

Revisiting the methods for detecting *Mycobacterium tuberculosis*: what has the new millennium brought thus far?

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Abstract

Tuberculosis (TB) affects around 10 million people worldwide in 2019. Approximately 3.4% of new TB cases are multidrug-resistant. The gold standard method for detecting *Mycobacterium tuberculosis*, which is the aetiological agent of TB, is still based on microbiological culture procedures, followed by species identification and drug sensitivity testing. Sputum is the most commonly obtained clinical specimen from patients with pulmonary TB. Although smear microscopy is a low-cost and widely used method, its sensitivity is 50–60%. Thus, owing to the need to improve the performance of current microbiological tests to provide prompt treatment, different methods with varied sensitivity and specificity for TB diagnosis have been developed. Here we discuss the existing methods developed over the past 20 years, including their strengths and weaknesses. In-house and commercial methods have been shown to be promising to achieve rapid diagnosis. Combining methods for mycobacterial detection systems demonstrates a correlation of 100%. Other assays are useful for the simultaneous detection of *M. tuberculosis* species and drug-related mutations. Novel approaches have also been employed to rapidly identify and quantify total mycobacteria RNA, including assessments of global gene expression measured in whole blood to identify the risk of TB. Spoligotyping, mass spectrometry and next-generation sequencing are also promising technologies; however, their cost needs to be reduced so that low- and middle-income countries can access them. Because of the large impact of *M. tuberculosis* infection on public health, the development of new methods in the context of well-designed and -controlled clinical trials might contribute to the improvement of TB infection control.

INTRODUCTION

State of the art

Methods for detecting *Mycobacterium tuberculosis*, the aetiological agent of tuberculosis (TB), in a given sample might have respectable sensitivity (i.e. the ability to detect the presence of bacillus in the specimen) and specificity (i.e. the ability to detect only the target bacillus). There are numerous commercial serological tests for the diagnosis of TB in many settings; however, they are not recommended because of their poor performance. Although we have acknowledged them, we have not revisited in depth any current immunological test for detecting *M. tuberculosis*

infection. Moreover, serological tests were found to be inconsistent and inaccurate, with widely varying values for sensitivity and specificity and high proportions of discordance. Hence, the World Health Organization (WHO) issued a guideline not recommending the use of such tests for the diagnosis of TB [1]. On the one hand, the only *in vivo* test available to evaluate *M. tuberculosis* infection is the tuberculin skin test, which has fair sensitivity but poor specificity [2]. On the other hand, the new interferon-gamma release assays are specific *ex vivo* tests. Both methods are based on the measurement of adaptive host immune response. However, none of these tests can accurately distinguish

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Abbreviations: AFB, acid-fast bacilli; AST, antimicrobial susceptibility testing; CPC, cetylpyridinium chloride; EMB, ethambutol; FCM, flow cytometry; FDA, fluorescein diacetate; FM, fluorescence microscopy; INH, isoniazid; LAMP, loop-mediated isothermal amplification; LJ, Löwenstein-Jensen; LMI, low- and middle-income; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; MS, mass spectrometry; MTD, *M. tuberculosis* direct test; NAAT, nucleic acid amplification tests; NTM, nontuberculous mycobacteria; qRT-PCR, Real Time Quantitative PCR reverse transcription; RFLP, restriction fragment length polymorphism; RMP, rifampicin; RT-PCR, reverse transcription polymerase chain reaction; SM, streptomycin; TB, tuberculosis; WGS, whole-genome sequencing; WHO, World Health Organization; ZN, Ziehl-Neelsen.

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Table 1. Commonly used methods for the detection of pathogenic mycobacteria

Assay	Reference
Polymerase chain reaction (PCR)*	[149]
Reverse transcription (RT)-PCR	[88, 150]
Enzyme-linked immunosorbent assay (ELISA)	[151]
Potentiometric biosensors	[152]
Surface plasmon resonance	[153]
Bioluminescence	[154]
Fluorescent labelling	[155]
Flow cytometry (FCM)	[156]
Transcriptomic	[115]

*Including TB-LAMP [84].

between latent and active TB [2–4]. Other diagnostic tools have been developed for the detection of *M. tuberculosis*, as well as drug susceptibility and viability, which can be evaluated by metabolic activity responsiveness (detection of respiration or mRNA synthesis), cell membrane integrity, or nucleic acid detection [5]. Along with these tests, conventional solid and new liquid media-based methods, which can obtain rapid results, have been developed; however, these tests are quite expensive [6]. Other methods have also been described for the detection of pathogenic mycobacteria (Table 1).

The aetiological agent of TB

Approximately 50 mycobacterial species cause human diseases. *M. tuberculosis* belongs to the family *Mycobacteriaceae* and is a member of the *M. tuberculosis* complex that includes *M. tuberculosis*, *M. canettii* and *M. africanum*, as well as other members that cause disease in other animal species (Fig. 1). All bacilli belonging to the family *Mycobacteriaceae* have a lipid-rich cell wall that confers resistance against chemotherapeutic agents but not against physical agents such as ultraviolet radiation and heat [7–10].

The gold standard for diagnosing TB is a positive *M. tuberculosis* culture [11]. *M. tuberculosis* is a rod-shaped, non-spore-forming and strictly aerobic bacterium. As a facultative intracellular pathogen, it is capable of living in human phagocytic cells. Because of its low growth rate, cell division occurs every 18–20 h, and it takes several weeks for the bacterium to be detected as visible colonies in solid culture media [12]. Importantly, this could be considered to be a major obstacle for rapid diagnosis.

The method of *Mycobacterium* sp. characterization from culture is more sensitive than ordinary bacilloscopy, such as the acid-fast bacilli (AFB) microscopy, which allows for the detection of 10–100 bacilli ml⁻¹ of a concentrated clinical sample [13]. The two most widely used culture media are the egg-based Löwenstein–Jensen (LJ) slopes and the Middlebrook series of agars (7H10 and 7H11), which are both solid-phase broths [14]. Among the two, LJ is more efficient for the detection of growth rate, whereas Middlebrook promotes faster bacterial growth [15]. Liquid culture media are used rationally to both increase the number of cells and store

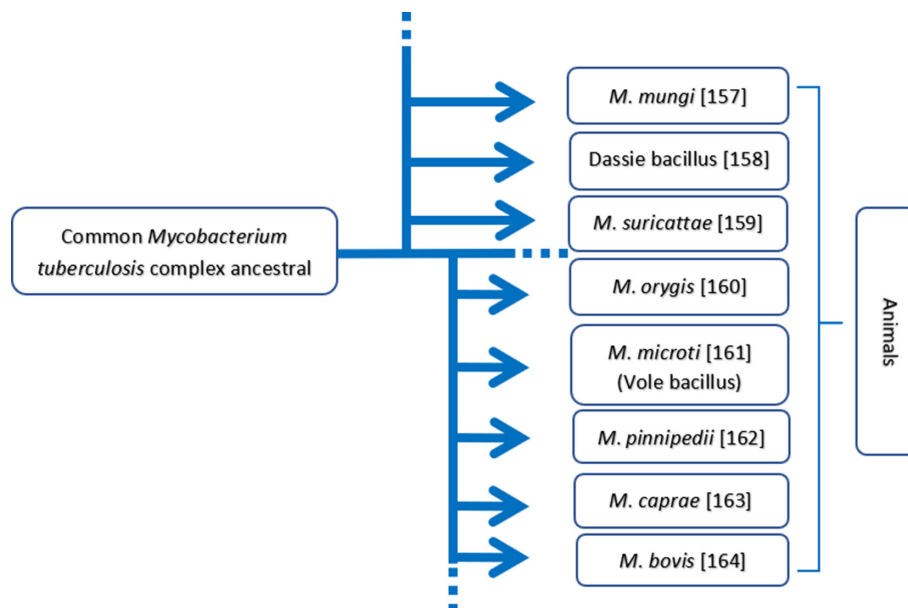


Fig. 1. Genealogical tree assembling a few members of the *Mycobacterium tuberculosis* complex causing disease to various animal species. The relevant references are shown alongside [157–164].

strains. The use of biphasic cultures in the same bottle enables more accurate analysis of the colony aspect [16]. In the case of samples obtained from co-infected TB/HIV patients, which usually have a high incidence of nontuberculous mycobacteria (NTM), species must be identified via biochemical tests or the use of specific genetic probes [17].

Following accurate diagnosis, patients must immediately begin TB chemotherapy with specific agents; however, as indicated earlier, drug resistance and compliance at this stage are a major concern. The inefficacy of treatment has been strongly related to the selection of mutant bacilli that are resistant to standard TB therapy as resistance to every anti-mycobacterial agent used is often caused by spontaneous mutations of *M. tuberculosis* in the target genes [18]. The two resistance mechanisms observed in *M. tuberculosis* are (i) overexpression of the drug target [19] and (ii) alteration of the drug target structure [20]. As indicated, a quicker diagnostic process, including the identification of resistant strains, is needed to initiate the most efficient therapeutic regimen. There are numerous target genes that can be potentially used to detect the resistant forms, such as *katG*, *inhA*, *mabA* and *ahpC* for isoniazid (INH), *rpoB* for rifampicin (RMP), *rpsL* and *rrs* for streptomycin (SM), *embA* and *embB* for ethambutol (EMB), and *girA* for fluoroquinolones. These genes can also be used to detect *M. tuberculosis* in clinical samples using molecular approaches [21].

Since the increase in the number of cases of treatment failure in active TB cases, the development of fast, reliable, simple and accurate methods for identifying the *Mycobacterium* sp. and its drug resistance has become paramount. Therefore, the detection of *M. tuberculosis* using a myriad of approaches has succeeded in the past 20 years. Because of a lack of review papers on this subject, we aimed to introduce the main tests developed in the current millennium and currently used for the detection of this pathogen in clinical samples and discuss their weaknesses and strengths.

DETECTION OF *M. TUBERCULOSIS*

Microscopic analysis

With regard to bacteriological analysis, the detection of AFB in fresh, stained smears of sputum of suspected patients examined microscopically provides initial evidence of the presence of mycobacteria in clinical specimens. In low- and middle-income (LMI) countries, diagnosis is made primarily via microscopic examination, but this method only has a sensitivity of 50–60% in cases of confirmed (bacillary) pulmonary TB and even lower sensitivity (< 30%) in HIV-positive or immunosuppressed patients and in children [22]. However, it is inexpensive, easy to perform and analyse, has a short time frame (1 day) and is correlated with the infectiousness of the case [23, 24]. In short, the traditional AFB is a method referred to for bacteria that are resistant to acid discoloration after staining procedures such as the Ziehl–Neelsen (ZN) and Kinyoun techniques. This property is due to the lipid component, which represents approximately 60% of the dry weight of the cell wall. The same lipid-rich structure is responsible

for both the slow growth and the resistance of bacteria to acids [25].

Among several factors related to the virulence of *M. tuberculosis*, the lipid profile of the cell wall has drawn much interest owing to its unique composition, which is implicated in giving the pathogen an advantage over the host [26]. This lipid-rich cell wall is a dynamic structure that is also involved in the regulation of the transport of anti-tuberculosis drugs [27]. In fact, *M. tuberculosis* also alters its fatty acid metabolism to survive the host conditions; this is reflected in a different cell wall composition in terms of lipids, thereby increasing its virulence. Furthermore, this profile has been shown to modulate the immune responses launched by the host [26]. Upregulated expression of the isocitrate lyase gene, indicative of a shift in the central carbon metabolism, has been observed in *M. tuberculosis* cultured on long-chain fatty acids in lipid-loaded macrophages [28]. Upregulation of lipid storage in this bacillus as a means to recover from reductive stress-induced damage was shown to result in a slower growth rate and a drug-tolerant phenotype in the lipid-rich *in vivo* milieu in a macrophage environment [28]. Two recent broad reviews on this fascinating topic have been published [26, 27].

Before the development of new diagnostic methods, mycobacteria were detected in morning sputum samples via direct microscopy using the ZN technique [21]. However, this method has serious drawbacks (Table 2). First, the time required for adequate detection is crucial; expert technicians take approximately 5 min to observe at least 200–300 microscopic fields in only 1 smear. This leads to exhaustion under the microscope (reading fatigue) and consequently to false-negative results; moreover, in overworked laboratories, the recommended time for analysis might not be followed [29, 30]. Second, this method has poor sensitivity: in 45% of patients with pulmonary TB and 75% of patients with EP-TB, mycobacteria are mostly not detected because of the minimum number of bacilli per sputum sample (10^4 ml⁻¹) required [31]. Lastly, specimens should be transported rapidly to the facility to avoid overgrowth of other contaminants. The cetylpyridinium chloride (CPC) method is widely used for the transport of sputum specimens; however, the detection of AFB via ZN staining can be significantly reduced in specimens preserved by CPC [32]. In addition, specimens treated with CPC should be preferentially inoculated in egg-based media as agar-based media have insufficient neutralizing activity for this quaternary ammonium compound. Sodium carbonate has been found to be a better preservative for sputum specimens for AFB smear microscopy and culture [33]. Nevertheless, aside from the weakness related to this method, a recent study has documented that DNA can be extracted from ZN smears and RMP resistance markers can be evaluated by a single polymerase chain reaction (PCR), namely nested PCR [34], thereby allowing more precise analysis of the sample, which is relevant for multidrug therapy. In a systematic review, the sensitivity of microscopy compared with that of culture ranged from 0–100% in induced sputum samples; only 8 of 23 studies reported on the species of mycobacteria isolated in culture [35]. Notably, in settings with a high level

Table 2. Overview of the most used methods to detect *M. tuberculosis* and its variants including cost-effectiveness

Assay	Accessibility/cost	Sensitivity	Quantification	Turnaround time*	Resistance identification
Bacilloscopy	High	Low	Intermediate	2–3 days	No
Solid culture	Cheap	Low	Intermediate	30–60 days	No
Liquid culture	Intermediate	Intermediate	Intermediate	15–30 days	No
Flow cytometry	Low	High	High	2–3 days	Yes
Nested PCR/RT-PCR	Low	Intermediate	Low	2–4 days	Yes
qRT-PCR	Low	Low	Intermediate	2–4 days	No†
GeneXpert MTB/RIF	Low	High	High	90 min	Yes
Fluorescence microscopy	Intermediate	High	High	1–2 days	Yes

*Time to detect the presence or absence of *M. tuberculosis*, according to the Foundation for Innovative New Diagnostics (FIND).

†Except for EP-TB [92].

of *M. tuberculosis* infection, the ZN technique is the cheapest method [36].

Fluorescence microscopy (FM) is another option to detect *M. tuberculosis* in a given sample. The use of auramine as a fluorescent marker was introduced during the 1940s [37], and the sensitivity of direct microscopy can be improved by concentrating the sputum in a sediment and applying auramine-O fluorescence staining, although this is not sufficient to distinguish *M. tuberculosis* from other mycobacteria [21]. In 2003, Kivihya-Ndugga and colleagues [38] compared the efficiency and cost-effectiveness of FM with those of the ZN method for the analysis of the sputum of patients with pulmonary TB. When considering cost-effectiveness, FM has been shown to have better sensitivity (78% vs 60% for ZN), a key factor leading to savings for both the healthcare system and the patient [38]. A summary of these findings is presented in Table 2. In fact, the use of FM is promising when compared with the poor sensitivity of light microscopy for children with TB [39]. It has been found to have poor performance for the identification of smear-negative TB in HIV-positive patients [40–43]. The increased sensitivity of FM compared with that of traditional light microscopy for the detection of pulmonary TB has recently been supported in a systematic review [44]. Additionally, a meta-analysis has found that FM might increase the sensitivity of sputum smears by 10% compared with the conventional method [45]. However, the equipment required for FM is expensive; thus, its use has been limited to regions that can afford it. In addition, the fluorescence fades with time. For this reason, the slides must be read within 24 h after development [39].

Fluorescent markers are particularly useful for *in vitro* and *in vivo* studies. They may be critical for understanding *M. tuberculosis* biology and disease progression as well as the development of new technologies for diagnosis and treatment, such as dyes used for flow cytometry analysis (as discussed below). A decade ago, a new near-infrared fluorogenic substrate for an endogenous mycobacteria enzyme was developed. This method using mycobacterial β -lactamase-sensitive

compounds is capable of faster detection *in vitro*, with a limit of 6×10^2 colony-forming units, as well as in the murine lung, with a limit of approximately 1×10^4 colony-forming units [46]. However, sputum may interfere during the detection of this enzyme, increasing the probability of false-positive results. Hence, it has been suggested that unknown β -lactamase present in the clinical samples might be cleaved by the fluorogenic substrate because the hypothetical enzyme could have a similar active site to that of the *M. tuberculosis* enzyme [47].

Solid and liquid media for culture

The gold standard recommended by the WHO for the diagnosis of TB is the use of the culture method and the identification of species based on their physiological and biochemical features as well as the time of culture growth [11]. In biological specimens, sputum has invariably been used for that purpose.

The introduction of prokaryotic cell culture techniques has enabled both clinical and research laboratories to identify the *Mycobacterium* sp. and its susceptibility to antibiotics, leading to more efficacious treatment for patients with TB (Table 2). Culture is much more sensitive than the prior microscopic examination: 50% of the pulmonary TB cases and an even greater proportion of documented EP-TB cases are negative under microscopy and are therefore only diagnosed via culture, as this method is capable of detecting few bacteria per millilitre [48, 49]. As stated previously, the traditional methods to determine the viability and growth of the *Mycobacterium* sp. are employed using solid agar media such as Middlebrook 7H10 or LJ; the latter has been employed for the culture of *M. tuberculosis* in LMI countries. Ogawa medium is another egg-based medium that is comparable to LJ in terms of its composition. It is more affordable because of the replacement of asparagine with sodium glutamate, an amino acid that is more readily available and much cheaper [50]. Nevertheless, in these solid culture media, it takes 6 weeks for *M. tuberculosis* to be readily detected by culture [51, 52].

In 2007, the WHO endorsed the use of liquid culture media as a gold standard for TB diagnosis based on the recommendations of international experts and studies that demonstrated that this method can be implemented in LMI settings to improve multidrug-resistant (MDR) TB diagnosis and AFB smear-negative pulmonary TB detection. The WHO's recommendation is consistent with a large body of scientific literature regarding liquid culture media. Studies have reported a higher rate of mycobacteria isolation in a short time frame when compared with the same specimens from solid culture media [53]. Hence, the latter have been ruled out from the clinical laboratory routine [54].

Currently, numerous liquid-based media for culture with automated incubation and reading methods are available, and several studies with regard to their use for antimicrobial susceptibility testing (AST) have been revised [6, 55]. Among methods involving liquid culture media, there are radiometric-based methods, such as the Becton Dickinson BACTEC 460 system [56], and colorimetric methods based on bacterial gas production, such as the bioMérieux MB/BacT System [57]. The BACTEC 460 procedure is based on the production of radioactive carbon dioxide from palmitic acid [58]. It is well established and extensively used for AST; it is now considered to be a standard method [59, 60]. Unfortunately, this method has drawbacks, such as logistics regarding radioactive waste disposal and the requirement for needle inoculation of specimens into the liquid culture medium. To overcome these problems, a nonradiometric colorimetric system was developed, namely, the BACTEC Mycobacteria Growth Indicator Tube (MGIT) 960, which seems to be more reliable than the BACTEC 460 for AST [61–64]. This method has the advantage of being fully automated. An oxygen-quenching fluorescent sensor is used for detection, and it eliminates the need for needles to be used. Another colorimetric system is the MB/BacT, which has been compared with the BACTEC 460 [59]. Strikingly, the results for AST produced good agreement and concordance with the values for INH (96.3%), RMP (98.8%) and EMB (98.8%) [65].

A study by Sorlozano and colleagues [66] comparing BACTEC MGIT 960 vs MB/BacT and the LJ solid culture media revealed 86.5% mycobacterial recovery from BACTEC MGIT 960 in clinical samples compared with approximately 79.5% mycobacterial recovery from the two others. The study also revealed that the combination of liquid-based and solid-based media exhibited better performance (95.5% recovery). The period taken to isolate mycobacteria is quite important to achieve rapid diagnosis [67]. Isolation in the BACTEC MGIT 960 method (15.3 ± 6.1 days) was shorter than that in the other two methods (20.1 ± 8.6 and 32.6 ± 11.8 days for MB/BacT and LJ, respectively). Detection with liquid-based media is faster and slightly more sensitive than with solid-based media, although the high sensitivity of that method is prone to contamination with environmental mycobacteria and other micro-organisms [68, 69]. This contamination may be associated with NTM because liquid-based media were found to be more effective than solid media for the detection of NTM [69].

Collectively, liquid-based media better support the growth of the *M. tuberculosis* complex than solid-based media [50]. Although both liquid- and solid-based media have fair sensitivity and specificity, their diagnostic values restrict them to qualitative analysis. Conversely, quantitative methods such as direct microscopic counting of AFB account for the exact number of mycobacteria present in the sample.

PCR and other molecular methods

Molecular identification has emerged as an alternative or a complement to traditional microbiological identification. It is now regarded as a promising approach [70]. Nucleic acid amplification tests (NAATs) such as based PCR, nested PCR, reverse-transcription (RT)-PCR and TB loop-mediated isothermal amplification (LAMP), use molecular probes that hybridize specifically with *M. tuberculosis* complex, *M. avium* complex, *M. kansasii*, or *M. goodii* [71]. For a comprehensive review see [5, 72]. These assays have sensitivities and specificities of almost 100% in the presence of at least 1×10^5 organisms, offering better accuracy than AFB microscopy and faster results than culture [73]. Accordingly, a comparative study found a 100% correlation between the IS6110-based PCR and the BACTEC MGIT 960 system, and the authors suggested both methods to be used as a combined protocol for routine clinical diagnosis [74]. In paucibacillary, smear-negative TB, the sensitivity of NAAT is invariably approximately 50–60%, but the specificity is almost 99% [75]. In general, PCR is less sensitive than culture, and its use is limited by costs and the need for laboratory expertise and infrastructure [76].

In the early 1990s, a pioneer NAAT study was conducted by Eisenach and colleagues [77] to detect *M. tuberculosis* using PCR, in which sensitivity, specificity and concluding results were available within 48 h. This method can also be employed to identify drug-resistant bacilli (Table 2). In fact, a broad review on the topic of drug sensitivity testing was performed in 2014 [78], and thus we intended to update this subject (Table 3). Accordingly, we conducted a phenotypic and genetic analysis of a drug-resistant phenotype and resistance-conferring mutations in patients at a referral hospital in Fortaleza, Brazil [79]. Primary resistance was high in the participants (50.9%), and analysis via multiplex allele-specific PCR and sequencing detected and identified mutations in *katG*, *rpoB*, *inhA* promoter and *gyrA* (Table 3). Spatial analysis revealed distinct isolates distributed in areas with low socioeconomic status in the city. Our results emphasized the importance of detecting resistance to TB drugs [79]. Previously, multiplex-PCR technology has been used to recognize INH-resistant *M. tuberculosis* from isolates in India [80]. Although PCR is unable to recognize viable vs non-viable bacilli (as discussed below), which is related to the inherent deficiency in quantifying the number of mycobacteria, NAAT approaches as a whole help to achieve a diagnosis of TB quickly and expedite the decision-making process for the best drug treatment protocol (Tables 2 and 3).

Table 3. Overview of the most recent methods (2014 onwards) for detecting *M. tuberculosis* drug resistance, highlighting the respective target and drug

Assay	Target gene	Referring drug	Reference
GenoType MTBDRplus*	<i>rpoB</i> , <i>katG</i> and <i>inhA</i>	INH and RMP	[165–170]
GenoType MTBDRsl*	<i>embB</i> , <i>gyrA</i> and <i>rrs</i>	EMB, fluoroquinolones, aminoglycosides and cyclic peptide	[171–173]
RT-PCR*	<i>katG</i> , <i>rpoB</i> , <i>MPB64</i> and <i>IS6110</i>	RMP and INH	[88, 150, 174–180]
Abbott Real-Time MTB RIF/INH†	<i>katG</i> , <i>inhA</i> and <i>rpoB</i>	RMP and INH	[181–183]
Multiplex allele-specific PCR*,‡	<i>rpoB</i> , <i>katG</i> , <i>inhA</i> , <i>pncA</i> and <i>embB</i>	RMP, INH, pyrazinamide, fluoroquinolones and aminoglycosides	[79, 184–192]
PCR-RFLP*	<i>katG</i> , <i>embB</i> , <i>rpsL</i> and <i>gyrA</i>	INH, streptomycin and fluoroquinolones	[193–195]
Genedrive*	<i>rpoB</i>	RMP	[196]
Anyplex Plus MTB/NTM*	<i>katG</i> and <i>inhA</i>	RMP and INH	[197–199]
AuNP-based lateral flow*	<i>katG</i>	INH	[200]
Electrochemical DNA sensors†,§	<i>rpoB</i>	RMP	[201]
Binary deoxyribozyme sensors†	<i>rpoB</i> , <i>katG</i> , <i>inhA</i> and <i>gyrA</i>	RMP, INH and fluoroquinolones	[202]
Luminex MicroPlex microspheres†	<i>rpoB</i> , <i>GyrA</i> and <i>inhA</i>	RMP and fluoroquinolones	[203]
Nipro Genoscholar†	<i>pncA</i>	Pyrazinamide	[204, 205]
Sequencing*	<i>rpoB</i> , <i>katG</i> , <i>embB</i> , <i>gyrA</i> , <i>gyrB</i> , <i>inhA</i> , <i>rpsL</i> and <i>rrs</i>	RMP, INH, fluoroquinolone and streptomycin	[206–214]

*Sputum samples.

†*M. tuberculosis* strain samples.

‡Distinguishes *M. tuberculosis* and *M. avium* complexes from other mycobacteria directly from clinical specimens [215].

§Based on polypyrrole/Fe₃O₄ nanocomposite-bearing redox naphthoquinone tag on PAMAM (spaNQ/PAMAM/PPy/Fe₃O₄).

In addition to PCR, there are three commercially available tests to directly identify the TB bacillus by 16S ribosomal transcripts, namely, (i) the *M. tuberculosis* direct test (MTD) using an amplicon detected using a DNA probe [81, 82]; (ii) the Amplicor using genus-specific primers by means of a colorimetric reaction [83]; and (iii) the TB-LAMP single-tube technique for the isothermal amplification of DNA or RNA [84]. The first two tests have been compared with the culture and clinical parameters, with both exhibiting high sensitivity and specificity in smear-positive specimens; however, low values were obtained in smear-negative specimens [85]. In addition, TB-LAMP is another low-cost alternative test for AFB ((84; [86]. TB-LAMP is simple, self-contained and efficacious for the early diagnosis of suspected cases of TB, with the advantages of having high throughput and no requirements for sophisticated equipment and complex biosafety facilities [84]. A meta-analysis found that additional factors, such as cost, feasibility and acceptability in settings that remain reliant on AFB, should be considered when deciding to implement this approach [86].

RT-PCR has been employed in cultured and clinical specimens. It is a scale-up technology employed to overcome the limitation of PCR in the quantification of samples. Its sensitivity ranges from 71–98% and its specificity is close to 100% [87]. The results are usually obtained 1.5 to 2 h after DNA extraction, and there is a low risk of contamination (reaction

and detection occur in a single tube). In 2006, Ortu and colleagues [88] employed RT-PCR to detect and identify *M. tuberculosis*. The authors suggested that this method can be useful for assessing the treatment response in patients with TB, as it is capable of quantifying the exact number of DNA copies of the pathogen, indicating the degree of infection in the patient. They also highlighted the risk of sensitivity reduction when samples contain small amounts of *M. tuberculosis* DNA contaminants.

As an inherent concern already highlighted, the detection of pathogen DNA via PCR does not distinguish viable from non-viable bacilli, and some NAAT studies have described the occurrence of false positivity due to contamination [89–91]. To minimize this possibility, real-time mRNA-based quantitative (q)RT-PCR, instead of DNA, might be useful to detect viable *M. tuberculosis* bacilli and for the diagnosis of active TB [92]. RNA has a short half-life [93] and it is predicted to be found in only viable cells. In addition, *M. tuberculosis* mRNA is quite stable, with a half-life of roughly 9 min [94]. In fact, the mRNA of Ag85B has only been detected in viable bacteria [95–97]. However, the sensitivity of the assay is low, and it is burdensome to work with RNA on a routine basis (Table 2). Thus, more effort is needed to develop simpler and more cost-effective PCR tests that can be used regularly in LMI countries to achieve efficient TB diagnosis [92].

Although PCR restriction fragment length polymorphism (RFLP) was developed in the last century, PCR-RFLP analysis of the genetic code has been employed to rapidly identify mycobacteria, including *M. tuberculosis*, in the current millennium [98]. In some cases, PCR-RFLP has been compared with conventional biochemical tests for diagnostic use [99, 100]. In addition, DNA microarrays have been used for the identification of *M. avium*, *M. chelonae*, *M. fortuitum*, *M. gordonae*, *M. intracellulare*, *M. kansasii*, *M. scrofulaceum*, *M. smegmatis*, *M. tuberculosis* and *M. xenopi* isolates [101]. We employed spoligotyping and georeferencing systems in an attempt to elucidate the genetic diversity of *M. tuberculosis* isolates circulating in patients with pulmonary TB from Fortaleza, Brazil [102]. Drug susceptibility testing and spoligotyping assay were both conducted, and the residences of the patients were georeferenced. Approximately 44.3% of isolates were resistant to at least one drug, whereas 55.7% were sensitive to all the drugs tested. A high frequency of resistance was observed in previously treated TB cases and among new cases. It was observed that the spoligopattern family distribution paralleled that reported for South America. A high case rate occurred among the resistant TB group because of transmitted and acquired resistance.

An automated method based on PCR technology has been described as a promising tool for fast and specific detection of *M. tuberculosis*. The Cepheid GeneXpert MTB/RIF system and its next-generation Xpert Ultra [103] uses a plastic cartridge containing all of the reagents required for DNA extraction to amplify the *rpoB* gene. GeneXpert MTB/RIF detects the amplicons in association with a point-of-care device, with results obtained in 2 h and with minimal hands-on technical time [104]. Recent studies have indicated the use of this approach to suppress the risk of bioaerosol infection as well as its use in point-of-care settings [105]. Accordingly, a single test using GeneXpert MTB/RIF might detect TB in 99% of patients with smear-positive and >80% of patients with smear-negative pulmonary TB [76]. Furthermore, GeneXpert MTB/RIF can detect RMP resistance with a sensitivity of 95.1% and a specificity of 98.4% [105], and a meta-analysis has suggested that this approach should be preferred in settings where resource and infrastructure requirements are adequate and where co-infection TB/HIV or drug resistance is a concern [86]. In addition, the updated Xpert Ultra test might detect HIV-associated TB with high sensitivity, thereby reducing TB-related mortality in co-infected TB/HIV patients [106]. The WHO initially recommended this technology in early 2011 [67]; the organization is monitoring the global rollout of this method to promote coordination [107]. In the past 3 years, one systematic and four literature reviews of the performance of GeneXpert MTB/RIF for the diagnosis of TB in several settings have been published [108–112]. Because comprehensive reviews on the diagnostic accuracy of the GeneXpert MTB/RIF system were published in 2013 [113], 2014 [114] and 2019 [103], we have not focused on this topic here. Accordingly, those reviews were made available for the detection of pulmonary TB and RMP resistance as part of a WHO process to develop structured guidelines on the use of the test.

Finally, the assessments of global gene expression measured in whole blood recently allowed for the diagnosis of TB. Accordingly, a pioneer study prospectively identified host-derived, blood-based 16-gene expression biomarkers in people from both South Africa and The Gambia who are at risk of developing active TB, thereby indicating the possibility of preventing the disease via a targeted intervention using non-sputum-based tests [115]. In a single year, the 16-gene signature predicted TB progression with a sensitivity of 66.1% and a specificity of 80.6%. In the same period, the risk signature in untouched groups exhibited a sensitivity of 53.7% and a specificity of 82.8%. Subsequently, another robust and simple PCR-based host blood transcriptomic signature, the so-called RISK6, was developed to identify individuals at risk of incident disease, to screen for subclinical or clinical TB and to monitor TB treatment [116]. Its performance in the diagnosis of subclinical and clinical diseases in HIV-uninfected and co-infected TB/HIV patients exceeded 85%. As a screening test for TB, RISK6 fulfilled the benchmarks established in the WHO target product profiles for those tests. The RISK6 scores were correlated with lung immunopathology activity and tracked treatment response. RISK6 predicted treatment failure prior to chemotherapy initiation. To obtain further details, two systematic reviews of the performance of the host blood global gene expression for diagnosing and predicting the progression of TB disease in different cohorts have recently been published [117, 118]. Collectively, these results indicate that both the 16-gene signature and RISK6 hold promise for worldwide applicability as field-friendly, point-of-care triage, diagnostic and predictive tests for TB based on the detection of biomarker profiles.

All the NAAT techniques described above have advantages over conventional techniques, such as their rapid detection and identification of TB, fast turnaround times for results, reliability and reproducibility. However, when using these techniques, additional equipment and trained personnel are needed. To date, these factors limit the implementation of these methodologies in LMI countries.

Flow cytometry

The evolution of methods to detect *M. tuberculosis* as described earlier has drastically reduced the time required for susceptibility testing from weeks to hours. Among these methods, FCM is a promising and potential tool that has become one of the best options for the rapid detection and quantification of various bacteria from the environment, food and clinical samples [119, 120]. An overview of its features is presented in Table 2.

In 1995, Norden and colleagues [121] described the use of FCM as a rapid test for drug susceptibility in a pioneer study. Using fluorescein diacetate (FDA), the authors tested *M. tuberculosis* strain H37Ra, which is susceptible to antimycobacterial agents, within 24 h and obtained similar results to those reported by others [122, 123]. Another study confirmed an agreement of approximately 94% between the agar proportion method and FCM (by detection of FDA

hydrolysis) for the INH-resistant strain, as well as total agreement for the EMB and RMP tests [124]. Although results can be obtained quickly using FCM, its biosafety remains an important drawback to its large-scale application owing to the production of infectious aerosols. Accordingly, Moore and colleagues [125] incubated specimens with paraformaldehyde before FCM analysis. However, it was observed that the tubercle bacilli trapped in the container could have escaped from the later treatment.

Another method to test the susceptibility of *M. tuberculosis* to drugs using FCM has been described to be fast and safe [126]. In this protocol, mycobacteria are heat-killed and probed with SYTO 16 stain, a non-symmetric cyanine with three positive charges that, when linked to pathogen nucleic acids, increases the intensity of the fluorescence signal and thus marks dead cells as bright green. After incubation of *M. tuberculosis* with SM, INH, RMP and EMB, the cells are stained and analysed using FCM. An excellent correlation between BACTEC MGIT 960 and FCM has been observed when compared with 12 to 15 days or 72 h of incubations, respectively, indicating that SYTO 16 enables clear distinction for drug susceptibility tests [126].

Recently, Qin and colleagues [127] discussed the importance of brighter fluorescent labels to improve the sensitivity of FCM and suggested the use of luminescent nanoparticles, as their superiority over conventional fluorophores in terms of fluorescence intensity and photostability was remarkable. In their study, an improved two-colour FCM approach was developed using a combination of Rubpy dye-doped silica nanoparticles with SYBR Green I dye to detect *M. tuberculosis*, thereby avoiding false positivity. However, neither drug susceptibility testing nor clinical sample detection was available in that setting. Another study recommended the use of FCM for the discrimination of live, drug-injured and dead *M. tuberculosis* [128]. Using SYTO 9, propidium iodide and ethidium monoazide (a compound that irreversibly binds to the DNA of dead cells), the authors could discriminate the effectiveness of anti-tuberculosis agents as targets to induce killing.

As FCM is a powerful, fast and safe tool that is essential for *M. tuberculosis* drug susceptibility testing in TB diagnostics that must remain simple and cost-effective. Janossy [129] claimed that there is a requirement to introduce this method in settings that have remained virtually untouched by contemporary laboratory technologies. Further, issues related to maintenance and special operational training restrict the wide use of FCM, particularly with regard to implementing the method in LMI countries.

Other nonconventional methods

Rapid detection of mycobacteria can be performed manually on the basis of the reduction of a tetrazolium salt indicator in a liquid-based medium [130]. This technique was compared with the use of the BACTEC 460 system and LJ solid culture media; it is a field-friendly device in which the antibiotic supplement is already incorporated, and easy and immediate

reading of the results is guaranteed [131]. However, no additional parameters, such as specificity and sensitivity, were reported in that study.

The ESP Culture System II (ESP II) is based on the detection of pressure changes in the culture medium [64]. This method has been evaluated by comparing its performance with that of the BACTEC 460 and Middlebrook 7H11 systems. ESP II is a less labour-intensive alternative to BACTEC 460 for detection of mycobacteria [64]. Similarly to BACTEC 460 or other liquid culture media, ESP II is recommended to be used in combination with another culture method rather than as a stand-alone system. ESP II plus BACTEC 460 yielded the highest mycobacterial recovery rate; however, in most laboratories, such a combination would probably be expensive [64].

In recent years, mass spectrometry (MS) has exhibited high efficacy in the identification of bacteria in routine clinical work [132, 133]. Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS was introduced during the 1980s [134], and in 2018 Cao and colleagues [135] conducted a systematic review and meta-analysis to confirm its accuracy in the identification of mycobacteria. The authors reported that the method might precisely identify 92% of *M. tuberculosis* isolates. In addition, several other studies corroborated that MALDI-TOF MS exhibits good sensitivity in the identification of *M. tuberculosis* [136–138]. However, this technique struggles to differentiate mycobacterial species with high genetic similarity [139]. In any case, MALDI-TOF MS is considered to be a promising diagnostic method that potentially accelerates the identification of slow-growing mycobacterial species and might also identify drug-resistant *M. tuberculosis* strains [140]. Besides the above-mentioned advantages, the drawback of MS for wide use in TB diagnosis is its high cost, especially when it is implemented in LMI countries.

The use of DNA sequencing began in the 1970s when Frederick Sanger developed the chain termination method [141]. However, despite its development, the Sanger technique presented limitations at that time, which made it impossible to generate a large amount of data at a low cost. Since then, scientific advances in the sequencing technique have emerged, which has led to the advent of new-generation sequencers [142]. Whole-genome sequencing (WGS) is becoming central in epidemiological investigations of TB because of its better resolution and cost-effectiveness compared with traditional typing approaches. Numerous systematic reviews on the performance of this technique were published in 2016 and 2017 in several settings, indicating the speed with which this predicted diagnostic tool has taken off in recent times [143–145]. In one systematic review, the authors revealed that WGS has an average sensitivity and specificity for detecting drug-resistant forms of *M. tuberculosis* strains of 98 and 97% for RMP and 97 and 96% for INH, respectively [145]. However, Witney and colleagues [146] reported that WGS may yield false-positive results when polymorphism occurs in regions correlated with RMP resistance. In another study, the authors concluded that there is still much to learn about

the origins of the growing genetic diversity that influence the interpretation from the understanding of *M. tuberculosis* transmission in each setting, and public health teams and researchers should combine epidemiological, clinical and WGS data to strengthen investigations of TB transmission [144]. Finally, besides the absence of a head-to-head comparison in *M. tuberculosis* isolates, a study found that WGS had greater discriminatory power than conventional genotyping and detected transmission events missed by epidemiological investigations [143].

Although this method exhibits high sensitivity, its implementation in the clinical laboratory routine is hindered by two factors: (i) the requirement for bacterial growth to obtain a certain amount of DNA necessary for analysis and (ii) the high cost of system maintenance. One of the major challenges of WGS in the diagnosis of *M. tuberculosis* is direct sequencing of sputum specimens (Table 3). In 2015, a study employed the biotinylated RNA technique specifically designed to detect *M. tuberculosis* genomes directly from the sputum of TB patients [147]. In 2018, the WHO published guidelines for the use of this technology for *M. tuberculosis* complex diagnosis and for the detection of drug-related mutations [148].

CONCLUSIONS

In this review, we demonstrated that the current microbiological tests for the diagnosis of TB in clinical samples are challenging. The most common and cost-effective and oldest test (developed over a century ago) for TB diagnosis is AFB sputum smear microscopy.

Currently, TB detection in LMI countries is performed using different methods, which involve clinical history, physical examination and complementary tests, such as sputum smears, *M. tuberculosis* culture, radiological examination, and histopathological and immunological approaches, all of which are supervised by well-trained healthcare workers who analyse the results of the complementary tests. On the other hand, in the modern laboratories of developed countries, suspected TB cases may be diagnosed by means of newer methods of cultivation using quicker molecular approaches, and this leads to accurate diagnosis in point-of-care settings [67].

New methods may be tested in well-designed and -controlled clinical trials, in addition to being used in high-incidence LMI settings. In this direction, both the 16-gene signature and RISK6 hold promise for worldwide applicability as field-friendly, point-of-care triage, diagnostic and predictive tests for TB based on the detection of biomarker profiles [117]. It is important to bear in mind that the time between the onset of TB disease and correct TB diagnosis and initiation of the correct anti-mycobacterial regimen is often protracted. In short, despite progress in the development of methods for mycobacterial detection helping to improve TB infection control, major gaps persist, and the diagnosis of TB requires more rigorous practices.

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Author contributions

Conceptualization – L.L.N., C.C.F., P.R.Z. A. Data curation – T.A.C., P.R.C.S., P.R.Z.A. Investigation – T.A.C., P.R.C.S., P.R.Z.A. Project administration – P.R.Z.A. Supervision – L.L.N., C.C.F., P.R.Z.A. Visualization – T.A.C., P.R.C.S. Writing (original draft) – T.A.C., P.R.C.S., L.L.N., C.C.F., P.R.Z.A. Writing (review and editing) – T.A.C., P.R.C.S., L.L.N., C.C.F., P.R.Z.A.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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