuberculosis* has reduced the required time for diagnosis methods; diagnosis by conventional culture requires several weeks[5]. Recently, many methods have been introduced to meet such needs and several other methods are now available for the detection of *M. tuberculosis*. Therefore, in this review, we discuss the advanced techniques for the detection of *M. tuberculosis* and resistance detection methodologies.

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Microscopy and Staining Methods

The primary method for TB diagnosis in low- and middle-income countries is the detection of acid-fast bacilli (AFB) using smear microscopy, namely ZN stain, since it is simple, cheap, and quick and also provides results only within hours[6]. However, the sensitivity of this test ranges from 20% to 60%[7].

Fluorescence microscopy with fluorochrome staining and light emitting diode technology improves the sensitivity of TB detection by nearly 10% compared with the conventional staining[7].

Culture Methods

The method of identification on culture media with growth in a solid or liquid medium is still considered the reference method for the diagnosis of *M. tuberculosis*. Traditionally, cultures used solid media such as Lowenstein-Jensen medium that provided results between 4 and 8 weeks and, additionally, cultures required equipped and specialized labs and highly skilled staff. Liquid culture systems using automated systems, such as BACTEC radiometric method (BACTEC-460), MB/BacT system, and mycobacterial growth indicator tube (MGIT) are even more sensitive and can detect growth in 1–3 weeks along with detecting 960 samples simultaneously[8]. The MB/BacT system (bioMerieux, Durham, NC, USA), a non–radiometric, fully automated, continuously monitoring liquid culture system, uses advanced colorimetric detection of carbon dioxide reduction and a sophisticated computer algorithm[9]. MGIT is based on the fluorescence detection of mycobacterial growth in a tube containing a modified Middlebrook 7H9 medium together with a fluorescence quenching-based oxygen sensor embedded at the bottom of the tube. As TB grows, free oxygen is utilized and replaced with carbon dioxide. The MGIT technology has a potential of yielding results within 4–13 days[10].

Molecular Methods in TB Diagnosis

The introduction of NAAT has been one of the major developments in the diagnosis of *M. tuberculosis* and real-time polymerase chain reaction (PCR) has been recently proposed for detection[11]. Over the last few years, new molecular methods have been introduced, which include PCR–restriction fragment length polymorphism, real-time PCR, DNA sequencing, and DNA strip assays as mycobacterial diagnostic tools, leading to considerable improvement of both speed and accuracy for identification. In 2009, Centers for Disease Control and Prevention (CDC) have recommended the use of at least one molecular technique per patient for *M. tuberculosis* detection[12].

1. Nucleic acid amplification

1) Cobas MTB test

CobasAmplicor MTB assay has been available since the 1990s and is one of the most widely utilized molecular tests for *M. tuberculosis* detection. The CobasAmplicor MTB assay for the direct detection of *M. tuberculosis* complex (MTBC) in pulmonary specimens is popular in developing and developed countries and recognized as a rapid and sensitive NAAT[13,14]. It has been reported that the overall sensitivity of Amplicor test (compared with culture) for respiratory specimens is 79.4%–91.9%, the specificity is 99.6%–99.8%, the positive predictive value is 92.6%–96.6%, and the sensitivity for smear negative specimens is somewhat lower, 40.0%–73.1%[15].

Roche Diagnostics (Taipei, Taiwan) has introduced a new system (CobasTaqMan MTB test), based on real-time PCR technology, to replace the CobasAmplicor MTB assay. The CobasTaqMan MTB test has recently replaced the well-established CobasAmplicor MTB assay which has been proven highly specific[16]. This method is a real-time PCR-based kit using TaqMan hydrolysis probes and primers that binds to a specific, highly conserved region of the *Mycobacterium* genome containing the gene for 16S rRNA. The CobasTaqMan MTB assay, which includes two major steps of DNA extraction and PCR amplification, is conducted according to the manufacturer's instructions[17]. Studies have shown that the CobasTaqMan MTB assay is at least as sensitive as its predecessor, the CobasAmplicor MTB system[18,19]. Commercial assay such as the CobasAmplicor MTB assay or CobasTaqMan MTB assay has been widely used in clinical mycobacteriology laboratories for direct specimen detection of MTBC DNA in the past 20 years[20,21]. These assays could significantly reduce the diagnosis time from weeks to hours, which can greatly improve patient care and infection control.

2) Abbott RealTime MTB automated assay

Recently, Abbott Molecular (Des Plaines, IL, USA) has introduced an automated real-time MTB PCR assay for the *in vitro* diagnostic purpose. This *in vitro* diagnostic (CE-IVD) marked assay includes an automated extraction step, which can manage a maximum of 96 specimens in a single batch and real-time PCR-based amplification that specifically targets two MTBC genes, the insertion sequence6110 (IS6110) and protein antigen b in a single reaction. The presence of multiple copies of IS6110 gene in some MTBC can certainly increase the diagnostic sensitivity of the assay[22,23]. The automated Abbott assay requires only very short manual handling time (0.5 hours), which could help improve the laboratory management. In a recent study, the overall estimates for the sensitivity and specificity of the Abbott RealTime MTB assay were both 100% among smear positive specimens, whereas the smear negative specimens were 96.7% and 96.1%, respectively; these sensitivity and specificity rates were high compared with Amplicor test for the smear negative specimens[24]. The new Abbott RealTime MTB assay has good diagnostic performance which can be a useful diagnostic tool for rapid MTBC detection in clinical laboratories.

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3) Amplified Mycobacterium Tuberculosis Direct

Amplified Mycobacterium Tuberculosis Direct test (AMTD; Gen-Probe, San Diego, CA, USA) was the first molecular assay to be approved by the Food and Drug Administration (FDA) for testing respiratory specimens, regardless of whether they were smear-positive or smear-negative[25]. The AMTD assay is an isothermal (42°C) transcription-mediated amplification method in which the target (mycobacterial 16S rRNA) is amplified by DNA intermediates. The overall sensitivity for respiratory specimens was found in the range of 90.9% to 95.2% and the specificity between 97.6% and 100%[26]. Also, Bergmann et al.[27] reported that the overall test sensitivity in respiratory specimens (compared with culture and clinical diagnosis) ranged from 85.7% to 97.8% and was shown to be higher for smear-positive specimens (91.7% to 100%), while dropping to between 65.5% and 92.9% in smear negative specimens.

4) BD ProbeTec ET TB System

The BD ProbeTec ET (DTB) is a semi-automated real-time system that allows simultaneous amplification of a gene sequence and detection of *M. tuberculosis*. Its amplification target is the mycobacterium-specific insertion sequence IS6110[28]. The literature has reported that DTB assay is a highly sensitive and specific technique, which gives an accurate result from a clinical specimen within 3 to 4 hours; its rate of sensitivity ranges from 98.5% to 100% for smear positive samples, but it is very variable (0.33%–100%) for smear-negative ones. On the other hand, this system is not yet approved by the FDA[29,30].

2. Molecular tests with drug susceptibility

1) Xpert MTB/RIF assay

In early 2011, World Health Organization (WHO) endorsed a novel, rapid, automated, with molecular beacon technology to detect DNA sequences amplified in a hemi-nested real-time PCR assay that can simultaneously detect *M. tuberculosis* and rifampicin (RIF) resistance. WHO has advocated universal use of Xpert MTB/RIF (Cepheid, Sunnyvale, CA, USA) for the diagnosis of TB. In the same multiplex reaction, five different hybridization probes are used; each nucleic acid probe is complementary to a different target sequence within the *rpoB* gene of RIF susceptible MTB and is labeled with a differently colored fluorophore[25]. Xpert MTB/RIF (Cepheid) tests are expensive, but marketed as point-of-care (POC) tests and require virtually no training and laboratory infrastructure. They are also designed for the rapid and simultaneous detection of *M. tuberculosis* and mutations associated with RIF resistance usually within 2 hours[26]. WHO has issued initial recommendations on Xpert MTB/RIF, especially for the individuals suspected of having multidrug-resistant (MDR)-TB[31]. This method has higher sensitivity for TB detection in smear-positive patients than in smear-negative patients; nonetheless, this test may be valuable as an add-on test following smear microscopy in the patients previously found to be smear-negative[32]. Recently, International Standards for TB Care have recommended that Xpert MTB/RIF and/or sputum cultures should be performed in the patients suspected of having pulmonary TB, who have negative sputum smears[33]. And also, a recently updated WHO policy has recommended that a repeated Xpert MTB/RIF test on a fresh specimen can be useful when Xpert MTB/RIF detects *M. tuberculosis* with RIF resistance in the patients considered to be at low risk of MDR-TB[34].

A recent review including 27 unique studies and involving 9,557 participants has reported Xpert MTB/RIF pooled sensitivity, in negative-smear microscopy result as 67% (95% credible interval [CrI], 60% to 74%) and pooled specificity of 99% (95% CrI, 98% to 99%; 21 studies, 6,950 participants). For smear-positive, culture-positive TB, Xpert MTB/RIF pooled sensitivity is 98% (95% CrI, 97% to 99%); also, for RIF resistance detection, Xpert MTB/RIF pooled sensitivity is 95% (95% CrI, 90% to 97%) and pooled specificity is 98% (95% CrI, 97% to 99%); compared with smear microscopy, Xpert MTB/RIF increases TB detection among culture-confirmed cases by 23% (95% CrI, 15% to 32%)[32].

2) Line probe assay

Line probe assay (LiPA) is NAAT that simultaneously detects infection with *M. tuberculosis* and amplifies the regions of drug resistance. LiPA can simultaneously identify MTBC and detect genetic mutations in the *rpo*B gene region related to RIF resistance that can be completed in about 12 hours[35]. RIF resistance in *M. tuberculosis* strains is conferred by a diverse group of mutations within a hypervariable region of *rpo*B gene, which codes for a beta subunit of RNA polymerase and more than 95% of RIF resistant isolates possess mutations within this hypervariable region[36]. A meta-analysis has summarized the results obtained for the LiPA RIF TB test and showed that LiPA has high sensitivity and specificity when *M. tuberculosis* isolates from culture are used in this studied 12 out of 14 studies in which the applied LiPA to isolates has sensitivity of greater than 95%, and 12 out of 14 have the specificity of 100%. The four studies that have applied LiPA directly to clinical specimens have 100% specificity and the sensitivity between 80% and 100%[35]. Despite their value as rapid and accurate diagnostic tools, tests based on the LiPA technology are not yet used in developing countries because of their high cost and the assumption that such sophisticated tests cannot be implemented in low-resource settings; on the other hand, LiPAs are not currently FDA-approved.

3) GenoType MTBDR*plus* assay

The GenoType MTBDR*plus* (Hain Lifescience, Nehren, Germany) assay is a commercially available assay that combines the detection of MTBC with the prediction of resistance

to both RIF and isoniazid (INH), thus can detect MDR-TB. In this assay, a multiplex PCR is followed by the hybridization of the DNA amplicons to membrane-bound probes and is the newer version of the GenoType MTBDR assay, which does not have the ability to detect INH resistance. The assay combines the detection of MTBC with that of the most common resistance mutations in *rpoB* (RIF resistance), *katG*, and *inhA* gene (INH resistance)[37,38]. The new GenoType MTBDR*plus* achieves better sensitivity for INH resistance (92% vs. 88.0% of the previous version). This assay is approved for use with the specimens growing in culture media and also for use with smear-positive sputum samples. The previous and new versions of the assay could correctly identify RIF-resistance in 98.7% of the cases, when compared with the conventional susceptibility testing[39]. MTBDR*plus* assay has good sensitivity and specificity with the turn-around time of less than 48 hours and may be a useful tool for the rapid detection of multidrug resistant TB in a tertiary care center. In India, a study showed that the sensitivity and specificity for the detection of resistance to RIF was 100% and 97.3%, and to INH was 91.9% and 98.4%, respectively, when GenoType MTBDR*plus* assay was used in comparison with the phenotypic drug susceptibility testing (DST)[40].

Other Resistance Detection Methodologies

1. Drug susceptibility test in liquid medium

The BACTEC 460TB system (Becton Dickinson Biosciences, Sparks, MD, USA) has been widely validated since several years ago in clinical laboratories for reliable and rapid testing of susceptibility of *M. tuberculosis* isolates to frontline drugs such as streptomycin, INH, RIF, ethambutol, and pyrazinamide, in accordance with the recommendations by CDC. Afterwards, BACTEC MGIT960 system, a developed nonradiometric, fully automated, continuous-monitoring system (Becton Dickinson Biosciences), has been introduced as an alternative to the radiometric BACTEC 460TB system[41,42]. Many studies have been now published on the application of MGIT960 and BACTEC 460TB systems for the rapid detection of resistance to the first and second line anti-TB drugs. For example, results of several studies have demonstrated that BACTEC 460 and BACTEC MGIT 960 systems are accurate methods for the rapid testing of the susceptibility of *M. tuberculosis* to the first and second line drugs[43,44].

2. Microscopic observation drug susceptibility culture

In 2010, WHO Strategic and Technical Advisory Group for TB endorsed and recommended a new policy on the use of noncommercial culture and DST methods[45]. Of these, the microscopic observation drug susceptibility (MODS) assay was recommended for screening patients suspected of having MDR-TB. It is based on the observation of the characteristic cord formation of *M. tuberculosis* that is visualized microscopically in liquid medium with the use of an inverted microscope. The MODS assay for the detection of TB and MDR-TB, directly from sputum, relies on three principles: first, *M. tuberculosis* grows faster in liquid medium than in solid medium; second, characteristic cord formation can be visualized microscopically in liquid medium at an early stage; and third, the incorporation of drugs permits rapid and direct drug-susceptibility testing concomitantly with the detection of bacterial growth[46]. The MODS assay is faster than the conventional proportion method performed on solid culture medium and is less expensive than the commercial liquid culture methods and molecular line-probe assays. The technique needs no more than 7 days for obtaining DST results. In Ethiopia, MODS detection of MDR-TB was excellent with the sensitivity and specificity of 95% and 100%, respectively, when compared with the 960/MGIT system[47]. Tests in several laboratories have demonstrated high concordance for INH (97%), RIF (100%), and fluoroquinolones (100%) compared with the reference standard techniques[47].

Mass Spectrometry Method

1. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry

The identification of bacteria by mass spectrometry dates back to the 1970s, where researchers first described the use of mass spectrometry to identify bacteria[48]. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) is a new technology for the routine identification of bacteria in clinical microbiology laboratories and this use of MALDI-TOF MS makes the diagnostic process approximately 24 hours shorter[49]. This method is a novel tool for rapid and reliable identification of microorganisms by the analysis of protein profiles from either disrupted cells or intact bacterial cells[50]. Also, MALDI-TOF MS is used to detect the probable proteins or oligonucleotides related to resistance based on the specific peptide mass fingerprinting in protein database, and also the drug susceptibility of *M. tuberculosis* can be resolved[51]. Some authors have used MALDI-TOF MS for the identification of mycobacteria and showed that it has the potential as a rapid and reproducible platform for the identification and typing of *Mycobacterium* species and detection of antibiotic resistance, but the global experimental data are still quite limited. However, detection of drug resistance by mass spectrometry is still at the initial stages; MALDI-TOF MS is a powerful, rapid, precise, and cost-effective method for the identification of intact bacteria compared with the conventional phenotypic techniques or molecular biology. In

some studies, MALDI-TOF MS combined with weak cationic exchange (WCX) magnetic beads has been used; e.g., such a method was applied in an investigation on patients with active TB and non-TB people. This model can discriminate patients with active TB from those with non-TB with the sensitivity of 98.3% and specificity of 84.4%. The test set is used to verify the performance, which demonstrates good sensitivity and specificity of 85.7% and 83.3%, respectively[52]. Thus, MALDI-TOF MS combined with WCX magnetic beads could serve as a diagnostic tool for active TB.

Interferon-Gamma Release Assays

Interferon (IFN)-γ release assays (IGRAs) are *in vitro* immune tests that have been introduced in recent years as an alternative to the tuberculin skin test (TST) for the diagnosis of latent tuberculosis infection (LTBI) based on detection of a T-cell immune response to the TB antigens (ESAT-6 and CFP-10), absent from most environmental mycobacteria and bacillus Calmette–Guérin (BCG), are of particular interest. IGRAs have excellent specificity that is unaffected by BCG vaccination[53]. Although IGRAs are intended for diagnosing LTBI, they have a potential role in supporting the diagnosis of active TB. Nevertheless, IGRAs cannot efficiently distinguish LTBI from active TB. In a recent meta-analysis, Chen et al.[54] showed that IGRAs had limited accuracy in diagnosing active TB in the human immunodeficiency virus (HIV)-infected patients. While IGRAs are recommended for the diagnosis of LTBI in many high-income countries, they are not recommended by WHO as a TST replacement for LTBI diagnosis in low- and middle-income countries[55]. Use of IGRAs to diagnose active pulmonary TB is particularly problematic. This concern equally applies to the use of TST for active TB. Nevertheless, in many high-burden countries, there is growing concern that IGRAs (and, to a lesser extent, TST) are being used off-label, particularly in the private sector, for the diagnosis of active TB[56,57].

There are two commercially available platforms that measure IFN-γ production following *ex vivo* antigen stimulation[58]. Two IGRAs, QuantiFERON-TB Gold In-Tube test (QFT-G-IT) and its predecessor the QuantiFERON-TB Gold (QFT-G) test and T-SPOT.TB (Oxford Immunotec, Oxford, UK), are now commercially available and their use is expanding[59]. In the QFT-G-IT and QFT-G test, the amount of IFN-γ released into the supernatant is quantified using an enzyme-linked immunosorbent assay (ELISA), whereas, in T-SPOT.TB test, the number of IFN-γ producing T cells is measured by ELISPOT[60]. In a meta-analysis, Diel et al.[61] showed that the overall specificity of IGRAs was 98%–100% in latent TB infection. In another meta-analysis, the evidence of QFT-G and T-SPOT.TB was directly evaluated on blood or extrasanguinous specimens for the diagnosis of active TB and it was found that, in blood and extrasanguinous fluids, the pooled sensitivity for the diagnosis of active TB was 80% (95% confidence interval [CI], 75% to 84%) and 48% (95% CI, 39% to 58%) for QFT-G, and 81% (95% CI, 78% to 84%) and 88% (confirmed and unconfirmed cases) (95% CI, 82% to 92%) for T-SPOT.TB, respectively[62].

Urine Lipoarabinomannan Assay

Lipoarabinomannan (LAM) is a structurally important 17.5-kD heat-stable glycolipid found in the cell wall of *M. tuberculosis*, which is a potential marker of active TB[63]. Detection of LAM antigens in urine has several potential advantages over the currently-used diagnostics. With its potential to be used as a simple POC test, lack of biosafety concerns, and use of a noninvasive, convenient patient specimen, the LAM assay is fast-tracked for commercial development. Urine samples are simple to collect and store. There are far fewer infection control concerns compared with sputum. Urine is a particularly attractive specimen in young children, who are often unable to produce sputum. Based on early data, a prototype urinary LAM detection test was produced by Chemogen Inc. (Portland, ME, USA) and a commercial version of this test is now marketed as the Clear view TMTB ELISA (Alere Inc. [formerly, Inverness Medical Innovations Inc.], Waltham, MA, USA)[64], which now supersedes a pre-commercial prototype (MTB LAM ELISA Test; Chemogen Inc.) first tested in 2005 in Tanzania[65].

HIV/TB co-infected children might benefit from LAM-based tests to aid early TB diagnosis and subsequent positive impact on morbidity and mortality. A systematic review and meta-analysis showed that, five studies that assessed accuracy in clinical and confirmed TB cases, sensitivity ranged from 8% to 80%, while specificity ranged from 88% to 99%. In five studies with results stratified by HIV status, sensitivity was 3%–53% higher in HIV-positive than HIV-negative subgroups; sensitivity was the highest with advanced immunosuppression[66]. In a recent study, the assays' sensitivity was higher in HIV-positive versus HIV-negative children: 70% (95% CI, 35%–93%) versus 13% (0%–53%) for MTB-LAM-ELISA and 50% (19%–81%) versus 0% (0%–37%) for determining TB-LAM. In 35 children (27%) with excluded active TB, both assays showed the specificity of 97.1% (85%–100%)[67]. Although highly variable diagnostic accuracy has been observed in different clinical populations, it is now clear that this assay has useful sensitivity for the diagnosis of HIV-associated TB in the patients with advanced immunodeficiency and low CD4 cell counts[68].

Conclusion

Improvement in the identification of TB spectrum, including active, drug-resistant strains and also latent TB, has the potential to have a profound effect on global health. For ex-

ample, a test with 85% sensitivity and 97% specificity in the identification of those with TB could prevent 392,000 deaths per year (22% of global TB deaths)[69]. Novel methods allow for the rapid diagnosis of active TB in the patients with negative sputum smears for AFB and enable prompt, highly accurate identification of drug-resistant strains of *M. tuberculosis* directly from respiratory specimens[70]. Recently, rapid isolation and detection of *M. tuberculosis* have improved the diagnosis of TB. Molecular techniques represent an important contribution to the detection of *M. tuberculosis* and and there are several advantages about molecular methods, such as reducing detection time, because they can provide results in a matter of hours, increasing case detection, rapid detection of mutations associated with drug resistance like Xpert MTB/RIF method with an impact on increasing case detection and reducing time to treatment. Despite the clear advantages that molecular methods have for detection and DST, they have some disadvantages and some systems are not yet approved by FDA. For example, rapid detection like molecular methods is mainly useful in smear-positive cases; in smear-negative patients, the amplification tests are recommended only when TB is suspicion high and always in relation to clinical data. Despite the clear advantages that molecular methods offer for DST, they all suffer from the problem that the genetic basis of resistance is not fully understood for any TB drug for all, so the test results must be always confirmed by phenotypic methods.

High cost of molecular tests is another disadvantage. Molecular methods have been developed for use in laboratories and clinics, but as yet little is available to assist early case finding in the community, because molecular techniques are of course considerably more expensive than traditional culture methods using either liquid or solid media. Although molecular methods are useful in the rapid detection of *M. tuberculosis*, methods like MALDI-TOF MS can be revolutionary techniques for the routine identification of *M. tuberculosis*, because this technique allows for easier and faster diagnosis of *M. tuberculosis* and may play a role in identifying mycobacterial species isolated from clinical samples, being faster than sequencing and hybridization-based techniques[71]. Furthermore, other methods like MODS assay offer a simple, rapid, economical, and feasible method for the detection of *M. tuberculosis* resistance to the first and second line drugs in resource-limited settings[60]. On the other hand, clinicians who manage patients with suspected TB should ensure that their diagnostic practices align with the guidelines for TB and use sputum smear microscopy and culture to investigate adult patients with suspected active TB[70].

At present that many of these techniques are only economically viable in the developed nations, it is hoped that recent advances will lead to the development of novel diagnostic strategies applicable to use in developing nations, where the burden of TB is maximum and effective intervention is most urgently required. Thus, more attention and research are needed to develop effective and cheap methods in different settings to overcome this worldwide problem and more evidence is required to assess the operational impact of non-commercial, low-cost, rapid diagnostic methods in field conditions.

Conflicts of Interest

No potential conflict of interest relevant to this article was reported.

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