Review Article

Cost-effectiveness in the diagnosis of tuberculosis: choices in developing countries

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Abstract
Tuberculosis remains one of the major causes of global death from a single infectious agent. This situation is worsened by the HIV/AIDS pandemic because one-third of HIV/AIDS patients are co-infected with *Mycobacterium tuberculosis*. Failure to control the spread of tuberculosis is largely due to our inability to detect and treat all infectious cases of pulmonary tuberculosis in a timely manner, allowing continued *M. tuberculosis* transmission within communities. Diagnosis of tuberculosis can be made using indirect and direct methods. The indirect tests, such as interferon-gamma release assays, provide a new diagnostic method for *M. tuberculosis* infection, but do not discriminate between infection and active disease. The most common direct method for diagnosing TB worldwide is sputum smear microscopy (developed more than 100 years ago), where bacteria are observed in sputum samples examined under a microscope. In countries with more developed laboratory capacities, cases of tuberculosis may also be diagnosed using culture methods (the current gold standard) or, increasingly, using rapid molecular tests. In this review, we discuss the traditional methods for the diagnosis of tuberculosis. We also discuss other inexpensive assays that can be used to detect the presence of *M. tuberculosis*.

Introduction

Tuberculosis (TB) is an ancient infectious disease caused by *Mycobacterium tuberculosis* (MTB) that typically affects the lungs (pulmonary TB), but can affect other sites as well (extrapulmonary TB). The disease is spread in the air when people who are sick with pulmonary TB expel bacteria by, for example, coughing. In general, 10% of people infected with MTB will go on to develop TB disease, whereas in 90% of the cases the subjects are asymptomatic and non-infectious because the host immune system contains the tubercular infection [1]. In these subjects, the infection persists in a latent form for the lifetime of the individual. Conditions such as HIV, malnutrition, and inflammatory diseases, which all require immunosuppressive drugs, may cause a latent tubercular infection (LTBI) to become an active TB infection [1]. In 2010, there were an estimated 8.8 million incident cases of TB (ranging between 8.5 million and 9.2 million) globally, equivalent to 128 cases per 100,000 of the population [2]. The greatest amount of the estimated number of cases in 2010 occurred in Asia (59%) and Africa (26%); smaller proportions of cases occurred in the Eastern Mediterranean Region (7%), Europe (5%) and the

Americas (3%) [2]. Of the 8.8 million cases of TB in 2010, 5.7 million were diagnosed and reported to national TB control programmers (NTPs). Among notified cases, there were an estimated 290,000 cases of multidrug-resistant TB (MDR-TB), of which only 53,000 (18%) were reported to have been diagnosed and enrolled in appropriate treatment [3]. Earlier and improved detection of TB and expanded capacity to diagnose cases of MDR-TB are thus global priorities for TB control. Diagnosis of tuberculosis can be made using direct and indirect methods. Indirect diagnosis can identify the subjects infected with MTB, but the assays that are now available do not discriminate between LTBI and active TB; consequently, diagnosis of active TB is still made using sputum smear microscopy, isolating MTB and identifying specific MTB DNA sequences.

Indirect diagnosis of tuberculosis
Tuberculin skin test

Historically, the diagnosis of tubercular infection was made using the tuberculin reaction test, also known as the Mantoux intradermal reaction. Tuberculin was developed over 100 years ago by Robert Koch and consists of a mixture of

approximately 200 purified proteins derivative (PPD) present in MTB but found also in *Mycobacterium bovis* Bacilli Calmette-Guerin (BCG) strain (used in vaccination) and in many environmental mycobacteria. Since its discovery, the PPD has been used to diagnose latent TB infection. When the PPD is injected intracutaneously, a classic delayed hypersensitivity reaction occurs in infected subjects, which manifests *in vivo* as a dermal infiltrate. The intradermal reaction may be indicative of a positive PPD reaction (when a 5 mm diameter in infants or immunocompromised subjects and a 10 mm diameter in adults is observed) or of a negative response. Unfortunately, the tuberculin skin test (TST) has several limitations: it has a low sensitivity, and the antigenic cross-reactivity resulting from the distribution of many antigens in BCG and in environmental mycobacteria is responsible for its low specificity. Consequently, a positive reaction can be indicative not only of contact with MTB but also of previous vaccination with BCG or contact with environmental mycobacteria. False positive results may also be caused by repeated tests as a consequence of the boosting effect of continuous sensitization with PPD. These different limits of the TST have encouraged scientists to develop an alternative assay that could overcome the PPD defects.

Interferon-gamma release assays (IGRAs)

Interferon-gamma release assays (IGRAs) provide a new diagnostic method for MTB infection based on detection of the interferon-gamma (IFN-γ) response to the TB antigens, which includes early secretory antigenic target-6 (ESAT-6), cultures filtered protein-10 (CFP-10), and a third antigen called TB7.7. These antigens are encoded by genes of region of difference-1 (RD1) which is present in MTB complex but absent in BCG and in most non-tuberculous mycobacteria (NTM). Two commercial IGRAs are now available: the enzyme-linked immunosorbent assay (ELISA)-based QuantiFERON-TB Gold In-Tube assay (QFT-GIT) and its predecessor, the QuantiFERON-TB Gold (QFT-G) test (Cellestis Ltd., Carnegie, Australia), in which interferon-gamma (IFN-g) is measured quantitatively by ELISA, and, the ELISpot-based T-SPOT.TBTM test (Oxford Immunotec Inc., Abingdon, UK) in which the number of IFN-gamma-producing T cells is measured by ELISPOT. The performance of IGRAs has been examined in numerous studies of immunocompetent subjects, HIV-infected individuals, and children. Overall, the data in the literature indicates that both IGRAs have a better specificity and correlation with exposure to MTB than the TST and equal or greater sensitivity [4]. The greater specificity of IGRAs in comparison to the TST is very important for the correct identification of infected subjects and for the administration of preventive therapy. In particular, this is very useful in immunocompromised subjects such as HIV patients and individuals with inflammatory disease. Overall, data indicate that sensitivity and specificity of both IGRAs in HIV-infected people is suboptimal when used alone to rule in or rule out active tuberculosis disease. HIV-associated immunosuppression, measured by circulating CD4+ T-lymphocytes, negatively affects the performance of QFT-GIT [5], and to a lesser extent, T-SPOT.TB. Moreover, the decrease in sensitivity of IGRAs in HIV-infected patients is largely due to high rates of indeterminate results that are due either to a high-background production of IFN-γ (negative control) or to a failure test caused by an insufficient number of peripheral blood mononucleated cells. Data concerning the performance of IGRAs in patients with inflammatory disease are limited and sometimes discordant. Recently, it was demonstrated that immunosuppressive treatment affected the TST result more than the IGRA result; to optimize the sensitivity of the diagnosis of tubercular infection, both assays should be used in these subjects [6]. However, considering the greater specificity of IGRAs over TSTs, immunological assays are preferable in areas where BCG coverage is high to avoid unnecessary preventive tubercular therapy. Concordance between IGRAs and TSTs in children is good; both tests may be used for diagnosis of tubercular infection in pediatric cases, but it seems that tubercular infection is detected more quickly by IGRA than the TST [7]. Moreover, immunological assays are not affected by the boosting effect and therefore can be used in a serial manner [7]. Screening to diagnose tubercular infection should be periodically assessed by the health workers who are at risk of tubercular infection. In these subjects, the results of TSTs and IGRAs were discordant due to the BCG vaccination that is obligatory in the health sector. The best strategy, in economic terms, is the one that provides the initial screening with the tuberculin test and then the execution of the IGRAs in all the subjects with positive skin tests. Studies of the diagnostic value of IGRAs in the diagnosis of active TB indicate that their sensitivity is not different from that of the tuberculin test and that IGRAs are not recommended as tests used to predict active tuberculosis [8]. IGRAs are an improvement over TSTs; they are less prone to false positives caused by non-tuberculous mycobacteria or

BCG, have an internal control, and do not require a follow-up visit to assess the reaction. However, their main limitations, as for the PPD, is that they are not able to discriminate between latent infection and active disease, and may not be used to monitor the effectiveness of tuberculosis therapy. It is likely that IGRAs, through the inclusion of new antigens of MTB in addition to those of RD1 region, may become a powerful means of diagnosis able to differentiate between latent tubercular infection and active disease.

Direct diagnosis

Direct diagnostic activity involves isolation of mycobacteria, species-level identification, and the determination of the isolated strain's drug sensitivity. Microbiological diagnosis of tuberculosis usually requires specific reagents, non-routine methods, more time, and more safety laboratory equipment related to bacteriological diagnostics. The specimens submitted for mycobacterial culture include a wide variety of different bodily fluids and tissues from various sites: sputum, urine, cerebral spinal fluid, aspirated gastric, tissue biopsy, or other samples from areas where the presence of TB infection is suspected. Ideally, the specimens should be obtained over three consecutive days, as a sample can be negative one day and positive the next. Mycobacteria are slow-growing and have an extended generation time (20 to 22 hours) compared to other bacteria (40 to 60 minutes); overgrowth of culture by other bacteria and fungi can occur in specimens obtained from non-sterile sources. The high

lipid content of the cell wall makes mycobacteria more resistant to strong acids and alkalis compared with other bacteria. This property has been used to develop the decontamination procedures with sodium hydroxide or chlorhexidine to eliminate commensal flora microorganisms while keeping the mycobacteria viable [9]. Once the samples have been decontaminated, they may be cultured and analyzed through microscopic and molecular examinations. Table 1 shows the different methods for direct diagnosis of TB with sensitivity, specificity, and the cost of commercial and non-commercial assays.

Direct microscopic examination

MTB is an acid-fast bacillus (AFB). Acid resistance is one of the main features of mycobacteria that allows for quick identification. The sensitivity and specificity of direct examination after coloration for the diagnosis of pulmonary tuberculosis ranges between 50% and 80% and 84.9% and 99.5%, respectively, in positive cultures. AFB sputum smear microscopy is inexpensive and rapid but it has several drawbacks including low sensitivity and significant differences in performance depending on the operator. Consequently, sputum culture remains the gold standard for TB diagnosis.

Isolation and culture

Culture remains the most sensitive method for the detection of MTB in clinical specimens and it is the gold standard for the isolation of the tubercular

Table 1: Direct methods of diagnosing tuberculosis

Method	Sensitivity (range)	Specificity (range)	Cost in US dollar USD (range)
Microscopical examination	50-80%	84.9-99.5%	0.50
Isolation and culture			
- Commercial (MGIT 960 system)	80-98.1%	89.6-100%	16-32
- Non-commercial (LJ, MODS, NRA, LRP)	85-97%	93-100%	4.56-11.13
Molecular identification			
- Commercial (Amplicor MTB, Cobas Taq Man MTB, BD Probetec ET, GeneXpert MTB/RIF)	77-100%	91.3-100%	25-30
- In-house NAAT	84-100%	83-100%	10
Susceptibility testing			
- Commercial (MGIT 960, Line Probe assay, Genotype MTB Dr plus)	75-98.1%	92-100%	15-56
- No commercial (MODS, NRA, LRP, etc.)	72-100%	91-100%	1.40-4.00
Other tests			
- LAM test	6-81%	88-100%	3.50
- Immunochromatografic assays	95.2-99.6%	97.6-100%	2.03
- *Cricetomys* rats	72-100%	92.23%	-

bacillus. Culture is capable of detecting as few 10^1 10^2 organisms/mL of specimen, surpassing the sensitivity of smear microscopy. While a number of culture options exist for clinical mycobacteriology laboratories, broth-based detection methods are preferable for the initial isolation of organisms from specimens. The liquid culture media is Middlebrook 7H9 and requires an automatized incubator allowing incubation with an atmosphere containing 5% to 10% of CO_2 and a regular and automatized detection of bacterial growth.

Different incubator system have been developed. The Bactec TB-460 device (Becton-Dickinson, Franklin Lakes, USA) was the first semi-automatized system introduced in 1980 for the inoculation of mycobacteria. This system uses a radiometric method for the detection of mycobacterial growth; however, disposal of the radioactive waste produced by this system presents a logistical problem and increases costs. In the last decade, several other manufacturers have marketed various tools for the automatized detection of MTB growth in laboratories, such as the Bactec 9000MB (Becton-Dickinson), the Bactec Mycobacterial Growth Indicator Tube 960 (MGIT 960, Becton Dickinson, Franklin Lakes, USA), the ESP Culture System II (Trek Diagnostic Systems, Sun Prairie, USA), and MB/Bact Alert 10 3D (Biomérieux, Craponne, France). Some are based on colorimetric methods that detect bacterial CO_2 production, such as the MB/BacT ALERT 3D System; others use pressure sensor or fluorometric methods to detect bacterial O_2 consumption, such as the ESP Culture System II and BACTEC MGIT 960 System and Bactec 9000MB, respectively. Numerous studies have favorably valuated the non-radiometric isolation system; generally, it has been observed that MGIT 960 showed the lowest detection time for MTB growth, with a mean of days that varies between 10 and 15 [10]. It has been reported that its sensitivity varies between 80% and 98.1% and its specificity varies between 89.6% and 100%. The MGIT 960 system is also used extensively for the antibiotic sensitivity *in vitro* test, and data obtained from different studies have shown that this system is a reliable and effective way to quickly determine the sensitivity to anti-tuberculosis drugs of a tubercular isolated strain [11].

Solid media are made of agar, egg, and malachite green to limit the growth of remaining contaminants (Lowenstein, Stonebrink, or Ogawa medium). The Middlebrook 7H10 and 7H11 media are semi-synthetic agar media for MTB culture under an atmosphere containing 10% CO_2. They are completely transparent and thus facilitate the early identification of MTB colonies.

Liquid media are considered the gold standard for the isolation of MTB culture and have a significant rapidity (between 10 and 14 days) and a better quality of isolation compared to solid media [12]; however, solid media still play an important role in the isolation of mycobacteria from clinical samples and are recommended for use alongside a liquid medium by the Center for Disease Control. Because liquid systems may fail to recover all mycobacteria, it is essential that standard solid-based culture methods be used in conjunction with automated systems to ensure recovery of *Mycobacterium* spp. that such systems may not detect.

Some simple manual TB culture-based methods are used with increasing frequency in developing countries. Among these are the microscopic observation of broth culture, nitrate reductase assay (NRA), luciferase reporter mycobacteriophage assay (LRP).

Microscopic observation of broth cultures is a low-technology but rapid testing method for tuberculosis [13]. When coupled with drug sensitivity testing, this assay has been called microscopic observation of drug sensitivity (MODS). MODS is a non-commercial TB liquid-culture method that is recommended by the World Health Organization for detection and drug susceptibility testing of MTB [14]. It is based on the observation of the cord formation of MTB grown in a liquid medium. Decontaminated sputum samples are inoculated into a 7H9 liquid medium in 24-well plates with or without antibiotics and incubated at 37°C in a CO_2 incubator. Plates are read using an inverted microscope to identify the typical MTB cord formation. Data in the literature indicates that MODS is an optimal alternative method for timely and affordable identification of MDR-TB in resource-limited settings. Chaiyasirinroje *et al.* analyzed 202 sputum samples of clinically diagnosed TB patients with MODS assay in relation to gold standard BACTECT MGIT 960 and Ogawa solid culture. The median time to culture positivity by MODS, solid, and liquid culture were 12, 30, and 6 days, respectively [15]. In another study conducted in Egypt, 115 smear-positive TB patients were simultaneously tested using MODS and the BACTECT MGIT 960. In this study, MODS detected 112 (97.4%) positive samples for MTB, and BACTEC MGIT detected 115 (100%) [16]. More recently, the diagnostic accuracy of MODS was estimated against a reference standard including Löwenstein-Jensen (LJ) media and BACTEC MGIT

culture. Overall MODS sensitivity for MTB detection was 85% and specificity was 93%; the authors also demonstrated that diagnostic accuracy did not significantly differ by HIV infection status [17]. The NRA is based on the ability of MTB to reduce nitrate to nitrite by the action of the nitrate reductase enzyme, which is indicated by the development of a dark rose to purple-rose color after the addition of the reagent. This method enables the rapid detection of bacterial growth [18].

The LRP assay is based on infection of MTB with phages harboring the luciferase gene. Viable mycobacteria will allow replication of the phages and production of light in the presence of cellular ATP when the substrate luciferin is added to the medium. Tests based on luciferase reporter phages have not been extensively evaluated. LRP assay proved comparably efficient to MGIT 960 at detecting MTB in a median of three days and with a sensitivity of 97%.

Molecular detection of M. tuberculosis

Molecular detection of MTB continues to change the landscape of TB diagnostics. Because of the slow growth rate of MTB, conventional methods for its detection, based on solid culture media, take several weeks to yield results. The introduction of liquid culture-based techniques was a great improvement for diagnosis, shortening the time to detection to about 10-14 days instead of the weeks that are needed when using conventional media. With the purpose of obtaining faster results and earlier diagnosis of tuberculosis, several molecular detection methods were introduced and have been evaluated in numerous studies. MTB-specific nucleic acid amplification tests (NAATs) are the most frequently used molecular tests for laboratory diagnosis of tuberculosis. Many in-house PCR methods have been proposed and many studies have been published describing their application for tuberculosis diagnosis [19,20]. In-house NAA tests use different targets, either DNA or RNA genus or species-specific targets, followed by a detection step performed on agarose or acrylamide gels, or hybridization in various formats. The most commonly used target for identification of MTB is the insertion sequence IS*6110* present in 4 to 20 copies of more than 95% of MTB strains [21]. Overall, the reported sensitivity and specificity are in the range of 84%-100% and 83%-100%, respectively in respiratory specimens. Lower sensitivity and specificity were observed in no respiratory samples. NAAT are also commercially available from different sources: the

first two and oldest in the market are the Amplicor *Mycobacterium tuberculosis* test (Amplicor) (Roche Diagnostic Systems Inc, Indianapolis, USA) and the Amplified Mycobacterium Tuberculosis Direct Test (MTD) (Gen-Probe Inc, San Diego, USA). The Amplicor MTB test is a DNA-based test that amplifies a segment of the 16SrRNA gene using genus-specific primers that are detected in a colorimetric reaction in a micro-well plate format. An automated version of the test is the Cobas Amplicor MTB test, which allows automation of the amplification and detection in one system. Recently, the COBAS TaqMan MTB test was introduced using real-time PCR. Sensitivity and specificity of the Amplicor MTB test was higher in respiratory samples and ranged between 83% and 92.4% and 91.3% and 100%, respectively. The Amplified MTD uses isothermal amplification of 16S ribosomal transcripts, which are detected in a hybridization protection assay with an acridinium ester-labeled MTB complex-specific DNA probe. Interpretation of results requires the use of a luminometer. Sensitivity ranged from 77% to 100%. BDProbe Tec ET is another commercial amplification method for the direct detection of MTB in clinical respiratory samples. It is based on a strand-displacement amplification, a technique of isothermal amplification that uses enzymatic replication of target sequences IS*6110* and the 16S rRNA gene. The most significant advance toward a molecular test for tuberculosis has come in the field of nucleic acid amplification with the launch of the GeneXpert MTB/RIF assay (Cepheid, Sunnyvale, USA) [22]. The GeneXpert multifunctional diagnostic platform is an automated closed system that performs real-time PCR, producing results in less than two hours. The assay simultaneously detects MTB and rifampin (RIF) resistance by PCR amplification of five overlapping probes that are complementary to the entire 81 base pair RIF resistance-determining region of the MTB *rpoB* gene, and subsequently probes this region for mutations that are associated with RIF resistance [23]. The PCR amplification process is hemi-nested, and the amplified target is detected in real time by six color fluorescent molecular beacons. The test is easy to use and does not require special training other than very basic computer knowledge. Assay sensitivity is higher than that of smear microscopy and close to that of culture [24]. When testing a single sputum sample, the assay detects 98%-100% of sputum smear-positive disease and 57%-83% of smear-negative disease among prospectively studied tuberculosis suspects [25]. In addition, the assay is useful in diagnosing

extrapulmonary tuberculosis from a range of samples from extrapulmonary sites, with sensitivities of 53%-95% [26]. The assay is also highly specific with no cross-reaction with non-tuberculous mycobacteria or normal flora of the respiratory tract. Despite the high sensitivity and specificity, several studies have since detected numbers of false-positive resistance when compared with other testing methods and *rpoB* gene sequencing [27] in settings with a prevalence of low RIF resistance. The current WHO implementation guide recommends use of a second testing method to confirm RIF resistance in these settings and to test for resistance to second-line drugs whenever multidrug resistance is detected [28]. Molecular methods, based on species-specific DNA probes that hybridize to rRNA, have also been developed for rapid identification of mycobacteria. The first of these methods was AccuProbe (Gen-Probe, Bedford, USA), which, with a sensitivity and specificity of more of 90%, identified MTB complex, *Mycobacterium avium* complex, *Mycobacterium kansasii,* and *Mycobacterium gordonae*. More recently, INNO-LiPA MYCOBACTERIA version 2 (Innogenetics NV, Ghent, Belgium), and the GenoType MTBC and GenoType Mycobacterium (Hain Lifesciences, Nehren, Germany) were introduced; all these are to be applied on positive cultures, and all are line-probe assays that detect different mycobacteria species including MTB.

A method useful for molecular identification of mycobacteria other than tuberculosis is Hsp65PRA (PCR-restriction enzyme pattern analysis), which combines the amplification of the gene encoding the heat-shock protein (hsp) from 65 kDa with the digestion of the same, with two restriction enzymes *BstE*II and *Hae*III. Using this method, it is possible to identify, in addition to *M. tuberculosis* complex, more than 50 species of mycobacteria other than tuberculosis on the basis of their characteristic restriction profile.

Therapy and susceptibility testing

Drugs administered for anti-tuberculosis therapy, called first-line drugs, were isoniazid (H), streptomycin (S), rifampicin (R), pyrazinamide (Z), and ethambutol (E). When an MTB strain is resistant to more than one antibiotic, in particular to isoniazid and rifampicin, the strain is considered multidrug-resistant (MDR). The presence of MDR strains has aggravated the already serious problem of tuberculosis, resulting in the use of alternative drugs that can be more expensive, less effective, and more

toxic than those used in traditional therapy. The World Health Organization (WHO) revised international guidelines for the treatment of tuberculosis in 2010, specifically responding to the growing evidence and escalating problem of drug-resistant disease worldwide. Earlier guidelines emphasized the use of two main standardized treatment regimens, one for new (previously untreated) cases and one for patients with sputum smear-positive disease who had previously received treatment (retreatment regimen) [29]. The drug combinations used in these two regimens differed only by the addition of a single drug. New patients with pulmonary TB should receive a regimen containing six months of rifampicin: two months HRZE and four HR. This regime also applies to extrapulmonary TB, but not to TB of the central nervous system, bone, or joint, for which some expert groups suggest longer therapy. Wherever feasible, the optimal dosing frequency for new patients with pulmonary TB is daily throughout the course of therapy [29]. There are two alternatives: new patients with pulmonary TB may receive a daily intensive phase followed by a three-times-weekly continuation phase (2HRZE/4(HR)3) provided that each dose is directly observed. Dosing three times weekly throughout therapy (2(HRZE)3/4(HR)3) is another alternative, provided that every dose is directly observed and the patient is not living with HIV or living in an HIV-prevalent setting. In populations with known or suspected high levels of isoniazid resistance, new TB patients may receive HRE as therapy in the continuation phase as an acceptable alternative to HR. TB patients whose treatment has failed or other patient groups with a high likelihood of multidrug-resistant TB should be started on an empirical MDR regimen. TB patients returning after defaulting or relapsing from their first treatment course may receive the retreatment regimen containing first-line drugs 2HRZES/1HRZE/5HRE if country-specific data show low or medium levels of MDR in these patients or if such data are not available [29].

In recent years, the problem of multidrug resistance has been aggravated both by the appearance of MTB strains defined as extensively drug-resistant" (XDR), which appear to be resistant to isoniazid and rifampicin in association with all fluoroquinolones and one of three injectable drugs, kanamycin, or amikacin, capreomicin. Strains that are resistant to all first-line and second-line drugs are considered XDR. Recently, six new drugs were developed and analyzed in clinical trials [30]. These new drugs include the diaryquinolone, TMC-207, which targets

mycobacterial ATP synthase; in a phase two clinical trial, it greatly increased sputum smear conversion in patients with MDR tuberculosis. Nitroimidazoles, such as PA-824 and OPC-67683, are equally active against drug-susceptible and drug-resistant tuberculosis and are also being assessed in clinical trials. Since nitroimidazoles are active against both replicating and non-replicating organisms, they could potentially shorten treatment of active disease and provide activity against latent tuberculosis infection. Culture-based and genotypic methods have been developed to detect drug-resistant tuberculosis. Automated liquid culture systems and molecular line probe assays are recommended by the WHO as the gold standard for first-line drug-susceptibility testing (DST) [31]. The Food and Drug Administration (FDA) has approved the BACTEC MGIT 960 for DST with first-line antibiotics. A number of studies have evaluated the BACTEC MGIT 960 system for DST of MTB [32]; the system was found to be a highly accurate and reliable tool for DST. The use of this system has not been cleared by the FDA for DST of second-line drugs, and thus most laboratories rely on agar proportion as the reference standard. Moreover, many studies have demonstrated that the performance of BACTEC MGIT 960 for DST of second-line drugs was comparable to the agar proportion method; the system is therefore a reliable tool for detecting resistance to second-line drugs [33]. With the purpose of detecting drug resistance in a shorter time, several molecular approaches have been proposed. There are currently two commercially available solid phase reverse hybridization assays for the rapid detection of drug resistance in MTB: the line probe assay (LiPA) (INNO-LiPA Rif TB Assay, Innogenetics, Ghent, Belgium) for detecting resistance to rifampicin and the GenoType MTBDR Plus (Hain Lifesciences, Nehren, Germany) for the simultaneous detection of resistance to rifampicin and isoniazid. Recently, the LiPA assay has been evaluated for its identification of MTB species and detection of mutation related to drug resistance in MTB [34]. The results of LiPA identification of *Mycobacterium* species in clinical isolates were almost identical to those of conventional methods; this assay also was used to directly test sputum specimens with a sensitivity of 85.6%. Compared with standard DST for the clinical isolates, LiPA showed a good sensitivity and specificity for detecting rifampin, isoniazid, pyrazinamide, and levofloxacin resistance of clinical isolates with a rate of 89.7% and 100%, respectively. Its sensitivity and specificity for detecting rifampin-, isoniazid-, and levofloxacin-resistant isolates in the sputum were both 100%, and those for detecting isoniazid-resistant isolates were 75% and 92.2%, respectively [34]. A meta-analysis carried out to assess the accuracy of GenoType MTBDR indicates that its sensitivity and specificity for rifampicin resistance were 98.1% and 98.7%, respectively; however, while specificity is excellent for isoniazid resistance (99.5%), sensitivity estimates were modest and variable. MTBDRsl assay (Hain Lifesciences, Nehren, Germany) is a new line probe assay for the detection of XDR TB. The test simultaneously detects resistance to ethambutol, aminoglycosides/cyclic peptides and fluoroquinolones through detection of mutations in the relevant genes. Its sensitivity for detection of ethambutol resistance was low, but its specificity was high for all drugs [35]. Besides these commercial kits for the diagnosis of drug TB resistance, different manual or experimental techniques were described in the literature. For example, MODS, NRA, or LRP were evaluated both for identification of MTB and determination of resistance to anti-tuberculosis drugs. Results of several studies have shown that the sensitivity of the MODS assay in detecting MDR varies from 72% to 100% [16,17,36]. Recently, the performance of NRA on Middlebrook 7H11 agar for detection of isoniazid and rifampin resistance has been evaluated and compared with the direct proportion method on LJ medium directly on smear-positive sputum specimens. The results of both methods were in 100% agreement for detection of R resistance while agreement for H was 96.4%. These data indicated that direct NRA testing on smear-positive sputum specimens by using 7H11 agar could be used as a fast, reliable, and inexpensive method in resource-starved settings [37]. Additionally, it has been demonstrated that the NRA method provided sensitive detection of resistance both to first- and second-line drugs and that it is capable of the simultaneous detection of MDR and XDR-TB [38]. Evaluations of commercial phage amplification assays yielded more variable estimates of sensitivity (range of 81%-100%) and specificity (range of 73%-100%) compared to evaluations of in-house amplification assays (sensitivity range 88%-100%, specificity range 84%-100%). Phage-based assays will require further development to maximize interpretable results and to reduce technical failures. Tests such as MODS, the nitrate reductase assay, and colorimetric reductase methods have been conditionally approved by WHO for use at national tuberculosis reference laboratory levels.

Different molecular methodologies to detect the anti-tubercular drug resistance were evaluated. Real-time PCR has been one of the most widely applied due to its rapidity, high sensitivity, reproducibility, and low risk of contamination. The most common real-time PCR assays for detecting drug-resistant mutations have been developed through two main methods. In the first method, a fluorescent signal is generated by hybridization of a probe to the target sequence at the end of each PCR cycle. The TaqMan probe- and molecular beacon-based allele discrimination assays belong to this category. Because most of these assays require the use of two different fluorophore-labeled probes for differentiation of one allele, the cost is relatively high and a real-time PCR instrument with multiple channels is required. In the second case, mutation detection is achieved by melting curve analysis. Two major assays, fluorescence resonance energy transfer (FRET) probe melting curve analysis and high-resolution melting curve (HRM) analysis, have been successfully applied to detect drug-resistant mutations in MTB [39]. Lou *et al.* developed a low-cost, widely applicable real-time PCR assay based on melting curve analysis of dually labeled probes, to rapidly detect the drug-resistant mutations of MTB. Six probes targeting the *rpoB* 81-bp core region, *katG315*, the *inhA* promoter, the *ahpC* promoter, and *embB306,* were designed and validated with clinical isolates. A comparison of the results with the sequencing data showed that all mutations covered by the six probes were detected with 100% sensitivity and 100% specificity [39]. Liu *et al.* developed a simple and widely applicable assay for detecting mutations associated with second-line drug resistance in *Mycobacterium tuberculosis*. Three dually labelled probes targeting gyrA, rrs, and the promoter of eis were designed to detect resistance to fluoroquinolones and second-line injectable agents (capreomycin, amikacin, and kanamycin). A triplex reaction with all three probes and corresponding primers was first tested against 13 isolates with different mutations in the targeted regions. The triplex assay was applied to 109 second-line drug-resistant isolates and the results were compared with the sequencing data. The detection results of 109 isolates were 100% concordant with sequencing data [40].

Another PCR-based method of detecting TB drug resistance is the single-stranded conformational polymorphism (SSCP) analysis, which involves amplification by PCR of a segment of the gene encoding for the specific drug target and comparison of PCR products of drug-sensitive and drug-resistant strains by SSCP, in which mutations usually result in an altered pattern. This technique is relatively simple, but its sensitivity and specificity have been questioned.

Pyrosequencing is a semi-automated sequencing method based on real-time monitoring of DNA synthesis, optimized to analyze short DNA sequences. It is based on the quantitative detection of released pyrophosphate during DNA synthesis. In a cascade of enzymatic reactions, light is generated at intensities proportional to the numbers of incorporated nucleotides [41]. Pyrosequencing has previously been described as a method for detecting drug resistance in MTB [41]. In comparison to phenotypic DST, the pyrosequencing method demonstrated high specificity (100%) and sensitivity (96.4%) for detection of MDR MTB as well as high specificity (99.3%) and sensitivity (86.9%) for detection of XDR MTB [41].

DNA microarrays have been applied widely in fundamental research, human genetics, infectious disease diagnosis, genotyping, genetic expression monitoring, pharmacogenomics, environmental control, and in the identification and detection of mutations in genes responsible for drug resistance. High-density oligonucleotide arrays have been used for parallel species identification and the detection of mutations that confer rifampicin resistance in mycobacteria, and more specifically for the detection of MTB strains resistant to rifampicin or isoniazid, kanamycin, streptomycin, pyrazinamide, and ethambutol. Aragon *et al.* developed a fast low cost and low density DNA microarray (LCD array) for the detection of mutations that confer isoniazid or rifampicin resistance in MTB isolates. They observed that LCD was 100% concordant with the sequencing data for H resistance and 93.8% concordant for R resistance [42]. A sensitive and specific microarray was designed by Yao *et al.* to detect mutations in the rifampin resistance determining region of rpoB and loci in katG and inhA associated with H resistance. They indicated that 100% of rifampicin-resistant MTB strains analyzed had rpoB mutations. Of the total 50 H-resistant isolates, 82% had a katG315 mutation and 18% had an inhA mutation. All the mutations detected by the microarray method were also confirmed by conventional DNA sequencing. It has been demonstrated that the microarray is an efficient, specialized technique and can be used as a rapid method for detecting rifampin and isoniazid resistance. More recently, Zhang *et al.* assessed the performance of the CapitalBio DNA microarray in the detection of H and R resistance in spinal tuberculosis compared with the BACT/MGIT 960 system. The DNA

microarray had a sensitivity of 88.9% and a specificity of 90.7% for R resistance, and a sensitivity of 80.0% and a specificity of 91.0% for H resistance. The mean turnaround time of MTB species identification and drug resistance detection using the DNA microarray was 5.8 hours [43].

Urine-baseddiagnostic test

M. tuberculosis antigen detection has long been viewed positively as an option for TB diagnosis. A number of mycobacterial antigens can be detected in the urine of patients with pulmonary TB; the most promising of these is the cell wall lipopolysaccharide lipoarabinomannan (LAM) [44]. An enzyme-linked immunosorbent assay (ELISA) that detects LAM has been developed. Although the sensitivity of this test has been disappointing in non-HIV-infected patients, moderate sensitivity and high specificity has been observed in HIV-infected patients with advanced immunodeficiency [45]. This assay could provide evidence of tuberculosis in patients with advanced HIV infection being screened in antiretroviral therapy clinics and in those with possible disseminated disease. Although the sensitivity of many tuberculosis diagnostic tests declines steeply in HIV-infected patients with more advanced immunodeficiency, paradoxically, the sensitivity of the LAM ELISA increases at lower CD4 lymphocyte cell counts [45]. The commercially available LAM ELISAs are 96-well plate format sandwich assays that employ highly purified polyclonal antibody preparations. This format of the assay is not suitable for use in resource-limited settings due to infrastructure limitations and the requirement for samples to be prepared and batches to be processed in centralized laboratories. However, using the same antibody preparations, a simple lateral-flow version of the assay has been produced as a point-of-care test. Determine TBLAM Ag (Determine TB-LAM, Alere, Waltham, USA) is an immunochromatographic assay in which the captured antibodies are adsorbed onto the nitrocellulose membrane of the test strip and the detection antibody is labeled by conjugation to colloidal gold particles. In a study evaluating this test as a tool for tuberculosis screening among patients enrolling in an antiretroviral therapy clinic in South Africa, the sensitivity was equivalent to that of the ELISA format of the assay, and specificity was 98% overall and stratified by CD4 cell count in all patient subgroups [46]. The same authors more recently demonstrated that the low-cost point-of-care urine test for LAM rapidly diagnoses a sub-group of cases with advanced HIV-associated TB

and poor prognosis, and if used in combination with laboratory-based diagnostics, treatment delays would decrease and survival might be improved [47]. Studies in appropriate clinical populations are providing new insights into the real utility of this assay; it is now emerging as an important tool that may serve to greatly expedite TB diagnosis and treatment in those with advanced HIV-associated immunodeficiency, potentially reducing mortality risk.

Immunochromatografic assay

Correct mycobacteria identification is crucial for the effective treatment of TB. Positive mycobacterial cultures do not necessarily mean they are positive for MTB complex; the culture needs to be identified to discriminate MTB complex from non-tuberculous mycobacteria (NTM).

Usually, most laboratories in resource-limited settings use the labor-intensive standard biochemical tests to identify MTB complex, which require subculture of mycobacteria on solid media and delays results for several weeks. This process increases the turnaround time for reporting positive results. Commercial diagnostic methods employing nucleic acid amplification may provide rapid results for the identification of MTB complex, but most of them are designed for semi-automated use; furthermore, they are still time consuming and require other laboratory equipment. There are currently three commercial tests for the rapid identification of MTB complex from positive cultures based on the same immunochromatographic technology: the Capilia test (Tauns Laboratories, Numazu, Japan), the SD Bioline TB Ag MPT64 Rapid Test (Standard Diagnostics, Inc, Kyonggi-do, South Korea), and the BD MGIT TBc Identification test (BD Diagnostic Systems, Sparks, USA). All detect the antigen MPT64 for rapid discrimination between MTB complex and NTM. The Capilia test is not widely available in the market; it is only available through FIND (Foundation for Innovative New Diagnostics, Geneva) and to a restricted number of countries, especially countries with a high TB burden. SD Bioline TB Ag MPT64 Rapid Test and the BD TBc ID test are available in the market and can be obtained without restrictions.

The Capilia test was evaluated in resource-limited settings for rapid identification of MTB from BACTEC MGIT 960 and BACTEC 9120 systems [48]. One thousand samples from pulmonary and disseminated TB were cultured in automated BACTEC MGIT 960 and BACTEC 9120 blood culture systems. The overall sensitivity and specificity

for Capilia TB assay for identification of MTB were 98.4% and 97.6%, respectively; additionally, Capilia TB was cheaper, easier to perform, and had a shorter turnaround time (20 minutes) [48]. A study in Zambia and South Africa demonstrated that sensitivity and specificity of Capilia TB assay was 99.6% and 99.5%, respectively, and that this test is inexpensive and can be used in resource-limited settings [49]. When the SD Bioline Ag MPT64 Rapid Test was evaluated for the identification of MTB, the assay showed excellent sensitivity and specificity. Martin *et al.* evaluated the performance of the TBc ID test. Ninety-two cultures were evaluated and the sensitivity and specificity of the TBc ID test was 98.5% and 100%, respectively. The researchers indicated that the assay might have yielded false negative results because of the mutation in the MPT64 gene or because the MTB cell numbers in the culture were too low to produce sufficient MPT64 antigen detection [50]. Po-Liang Lu *et al.* reported less sensitivity and specificity (95.2% and 99.2%, respectively) and also indicated that false negative results can occur when there is a mutation in the MPT64 gene or when its amount is low [51]. Overall data indicate that the TBc ID test is a very simple way to identify the presence of MTB complex in liquid cultures, which could provide a good alternative to the time-consuming biochemical identification tests and more expensive molecular tests. Moreover, integration of solid and liquid cultures and using an additional molecular method can yield a sensitivity up to 100% for MTB complex detection. All immunochromatografic assays have several advantages and are destined to be used in all laboratories, particularly in endemic areas.

Cricetomys rats: a potential diagnostic system

Tuberculosis diagnosis in regions with limited resources depends on microscopy with insufficient sensitivity. Rapid diagnostic tests of low cost but high sensitivity and specificity are needed for better point-of-care management of TB. Giant African pouched rats (*Cricetomys gambianus*) are used operationally to detect land mines. A nonprofit humanitarian organization, APOPO, located in Morogoro, Tanzania, is responsible for the use of rats as mine-detection animals and is also exploring other humanitarian applications for the rats, using the animals to detect tuberculosis in humans. These large and long-lived rats, which are native to much of Africa and have an excellent sense of smell, detect TB by sniffing sputum samples. They are trained to respond consistently in one way (pause) if the samples contains the TB bacillus (positive) and respond in another way (not pause) if the sample does not contain the bacillus (negative) [52]. Each rat can test hundreds of samples each day, allowing for inexpensive testing. The odor uniquely associated with MTB constitutes a signal for the rats that emit an easily observed indicator response when they smell a sputum sample positive for MTB. The training of *Cricetomys* rats for TB diagnosis commences at four weeks of age. The training duration required for rats to qualify for diagnosis of TB ranges between six and nine months. Several studies have described the use of rats as TB detectors. Weetjens *et al.* used a cage with 10 sniffing holes and put in a cassette carrying 10 sputum samples. Rats were trained to sniff each consecutive sample and indicate TB positives by fixing their nose for five seconds at the sniffing hole. Eighteen rats were able to discriminate positive from negative sputum with a daily sensitivity ranging from 72% to 100%. Daily false positives ranged from 0.7% to 8.1% [53]. Poling *et al.* evaluated 23,101 sputum samples; the specimens were first evaluated by microscopists and then by rats. Microscopists identified 2,487 sputum samples as TB positive; the rats verified these findings and identified 2,274 samples as TB positive. The rats also identified as positive an additional 927 TB samples initially indicated as negative but found in second microscopy to contain the bacillus. The authors concluded that the use of pouched rats for TB detection in developing countries certainly warrants further research [54]. Mgode *et al.* evaluated rats' ability to discriminate between clinical sputum containing other *Mycobacteria* spp. and non-mycobacterial species of the respiratory tract. The authors indicated that MTB produces specific volatiles that are not produced by NTM and other respiratory microorganisms investigated in this study [55]. They suggested that a defined blend of MTB-specific volatiles apparently allows trained rats to discriminate TB-positive from TB-negative sputa for accurate odor diagnosis of TB. These findings are potentially relevant to initiatives to develop a point-of-care test for rapid TB diagnosis. Another recent study investigated the odor volatiles of MTB detected by rats in reference to MTB, non-tuberculous mycobacteria, *Nocardia* sp., *Streptomyces* sp., *Rhodococcus* sp., and other respiratory tract microorganisms spiked into MTB-negative sputum. Thirteen compounds were specific to MTB and 13 were shared with other microorganisms. Rats discriminated a blend of MTB-specific volatiles from individual and blends of shared compounds. The rats' sensitivity for typical TB-positive sputa was 99.15%,

with 92.23% specificity and 93.14% accuracy. Overall, these data indicated that the use of trained sniffer rats for TB detection is a potentially faster screening method and is at least as sensitive as smear microscopy. This method could therefore be suitable for active case finding, especially where large numbers of samples are to be analyzed in resource-limited settings, to complement existing diagnostic techniques.

Discussion

Tuberculosis is one of the main causes of mortality worldwide and most cases occur in resource-limited as areas of Asia and Africa. Poverty is one of the biggest factors influencing TB diagnosis in these countries. Consequently, the implementation of new diagnostics for the more accurate and rapid diagnosis of TB can be extremely costly for resource-poor countries.

In many low-resource high-burden countries, microscopic examination of Ziehl-Neelsen stained sputum specimens is often the only tuberculosis test available due to its low cost (0.50 US dollar) for two sputum smear examinations. However, this method lacks sensitivity and performs poorly in young children and individuals who are immunocompromised. Also in these countries, many smear microscopy laboratories are single rooms and understaffed with poorly maintained microscopes, and some of these laboratories lack consistent sources of electricity and clean water.

Culture remains the gold standard for the diagnosis of TB. Different liquid broth automated systems have been developed and they have been essential for strengthening TB diagnoses, as they have a good sensitivity for isolation of mycobacteria and treatment in many countries [56]. The WHO recommends expanded use of liquid culture systems in resource-constrained settings; however, purchasing, maintaining, or providing large rooms to safely accommodate these instruments can be extremely difficult for most of the public health laboratories in these countries. Consequently, most resource-poor countries depend on LJ egg-based solid medium for the detection of growth of MTB isolates. LJ medium can be prepared locally, has good buffer capacity, has a shelf-life of several months when refrigerated, and supports the growth of most mycobacterial species. Chihota *et al.* compared the MGIT system with LJ medium, considering MGIT's cost effectiveness. Cost per culture on LJ, MGIT, and MGIT+LJ was, respectively 12.35, 16.62, and 19.29 US dollars [57]. These costs per culture were lower than estimates from

an another study in Zambia, where base-case throughput was substantially lower and overhead costs, which included transport and all resources not directly involved in performing the culture, were substantially higher. Comparable costs in this study were slightly higher than estimates from Dowdy *et al.,* probably due to the assumption of available infrastructure capacity with no additional cost used in the Dowdy study [58]. Generally, MGIT costs are high, but MGIT gives a higher yield and faster results compared with solid media. The MGIT system is sensitive to throughput and its use facilitates high throughput that few manual systems can match, and is therefore well suited to busy regional laboratories but is clearly less appropriate for a district laboratory receiving 30 samples per week.

Kidneye *et al.* compared micro-broth culture in microplate wells, where MTB growth was detected by microscopic observation, with LJ solid media. The first method is early, feasible, and inexpensive for detection of pulmonary tuberculosis. The cost was 4.56 US dollars per sample versus 11.35 US dollars for LJ solid media [59]. A limit of micro-broth culture is that an inverted light microscope to visualize the characteristic cording growth of MTB is required, which is not a standard piece of lab equipment, and few labs have this tool. Consequently, despite its low cost and good performance in the detection of MTB growth, the routine use of the broth culture method in resource-poor settings depends on the lab availability of the inverted light microscope. Several rapid molecular tests have been proposed for the rapid diagnosis of tuberculosis. Both in-house and commercial assays are available, and both have been evaluated in numerous studies performed in different settings with good sensitivity and specificity. Molecular test are also available for the detection of drug resistance; they have been evaluated both for first- and second-line antitubercular drugs. Xpert MTB/RIF is a new molecular assay that provides simultaneous detection of MTB and R resistance. This assay was specifically recommended for use as the initial diagnostic test for suspected drug-resistant or HIV-associated pulmonary tuberculosis. By June 2012, two-thirds of countries with a high tuberculosis burden and half of the countries with a high multidrug-resistant tuberculosis burden had incorporated the assay into their national tuberculosis program guidelines. The Foundation for Innovative and New Diagnostics negotiated a discounted pricing structure applicable to145 high-burden and developing countries. A four-module GeneXpert platform and

linked computer costs about US$17.00 (over 60% cheaper than elsewhere).

Thanks to funding from different organizations, the cost per cartridge was set at 9.98 US dollars beginning Aug 6, 2012, for the next 10 years [60]. Xpert MTB/RIF assay implementation for routine use in resource-limited settings has potential benefits as well as challenges. The most important benefit is the increase in TB case detection with reduction in mortality, morbidity, and TB transmission. Molecular platforms can also reduce the time to diagnosis and treatment, and reduce the need for culture and biohazard. However, implementation of the Xpert MTB/RIF in resource-limited settings, especially in peripheral health facilities, has required investments in training of operators and laboratory staff. Environmental limitations (stable and regular electricity, adequate room temperature) and difficulties involved in supply and maintenance are others factors that limit the use of this method. It should be possible to offer the test fairly rapidly at the main provincial or regional referral hospitals, where more services can and should be offered to patients. Alternative methods for diagnosing drug resistance such as NRA assays and the MODS method could be implemented if an inverted light microscope is available. Both can also be used as culture assays, and both are low cost non-commercial tests (ranging in cost from 3.00 to 4.00 US dollars and 1.40 to 3.50 US dollars, respectively), have standardized methods and protocols, use minimal equipment, and are readily available. Nevertheless, NRA on solid media uses a modified LJ media and should thus be straightforward for laboratories already performing LJ cultures, although the need for repeated opening of cultures demands category III level laboratory biosafety. With the MODS assay, biosafety is good because cultures are never opened; thus, it can be performed in a category II facility. Other tests such as the urine-based diagnostic assay and immunochromatografic test are rapid and inexpensive methods (3.50 and 2.03 US dollars, respectively) for diagnosing tuberculosis. In particular, the urine-based test, despite its apparent sub-optimal sensitivity, may be an important tool that my serve to greatly expedite TB diagnosis and treatment in those with advanced HIV-associated immunodeficiency, potentially reducing mortality risk. In recent years, operant discrimination training procedures have been used to teach giant African pouched rats to detect tuberculosis in human sputum samples. Available data suggest that pouched rats, which can evaluate many samples

quickly, are sufficiently accurate in detecting TB to merit further investigation as a diagnostic tool.

Budgetary constraints are a major consideration that has affected the choice of diagnostic assay in developing countries. In limited settings, a cost-effectiveness analysis of the introduction of a new TB diagnostic is very important. The choice between five 10 US dollars tests or one equally reliable 50 US dollars test might appear straightforward. However, if the 50 US dollars test provides the result for one patient at particular high risk in one day, this might be more useful than having results for five people one week later.

Modeling studies have estimated that 400,000 lives could be saved each year with the introduction of an accurate, rapid, and widely available TB diagnostic system with a sensitivity greater than 85% for both smear-positive and smear-negative cases, and 97% specificity. In addition, modeling has shown that expanding TB culture and drug susceptibility testing could lead to reduced mortality, especially for those patients infected with MDR-TB, and there is significant potential that TB culture and new diagnostics could be effective and cost-effective for testing HIV-positive patients in resource-poor settings.

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