



Tuberculosis in Resource- Limited Settings: A Need for Diagnostic Algorithm

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Abstract

In spite of huge burden of tuberculosis (TB) worldwide, detection of infectious cases continues to be low, thus jeopardizing global TB control efforts especially in resource-limited high burden countries. It is no longer news that vast majority of global TB is diagnosed by a century old method - smear microscopy. Though the method is inexpensive and simple to use, it performs poorly for the diagnosis of TB/HIV co-infections, childhood TB, extra-pulmonary TB and detection of multidrug resistant *Mycobacterium tuberculosis* (MDR-TB). Although, isolation of the causative pathogen in pure culture is the definitive bacteriological diagnosis of TB, it takes days or weeks to accomplished, thus leading to delayed therapy. Rapid diagnostic tools such as Line probe assay (LPA) and GeneXpert/Rif have revolutionarized TB diagnosis in recent times. However, apart from high cost of equipment and consumables, LPA requires elaborate laboratory space and trained laboratory workers. The major limitation to use of GeneXpert/Rif is the high cost of the equipment and the need for annual calibration. This review aims at highlighting the various methods employed in the diagnosis of active TB and their limitations. It consists of literature search of relevant journals and chapters in books and focuses on microscopy, culture and rapid diagnostic methods in TB. The review has revealed the need to develop an algorithm for use of TB diagnostic tools in resource -limited high burden countries in order to reduce the burden of the disease in the community.

Keywords: Tuberculosis, Diagnosis, Algorithm, Resource-limited settings

INTRODUCTION

Mycobacterium tuberculosis, the aetiological agent of tuberculosis (TB) is considered to be under- diagnosed despite the burden of the disease worldwide. About one-third of the world's population is affected by TB (Lauzardo and Ashkin, 2000) while nearly 2 million out of the estimated 9 million people that developed the disease died from the infection each year (Corbett *et al.*, 2003; WHO, 2005). It is estimated that 1.7 million people died from TB in 2009, many of whom were undiagnosed (WHO, 2011). In spite of this huge global TB burden, detection of cases continues to be low (WHO, 2011) thus, posing major impediment to TB control strategies especially in high burden countries with limited- resources.

Conventional TB diagnosis still relies on smear microscopy, isolation of the causative organism on culture and radiological findings. These tests are known to have limitations (Foulds and O'Brien, 1998; Perkins, 2000; Perkins and Small, 2006). For example, while smear examination for acid-fast bacilli (AFB) is the most widely used method for diagnosis of pulmonary TB (PTB) in developing countries, the test performs poorly for diagnosis of extra-pulmonary TB, childhood TB and TB/HIV co-infections. Furthermore, smear AFB examination is not good enough for monitoring of patients on TB treatment and also for detection of MDR-TB which is of great public health importance.

Modern rapid diagnostic tools including molecular techniques such as the line- probe assay (LPA) and GeneXpert MTB/RIF (Xpert) are not readily available in many TB centres in developing world. The need for a more sensitive and cost - effective test for diagnosis of infectious TB including drug resistant strains is a task that needs to be addressed by all stakeholders (Pai *et al.*, 2006). This review therefore attempts to provide information on available TB diagnostic tools that can be adapted for use in high burden countries with limited resources.

Microscopy

It is generally known that accurate and early diagnosis of TB is crucial for effective treatment of infectious cases and global TB control strategy. Smear microscopy for AFB is the World Health Organization (WHO) recommended method for diagnosing TB in high burden countries. This method has low specificity as it does not distinguish between pathogenic mycobacteria from non-pathogenic species. Also, it is inadequate for diagnosing TB in patients with TB/HIV co-infections, extra-pulmonary TB and TB in children. Despite these limitations, smear microscopy remains the cornerstone for diagnosis of TB in resource-limited settings (WHO, 2011).

Systematic reviews have provided strong evidence that fluorescence microscopy (auramine stained smear microscopy) is more sensitive than conventional light microscopy (Ziehl - Neelsen (ZN) stained smear microscopy) without significant loss in specificity (Steingart *et al.*, 2006). Furthermore, the use of bleach or centrifugation has been shown to be effective in increasing the yield of smear

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microscopy (Steingart *et al.*, 2006; Pai *et al.*, 2008). In addition to informing evidence-based TB diagnosis, systematic reviews have also helped to inform policy decisions on TB (Pai *et al.*, 2008). For example, it has been shown that smear microscopy can be optimized by using three approaches namely: (i) chemical and physical processing for concentration of sputum, (ii) use of fluorescent microscope as against conventional light microscope and (iii) examination of two sputum specimens instead of the traditional three samples (Steingart *et al.*, 2006a, b; Mase *et al.*, 2007). These findings which have been incorporated into the International Standard of Care (Hopewell *et al.*, 2006) have helped to inform policy guidelines on the diagnosis of smear-negative TB in TB/HIV co-infected individuals (Getahun *et al.*, 2007). Mase *et al.*, in 2007 reported that the average incremental yield or increase in sensitivity of examining a third sputum specimen ranged between 2% -5% . This indicates that reduction of the number of recommended number of specimens examined from three to two does not significantly reduce the sensitivity of smear microscopy (Pai *et al.*, 2008).

The WHO revised its policies on smear microscopy based on these findings (WHO, 2008). Thus, WHO now recommends that the number of specimens to be examined for diagnosis of TB cases be reduced from three to two, especially in settings where there is (i) a well -functioning external quality assurance system,(ii) very high laboratory workload and (iii) limited human resources (WHO, 2008a). The revised WHO case-defining definition for a new sputum smear-positive TB is based on the presence of at least one AFB in at least one sputum specimen (WHO, 2008b).

This new WHO policy has significant impact in centres where smear microscopy is the only available diagnostic tool. Omitting the third sputum specimen will potentially reduce costs, reduce time to diagnosis and alleviate the workload of laboratories especially in settings with inadequate manpower. Furthermore, adopting the two smear policy may create opportunity to examine both smears during patient's presentation to a health care facility hereby, reducing the number of patients who might probably drop- out during diagnostic process (Squire *et al.*, 2005). Hence, there is a need to collect more data to further provide support for this policy.

Both ZN and auramine smear microscopy are unable to differentiate viable bacilli from dead bacilli hence they are not useful for treatment monitoring. Fluorescein - diacetate (FDA) smear microscopy is a simple and instant method used for detecting viable from dead bacilli (Schramm *et al.*, 2012). It is useful to monitor response to TB treatment thus, help to differentiate those patients that still have viable bacilli following treatment (drug resistant strains) from those with dead bacilli who are responding to treatment. This becomes imperative because of the urgent need to diagnose multi-drug resistant *M. tuberculosis* (MDR-TB) and also not to prolong drug use for patients who are responding to treatment. FDA serves as a better alternative to mycobacterial culture that requires longer incubation period and significant laboratory infrastructure. This culture facility is not readily available in many centers in resource- limited settings.

Culture on solid media

Culture of the causative organism is the definitive diagnosis of TB. Isolation of *Mycobacterium tuberculosis* in pure

culture is regarded as gold standard and forms a baseline for species identification, determination of resistant strains and genetic epidemiological studies. Conventional solid culture medium makes use of egg-based Lowenstein - Jensen (L J) medium. Glycerol incorporated into egg-based L J medium is used for isolation of *Mycobacterium tuberculosis* while *Mycobacterium bovis* requires L J medium containing sodium pyruvate (Moore *et al.*, 2006a) . Isolation on LJ medium requires 6 - 8 weeks period of incubation because of the slow doubling time of mycobacteria species (15-20 hours) compared with other bacteria (Caviedes and Moore, 2007). The major limitation of use of solid culture method is the diagnostic delay arising from the need for long incubation period. This leads to delay in initiation of therapy and subsequent dissemination of the infection. Apart from this drawback, culture method requires significant laboratory infrastructure which is not available in many centers in high burden countries.

Rapid solid culture system for example TK medium (Salubris, InC., MA, USA) used for TB pathogen isolation has been recommended to replace convectional L J medium because of its' rapid turnaround time, easily practicability, low cost and simplicity of use. However, published evidence on the test is limited (Bayan *et al.*, 2004; Kocagoz *et al.*, 2004).

Culture in Liquid media

Automated liquid culture systems were introduced for isolation of mycobacteria species in order to circumvent the diagnostic delay associated with solid culture method. BACTEC 960 MGIT (Mycobacterium Growth Inhibitory Tube) is a modification of BACTEC 460TB system (Becton Dickinson InC., NJ, USA). It is a state of the art, non-radioactive, non-invasive in-vitro diagnostic instrument used for rapid detection of mycobacteria and drug susceptibility testing (DST). It utilizes a modified 7H9 Middlebrook broth base with 0.25% glycerol (7ml) with an oxygen quenching fluorescent sensors embedded in silicon at the bottom to detect microbial growth directly from clinical specimens. However, high cost of equipment including consumables and erratic power supply may hinder its' use in resource -limited environment.

The urgent need for improved diagnostic tools for detection of TB and MDR-TB has been previously highlighted (Caviedes and Moore, 2007). To have an impact on TB control and patient care in resource-limited high TB burden settings, such tool needs to be affordable, rapid, robust and technically straightforward. The microscopic observation drug susceptibility assay (MODS) (Moore *et al.*, 2004; Moore *et al.*, 2006a) may be useful in this regard.

MODS depends upon three principles namely: (i) *Mycobacterium tuberculosis* grows faster in liquid (broth) than on solid medium, (ii) *M. tuberculosis* grows in visually characteristic manner (tangles, cording) in liquid culture which can be observed under the microscope long before the naked eye could visualize colonies on solid agar, (iii) incorporation of anti-TB drugs into broth cultures at the onset of the test enables direct susceptibility testing from sputum samples. MODS is a tissue-culture plate based- assay utilising observation of Middlebrook 7H9 cultures with an inverted light microscope to detect the characteristic tangles of *M. tuberculosis* in liquid media. Drug - containing and drug-free control wells allow concurrent DST for rifampicin

and isoniazid. Moore *et al.* in 2006(a) while evaluating MODS for diagnosis of TB reported 98% sensitivity for MODS compared with 84% sensitivity for L J while speed of detection was found to be seven days compared with 26 days for L J.

The MODS methodology is straightforward and the standard operating procedure is simple to follow. The method is associated with a significantly lesser workload than conventional cultures and contamination rate was reported to be 50% that of L J medium (Caviedes and Moore, 2007). Concerns about biosafety which is of major issue with other liquid cultures do not arise when using MODS method because spillage of mycobacterial "soup" do not occur as inoculated MODS plates are permanently sealed in zip-lock polythene bags through which microscopic examination takes place. Furthermore, the test is associated with low frequency of cross-contamination and aerosolisation because the method employs direct DST which does not require any secondary sub-culture or any other manipulation (Moore *et al.*, 2006b). However, being a mycobacterial culture, it is recommended to use a biological safety cabinet. The high cost of an inverted light microscope may limit its use in resource-limited settings.

Radiology

Diagnosis of PTB, the commonest manifestation of TB relies on chest radiological investigations in settings with little or no laboratory services. In such settings however, non-availability of specialist radiology support, financial constraints and erratic power supply are some of its limitations. A survey in Malawi showed that medical officers misdiagnosed about one-third of suspected PTB patients based on radiological findings (Nyirenda *et al.*, 1999). This shows the inadequacies of chest X-Ray as a TB diagnostic tool. In addition to this, the test may not be helpful for diagnosis of smear-negative PTB in TB/HIV co-infected individuals due to lack of typical radiological findings (Siddiqi *et al.*, 2003; Kehinde and Dada-Adegbola, 2013).

Molecular methods

Nucleic acid amplification (NAA) tests : The advent of molecular methods have revolutionized TB diagnosis. Nucleic acid amplification (NAA) tests amplify target nucleic acid regions that uniquely identify *Mycobacterium tuberculosis* complex (MTBC). Nucleic acid amplification tests are also called direct amplification methods because they can be applied directly on clinical specimens. They can be categorized into two namely: commercial kits and in-house assays. Examples of commercial NAA tests include Amplicor MTB tests (Roche Diagnostic Systems, Branchburg, NJ) and Amplified *Mycobacterium tuberculosis* Direct test (MTD) (Gen-Probe, InC., San Diego, CA) while in-house assays are laboratory-developed PCR assays. The amplicor MTB test is based on PCR. The mycobacterial DNA is amplified with genus-specific primers formulated on the basis of the 16S rRNA. The results from amplicor MTB assay are available in about 7 hours (Soini and Musser, 2001). The sensitivity of the test compared with culture is 79.4 - 91.9%, while the specificity is 99.6 - 99.8% (Soini and Musser, 2001).

The reliability and accuracy of NAA tests as TB diagnostic tool has been extensively studied (Pai, 2004; Brodie and Schluger, 2005; Nahid *et al.*, 2006). The sensitivity of NAA tests is high in smear positive PTB suspects while it is reported to be low in paucibacillary forms of TB (smear-negative PTB) (Nahid *et al.*, 2006). Current evidence suggests that NAA tests should be used and interpreted in conjunction with conventional tests (smear microscopy and culture) and clinical data and not in isolation (Flores *et al.*, 2005). NAA tests have high specificity and positive predictive value, and these characteristics confer value in terms of its ability to confirm *M. tuberculosis* especially in PTB patients with smear positive sputum (Brodie and Schluger, 2005). These tests however, have relatively lower sensitivity and negative predictive value, particularly in PTB patients with smear negative sputum (Nahid *et al.*, 2006). Thus, a negative test does not rule out diagnosis of PTB. Therefore, NAA tests are not of use whenever sputum smears are negative and there is low clinical index of suspicion. NAA tests are not useful for monitoring of patients undergoing treatment because the tests amplify both live and dead mycobacteria thus, does not distinguish between viable and non-viable bacilli.

Line-probe assay : Line probe assays (LPAs) are a family of novel strip tests that use PCR and reverse hybridization methods for rapid detection of MTBC and simultaneously detect genetic mutations related to drug resistance to isoniazid and rifampicin. The magnitude of MDR-TB -that is, *M. tuberculosis* isolates that are resistant to both rifampicin and isoniazid is largely unknown in many of the resource-constrained high burden countries because of inadequate diagnostic facilities (WHO, 2013a). Line probe assay is performed by extracting DNA from cultures or directly from clinical specimens. Rifampicin resistance is determined by amplification of the *rpoB* gene sub-region while isoniazid resistance is detected by the presence of the amplified *katG* gene and *inhA* gene using PCR. The PCR products are then hybridized with the immobilized probes and results determined by colometric method. Commercially available LPA kits include the INNO-LiPA Rif. TB kit (Innogenetics, Gent, Belgium) and the GenoType MTBDR assay (Hain Lifescience, Nehren, Germany).

Line probe assays have been evaluated as TB diagnostic tool using smear positive samples, smear negative samples and culture positive specimens in a variety of settings (Hillemann *et al.*, 2005; Ling *et al.*, 2008; Crudu *et al.*, 2012). LPAs have been endorsed as a diagnostic tool by the WHO (WHO, 2008). The high cost of LPAs kits, the need for sophisticated laboratory infrastructure and non-availability of skill manpower limit its use in resource-poor settings.

Gene Xpert MTB (Rif): To respond to the urgent need for simple and rapid diagnostic tool at the point of care in high burden countries, a fully automated molecular test for TB case detection and drug resistance testing was developed through public-private partnership (WHO, 2008). GeneXpert MTB (Rif) is a real-time PCR assay that amplify an MTB-specific sequence of the *rpo B* gene, which probed with molecular beacons for mutations within the rifampicin-resistance determining region (Piatek *et al.*, 1998; El-Hajj *et al.*, 2001; Urdea *et al.*, 2006). Testing is carried out on the MTB/RIF test platform (GeneXpert, Cepheid). This platform

integrates sample processing and PCR in a disposable plastic cartridge containing all reagents required for bacterial lysis, nucleic acid extraction, amplification and amplicon detection (Raja *et al.*, 2005). The only manual step is the addition of a bactericidal buffer to sputum before transferring a defined volume to the cartridge. The cartridge is then inserted into the GeneXpert system, which provides results within 2 hours. GeneXpert assay was found to identify more than 97% of all patients with culture-confirmed TB including more than 90% of patients with smear-negative disease according to a study done by Boehme *et al.*, in 2010. In view of the low sensitivity of smear microscopy for the diagnosis of PTB in patients with HIV infection, the increased sensitivity associated with GeneXpert (Boehme *et al.*, 2010), notably among patients with smear-negative disease is one of the important strengths of this test as a TB diagnostic tool.

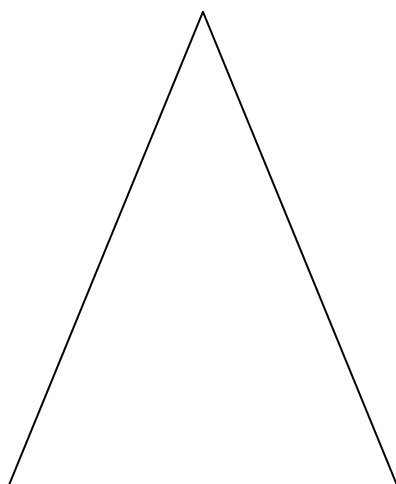
The need for annual calibration, coupled with high cost of the equipment have been identified as challenges for implementation at peripheral levels especially in resource-limited areas (Boehme *et al.*, 2010). The WHO through one of its agencies - Foundation for Innovative New Diagnostics (FIND) has negotiated the downward price of the test in order to make it affordable and accessible to majority of patients in poor-resourced settings (WHO, 2013a).

Globally, ineffective TB detection and the rise of multidrug resistance and extensively drug-resistant TB have led to calls for dramatic expansion of culture capability and drug susceptibility testing (DST) in high burden countries (WHO, 2006). Unfortunately, the infrastructure and trained personnel required for such testing are not available except in few reference centers. Laboratory results often take some months to be available which dramatically reduce its clinical utility (Yagui *et al.*, 2006; Urbanczik and Rieder, 2009).

Other advantages of GeneXpert test include (i) Little chance of amplicon contamination because the automated DNA extraction, amplification and detection take place inside a test cartridge which is not opened frequently; (ii) Its use eliminates the necessity for a biosafety cabinet because specimen processing is simplified to a non-precise step that both liquefies and inactivates sputum which eliminates generation of infectious aerosols. These features of simplicity and safety of use allow for cost-effectiveness and highly sensitive detection of TB and DST outside reference centers. This may ultimately increase access to testing and decrease delays in diagnosis, without the need to build large number of laboratories equipped with advanced bio-safety equipment.

Table 1.
Advantages and limitations of available bacteriological tests

Test	Advantages	Limitations
A. Smear microscopy (i) Light microscopy (ZN) (ii) Fluorescence microscopy (FM) (iii) Fluorescein-diacetate stained microscopy (FDA)	Rapid, cheap, specific in high TB - burden settings, easy and simple to implement. More rapid and more sensitive than ZN. Can detect viable bacilli.	Low sensitivity. Not useful for detecting resistant strains eg MDR (TB). Cannot detect viable bacilli. Requires additional cost and more training than ZN. Cannot detect viable bacilli. Still at research level. Not routinely used on bench.
B. Culture (i) LJ (ii) MGIT	More sensitive and more specific than smear microscopy. Can detect viable bacilli. Can detect MDR-TB. More rapid and more sensitive than LJ. Shorter incubation period compared with LJ. Can detect viable bacilli. Can detect MDR-TB.	Long incubation period. High cost. Procedure is more risky. Requires skilled personnel. More expensive than LJ. Higher rate of contamination. Requires steady electricity.
C. Molecular (i) Gene Xpert/Rif (ii) Line prone assay (LPA)	More rapid than culture. Rapid detection of (MDR-TB). Rifampicin as indicator. No need for skillful personnel. No need for biosafety laboratory. Point of care diagnostics. Rapid detection of MTBC and NTM. Rapid detection and confirmation of mono-resistance and MDR-TB.	Less sensitive than culture. Very expensive than culture. Need for annual calibration. Short shelf-life of reagents (15 months). Not good for follow-up. Need constant electricity. Requires big laboratory space (demarcated 3 cubicles). Highly trained personnel. Needs constant electricity.



THC (Good ZN+ FM+ Gene Xpert + Culture + FDA+LPA)
 SHC (Good ZN+ FM + Gene Xpert)
 PHC Facility (Good ZN/±FM)

Key: THC: Tertiary healthcare facility/Research Lab/National reference Lab

SHC: Secondary health care facility/ General hospitals

PHC: Primary health care facility

ZN: Ziehl-Neelsen smear light microscopy

FM: Auramine stained fluorescent microscopy

Gene Xpert: Gene Xpert/Rif

FDA: Fluorescent-diacetate stained smear microscopy

LPA: Line probe assay

Fig 1. Recommended diagnostic algorithm according to Health care facility

Being a NAA based test, GeneXpert is not useful for follow-up of cases on TB treatment as the test is very sensitive to detect DNA from dead bacilli in a patient on treatment. GeneXpert detects rifampicin resistance, a surrogate marker for MDR-TB strains. Hence, for confirmation of MDR-TB or rifampicin mono-resistance, there is a need for culture and DST using a second sputum sample making use of another method like LPA. This will allow for testing for isoniazid and other first-line drugs and possibly second-line drugs.

Since GeneXpert detects about 60-70% of the smear negative culture positive TB patients as seen in TB/HIV co-infected individuals (Boehme *et al.*, 2010), it then becomes imperative that TB suspects who are GeneXpert negative but HIV positive should have culture and DST done by LPA using a second sputum specimen. Culture may also be required for patients who are both HIV and GeneXpert negative. Such patients may benefit from antibiotic treatment for pneumonia and then reassessed for TB infection after full course of antibiotic therapy (Boehme *et al.*, 2010).

GeneXpert can be used for diagnosis of TB in children with improved sensitivity especially in children who are able to produce sputum, or whom induced sputum can be obtained (WHO, 2013b). Thus, low bacteriological yield often obtained from children suspected with TB using conventional methods (Kehinde *et al.*, 2008) can be overcome with the use of GeneXpert (WHO, 2013b).

GeneXpert is cost-effective and particularly useful in settings that have inadequate capacity to rapidly diagnose smear negative culture positive TB, high prevalence of HIV co-infection and MDR-TB (Vassall *et al.*, 2011).

Table 1 shows advantages and limitations of the available bacteriological tests for TB diagnosis in resource-limited countries. It is suggested that good quality assured smear microscopy (ZN stains) with or without fluorescent microscopy (FM) should be available in all primary health care facilities while culture and molecular-based assays such as line probe assay should be reserved for tertiary health care center and National reference laboratory. Fig 1 illustrates the diagnostic tests that can be used according to health care level.

In conclusion, TB diagnostic algorithm in resource-limited settings must take into consideration the burden of the disease, associated HIV co-infection, prevalence of drug-resistant strains, laboratory infrastructure and availability of skilled workers.

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