Review Article

A REVIEW ON LABORATORY DIAGNOSIS OF *MYCOBACTERIUM TUBERCULOSIS.*

Abstract. Tuberculosis remains the major public health concern worldwide responsible for about 1.6 million deaths and 0.3 million co-infected with Human immunodeficiency virus (HIV) annually. *Mycobacterium* is the causative agent of tuberculosis infection and is transmitted principally through air when an infected person coughs, talks, sneezes etc. This infection can be diagnosed using different Microbiological, Molecular and Immunological techniques including, sputum smear microscopy, sputum culture, nucleic-acid amplification test (NAAT), genotyping assay, tuberculin skin test (TST), interferon-gamma release assay (IGRAs) etc. These techniques vary in sensitivity and specificity as well as the ease with which they are carried out. World Health Organisation (WHO) encourages the use of techniques that are sensitive, patient-friendly, and those which produce accurate results in any clinical setting world-wide. Hence, this review highlights smear microscopy and incorporation of more rapid and sensitive diagnostic techniques such as Gene Xpert, IGRAs and urinary antigen analysis in clinical setting in the detection of *Mycobacterium.* These techniques show high sensitivity, are less time consuming do not require a repeat for a single result, some are able to differentiate latent and active TB infections, and have the capacity to be used to screen people unable to expectorate. This review encourages the incorporation of smear microscopy, GeneXpert, IGRAs, urinary antigen analysis into routine laboratory diagnosis especially in high TB burden countries. It is believed that high level of sensitivity and less time used in producing results display by these techniques will yield reduction in mortality rate, decline in static nature of TB status and possibly zero TB 2020 proposed by WHO.

Keys Words: *Mycobacterium tuberculosis,* Laboratory techniques, Challenges in MTB diagnosis, Sensitivity, and Limitations.

Introduction

Mycobacterium tuberculosis is a non-motile, non-spore forming, obligate aerobe and acid fast bacillus [1]. It is a pathogenic organism belonging to the family Mycobacteriacea. The family is divided into *Mycobacterium tuberculosis* complex (MTC), *Mycobacterium avium* complex (MAC) and non-tuberculosis Mycobacteria. Members of each group are shown in Table 1 below.

Table 1. Members of *M. tuberculosis and M. avium complex*

***Mycobacterium tuberculosis* complex (MTC)**	***Mycobacterium avium* complex (MAC)**
M. africanum	*M. avium*
M. bovis	*M. avium silvaticum*
M. bovis-BCG	*M. avium hominissiuis*

M. canetti
M. caprae
M. microti
M. mungi
M. orygis
M. pinnipedii
M. suricatae and M. tuberculosis
M. colcmbiense
M. indicus pranii
M. intracellulare and M. avium paratuberculosis

The non-tuberculous Mycobacterium (NTB) group causes pulmonary disease similar to tuberculosis [2].

M. tuberculosis was first discovered in 1882 by Robert Kock [3] as organism with several notable features such as: ability to enter non-replicating states for long period and cause latent infection, possession of waxy cell wall, slow growth rate in culture, intrinsic drug resistance and antibiotic tolerance [4]. Like all Mycobacteria, it is distinguished by its ability to form stable mycolate complexes with acryl methane dyes (Carbolfuchsin, auramine, and Rhoda mine).

The report of James *et al.* (2016) [1], states that about 98 % of TB cases are transmitted through aerosol when a person with pulmonary disease coughs, talks, sneezes etc. Once an infected droplet is inhaled, *M. tuberculosis bacilli* land in the alveoli where they are engulfed by alveolar macrophages. In some individuals, the immune system is able to clear the infection without treatment. In others, it subverts the alveolar macrophages and replicates inside the phagocyte for several weeks [5]. As the bacilli multiply, they are frequently carried into the regional lymph nodes by alveolar macrophages and can spread haematogenously to other sites including the lung apices, vertebrae, peritoneum, meninges, liver, spleen, lymph nodes and genitourinary tract.

WHO Report (2018) states that tuberculosis is one of the top 10 causes of death worldwide, adding that 10 million people were infected with TB in 2017 and 1.6 million lives were lost including 0.3 million co-infected with HIV [6]. Despite the 22 % fall in TB death rate between 2000 and 2015, WHO still regrets that there are information gaps due to under-reporting of TB cases, especially in countries with large unregulated private sector and under-diagnosis in countries with major barriers to accessing health care facilities; thus, causing reported reduction in rate of TB infection to remain static at 1.5 % from 2014-2015.
The National tuberculosis and Leprosy Control report in (2016) states that Nigeria was ranked 4th country with the highest cases of tuberculosis world-wide. The statistics also showed that over 80% of tuberculosis cases in Nigeria were still under-detected due to poor diagnostic techniques, as a result, over 1.6 million lives are lost annually in the country due to this infection.
The 2015 global TB report records that Nigeria and five other Countries, namely, India, Indonesia, China, Pakistan, and South Africa accounted for 60 % of total TB cases worldwide with 10.4 million new cases, about 1.8 million deaths and 0.4 million HIV co-infections. WHO in its 2016 “Global tuberculosis Report” pointed out that for the above mentioned countries to meet up with the global targets of disease eradication, quick attention need to be given to preventive, diagnostic and treatment procedures. Appropriate attention on these areas will move these countries from the current static TB status (1.5 %) to accelerated 4-5 % annual decline, the first mile stones of World Health Assembly approved “End TB Strategy” 2020. The present review seeks to evaluate commonly used laboratory techniques for detecting the presence of TB bacilli, their limitations base on time used in producing result.

Techniques Used In TB Diagnosis

Microbiological Techniques

Sputum Smear Microscopy

This technique has been the primary method for diagnosis of pulmonary tuberculosis in low and middle income countries where nearly 95-98% deaths due to TB occurs. It is a simple, rapid, and inexpensive technique that is highly specific in identifying highly infectious person. It is also very useful in areas with very high tuberculosis prevalence and widely applicable in various populations with different socio-economic levels, hence, has served as an integral part of the global strategy for TB control.

In this method, productive cough (sputum) is first digested in potassium hydroxide (KOH), then neutralize with hydrochloric acid (HCL) and concentrated by centrifugation. The sediment is smeared on a grease free slide, heat fixed and stained using Ziehl-Nelsen technique. The slides are subsequently examined for acid fast bacilli under the microscope [7].

Sputum Culture

Sputum culture is another microbiological technique used in detecting and identifying *Mycobacterium* in solid or liquid medium. In this technique, a loop-full of KOH digested sputum specimen is inoculated unto already prepared Lowenstein-Jensen (LJ) slopes in a screw-capped universal bottle and incubated aerobically at 37 ^{0}C for 8-10 weeks and observed regularly for growth. Slopes with insignificant growth after 10 weeks of incubation are not considered as positive result. Brown granular colonies (sometimes called "buff, rough and tough") indicate the presence of *M. tuberculosis.* Prominent and suspected colonies are smeared on slide and stained using Ziehl-Neelson (ZN) technique. This technique also give room for determination of sensitivity of the bacterium to different TB drugs [8].

Immunological Techniques

Tuberculin Skin Test (TST)

TST was the first assay introduced by Charles Monteux in 1908 and is still widely used as an important screening test for tuberculosis [10, 11]. It is used in determining previous infection with M.TB based on the principle of delayed hypersensitivity reactions to tuberculin antigen (purified protein derivative (PPD)). PPD is a cell-free purified protein fraction obtained from a human strain of M.TB consisting of more than 200 proteins. The reaction produced by TST may occur in patients with active tuberculosis, latent tuberculosis infection (LTBI) or in those previously immunized with Bacillus Calmette Guerin (BCG) vaccine. The delayed hypersensitivity reaction, however is not specific for all Mycobacterial infections. In this method, a vial of PPD usually 0.1 tuberculin unit (TU) is injected intradermally into the forearm of an individual. Previous exposure to *M. tuberculosis* induce an immune response in the skin injected and inoculated with the bacterial proteins within 48-72 hours after injection. Diameter of (a palpable raised, hardened area) of 5-15 mm across the forearm perpendicular to the long axis in millimetres is considered positive (Monteux tuberculin skin test DVD Transcript and Faculitor Note, 2003).

Interferon-gamma Release Assay (IGRAs)

Recently, Food and Drug Administration (FDA) US, approved three whole blood immunodiagnostic assays: QuantiFeron-TB Gold in Tube (QFT-GIT), T-SPOT and QuantiFeron Gold QFG). QuantiFeron-TB Gold in tube (QFT GIT) assay is based on region of difference-1 (RD-1) specific peptides of TB antigens (ESAT-6, CFP-10 and TB7.7). These antigens are made available commercially in tubes format for use in the screening of *in-vitro* specific immune response to *M. tuberculosis* [12]. QFT-GIT was the first whole blood test approved for the diagnosis of LTBI. However, the three techniques make use of blood but are different in screening ability and are reagent based. According to Centre for Disease Control (CDC) guidelines, these techniques can be used in all circumstances for which TST is currently being used, including evaluation of contacts of TB cases, recent immigrants vaccinated with BCG, differentiating infection by other M.TB complex and tuberculosis screening of health-care workers etc. QFT-GIT assay rely on the production of interferon gamma (IFN-Y), a potent pro- inflammatory cytokine released by T-cells and natural killer (NK) cells as a function of activation of macrophages. It is a reflective of adaptive T- cell responses to TB antigen.

A prominent vein of a consenting individual is aseptically swabbed and 1 mL of whole blood is collected into the three different tubes. One coated with ESAT-6, CFP-10 and TB7.7 TB antigen (detect CD4+ T cell responses to TB antigen), the second tube is a positive control mitogen that induces low response indicating inability to generate IFN-Y and a negative control tube with heparin (anticoagulant) alone. Tubes are inverted severally (9-10) times to solubilize contents. The blood is incubated for 16-24 hours at 37 ^{0}C. The amount of IFN-Y released from harvested plasma after incubation is quantified using Enzyme-linked Immunosorbent assay (ELISA) reader. The reading of the second and third tubes is subtracted from the first tube (nil tube), values equivalence to 35 International unit (IU) is regarded as positive for IGRAs (WWW.Qiagen.com). According to literature, this technique offers a more sensitive approach than the conventional TST in detecting LTB infection [13]. It is also believed to be a better indicator of the risk group of *M. tuberculosis* infection especially among BCG-vaccinated individual. Apart from its sensitivity, it is the most preferred in screening different calibres of people especially children and people that are very sick and have difficulty in expectorating sputum for microscopy and culture.

Urinary antigen detection

A promising immune-based approaches of directly detecting *M. tuberculosis* antigen known as lipoarabinomannan (LAM) in urine. LAM is a lipopolysaccharide in the cell wall of *M. tuberculosis* that is released from the M.TB and excreted in urine of an infected individual. It is a heat stable glycolipid specific to mycobacteria that is release by metabolically active bacteria, filtered by the kidney and found in the urine of patient with active TB. LAM was originally detected in serum, but this test was limited by immune complex formation.

For convenience, urine is easy to collect compared to sputum and may be less variable in quality and safer to handle. Recent studies have evaluated commercially available tests that detect LAM in urine by antigen capture ELISA for the diagnosis of tuberculosis. The LAM ELISA sensitivity ranged from 38 % to 50.7 % for TB cases, with a specificity range of 87.8 % to 89 %, as confirmed by smear microscopy, solid culture and liquid culture. The commercially available generation of lipoarabinomannan- enzyme-linked Immunosorbent assay (LAM ELISA) has adequate specificity but suboptimal sensitivity and does not appear to be useful as an independent diagnostic test to confirm or exclude pulmonary TB in either HIV-infected patients. However, these assays could increase case finding if combined with smear microscopy and culture in settings of high HIV prevalence and could be of particular

value in diagnosis TB in HIV-co-infected patients with CD4 cell count of less than 100 cells/ml.

Urine LAM assay testing is usually carried out on urine samples using Determine[d] TB LAM test (Alere Inc., Waltham, USA). Urine samples are centrifuged at 10,000 rpm for five minutes, 60 µl of clear supernatant is transfer to the test strip with two readers ((PKD) and LG). The result is interpreted after 25 minutes as positive for LAM assays graded from low band intensity to high band intensity (1-5) or invalid for LAM assays without positive control lines [14].

Volatile Marker

Volatile organic compounds (VOCs) represent a wide range of stable chemicals that are detectable in exhaled breath, urine, faeces, and sweat of an infected person. Shneh *et al.*, (2013) [15] reported that *M. tuberculosis*, among other microorganisms was listed as producing a characteristic foul smell. Volatile organic compounds from *in-vitro* cultured Mycobacterium species revealed several metabolites of nicotinic acid, such as methyl phenyl acetate, methyl phenyl arsonate, methyl nicotinate, and o-phenylanisole, which were considered specific for *M. tuberculosis* complex strains. These compounds represent derivatives of nicotinic acid with characteristic unpleasant smell. This diagnostic technique offers an option for developing rapid and potentially inexpensive disease screening tools. It based on detection of volatile organic compounds (VOCs) that are emitted from infected cells and released in exhaled breath. Exhaled breath is collected into a Tedlar bag connected to the inlet port of a micro reactor fused silica tube. The exit port of the micro reactor is connected to a vacuum pump through the other fused silica tube on the reactor. The setup for capturing of volatile organic compound includes a vacuum pump to pull gaseous breath samples from a Tedlar bag through the atmospheric-coated reactor. The pulled gaseous breath is evacuated into a vacuum. The volatile organic compound adducts are eluted and directly analysed by Fourier Transform-ion cyclotron resonance mass spectrometry (FT-ICR-MS). A known concentration of methanol was added to the eluent as an internal reference (IR). The concentrations of all volatile organic compounds in exhaled breath were determined by comparison of the relative abundance with that of added atmospheric (ATM) -acetone [16].

Bead-based Method for Diagnosing TB

This technique makes used of immune-magnetic beads detecting and identifying bacteria in routine clinical setting. In this approach, beads are coated with either monoclonal, polyclonal antibodies or with non-specific markers such as lectin. These serve to capture or target bacterial pathogens which are then concentrated and detected by different system.

Microsens Medtech Ltd (London, UK) developed a kit with paramagnetic beads coated with chemical ligand that binds to mycobacteria and capture *M tuberculosis* complex cells from the sputum of TB positive patients. Magnetic micro-particles have also been coated with anti-mycobacterial polyclonal antibodies. This can concentrate mycobacteria for direct identification by PCR or other rapid techniques. More recently, core-shell magnetic nanoparticles coated with anti-BCG monoclonal antibodies were used to target *M. bovis* BCG cells spiked into human sputum. Immuno-magnetic bead is an ideal point-of-care diagnostic tools especially in resource-limited setting.

Molecular Techniques

Cartridge Based Nucleic Acid Amplification Test (CB-NAAT)

This technique is used in the detection of *M. tuberculosis* DNA and Rifampicin-resistance mutation in 81-bp region of rpoB gene determining region (RRDR). A deep productive cough is collected from a suspected patient, 2:1ratio of the sample buffer is added to the specimen, shaken and allow to stand for 15 minute at room temperature. 2mL of inactivated material (equivalent to 0.5 ml of decontaminated pellet) is transferred to cartridge and inserted into MTB-RIF test platform for automatic filter and washed. The filter is captured for ultrasonic lysis to release the deoxyribonucleic acid (DNA) of M.TB. The DNA molecule is mixed with dry Polymerase chain reaction (PCR) reagent for amplification and detection. The result is ready in 1 hour 45 minute showing MTB/ Rifampicin (RIF) resistance detected in positive samples, not detected in negative samples and not clear for repeat [9]. Other molecular techniques include Genotyping and Spoligotyping not reviewed in detail here.

Table 2. Challenges in Laboratory Diagnosis Techniques in Detecting TB

Diagnostic Methods	Sensitivity and Limitation
Smear Microscopy	The sensitivity of this technique for the detection of *Mycobacterium tuberculosis* is approximately 45 to 75% [17]. Bacterial load impaired sensitivity rate especially when bacterial load is less than 10,000 organisms/mL of sputum sample. Poor track record in extra-pulmonary tuberculosis, paediatric tuberculosis and in patients co-infected with HIV. Requirement of series of sputum examinations make some patients diagnostic defaulters due to inability to come back for repeated sputum examinations. Some do not come back for results, and are lost to treatment and follow up.
Sputum Culture	Sputum culture has a higher sensitivity of between 81-97 % compare to sputum smear [18]. Limitation include; high risk due to the infectiveness of the bacillus. It can only be done in high containment laboratories operated by highly skilled personnel. High cost and skilled personnel are required. It takes long time to complete and issue results due to the slow growing nature of the bacillus.
Gene Xpert	The report of Arzu *et al.* (2011) [19] states that there is similarity in the level of sensitivity between MTB/RIF and smear positive culture and less sensitive with smear negative specimen. This technique requires a stable power supply, this often limits its value in poor resource countries that need it the most. Test equipment is capital intensive, shelf life of the cartridges is only 18 months. The instrument needs to be recalibrated annually and the temperature ceiling is critical. Provides limited information about TB drug resistance except rifampicin.
Tuberculin Skin Test	The work of De Keyser *et al.*, (2014) [20] reported that the specificity of TST and QFT is between 75-64% in smear negative specimens. There are limitations in result interpretation, making it difficult and controversial due to influences by various factors including age, immune status, mixed infections etc. Thus, TST has a lower specificity in populations with high BCG coverage and Non tuberculosis Mycobacterium (NTM). False positive tuberculin reactions often occur in individuals with other Mycobacteria infections caused by some antigens shared within the genus.
Interferon gamma release assay (IGRAs)	Both the machine and the reagents are capital intensive
Urinary antigen detection	High negative predictive value usually leads to negative result used as evidence against active TB.
Volatile marker	The origin of VOCs compound which is derivatives of nicotinic acid can be miss

	track.
Bead-based methods	It requires extensive specimen processing, the use of complex measurement setups, and are not easily scalable for clinical demands. Only TB positive sample is screen.

Conclusion

Laboratory diagnosis is an acceptable clinical routine practice used in screening and detection of a causative agent(s) of a particular infection. Different laboratory techniques are employed in detecting the presence of this organism in routine clinical practice. However, some of these techniques have limitations that serve as a contributing factor to increase in mortality rate as a result of delay in isolation, identification and treatment. The speed of spread and invasive nature of the infection posed serious threat to public health. Thus, WHO regular evaluation of TB diagnostic techniques encourages the introduction of rapid, more sensitive techniques in routine clinical setting to combat delay in TB detection and treatment. The present review encourages the use of rapid and simple techniques such as smear microscopy, GeneXpert, IGRAs, urinary antigen analysis in routine laboratory techniques. This will serve as a better strategy for early TB detection and commencement of treatment, reduction in mortality rate as well as actualization of WHO dream of zero TB 2020.

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