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## Advancement in the diagnosis of Tuberculosis

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### Executive Summary

Tuberculosis is still one of the leading causes of mortality and morbidity among the infectious diseases in developing and in developed countries. The domestication of cattle, thought to have occurred between 10,000 and 25,000 years ago, would have allowed the passage of a mycobacterial pathogen from domesticated livestock to humans, and in this adaptation to a new host, the bacterium would have evolved to the closely related *M. tuberculosis*. Delayed or misdiagnosis and poor treatment, particularly in resource-poor settings, has aggravated the transmission of TB. Inability to detect and treat all infectious cases of pulmonary tuberculosis in a timely fashion has allowed continued *M. tuberculosis* transmission within communities and caused failure to control its spreading. Currently there has been a marked progress in the development of drug and testing tools to detect TB complex and of the resistant isolate. These diagnostic tools are expected to provide rapid and accurate information for detecting resistance to guide selection of the treatment regimen for each patient. The contribution of globally representative genotypic, phenotypic, and clinical data in achieving the need of accurate diagnosis is tremendous. The advancement of new diagnostics facilitate clinical decision making, and improve surveillance for drug resistance.

**Key Words:** Diagnosis,HIV, MDR, Treatment, Tuberculosis

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## Background

Tuberculosis (TB), one of the oldest recorded human afflictions, is still one of the biggest killers among the infectious diseases, despite the worldwide use of a live attenuated vaccine and several antibiotics. New vaccines and drugs are needed to stem the worldwide epidemic of TB that kills two million people each year (1).

TB has many manifestations, affecting bone, the central nervous system, and many other organ systems, but it is primarily a pulmonary disease that is initiated by the deposition of *Mycobacterium tuberculosis*, contained in aerosol droplets, onto lung alveolar surfaces. From this point, the progression of the disease can have several outcomes, determined largely by the response of the host immune system. The efficacy of this response is affected by intrinsic factors such as the genetics of the immune system as well as extrinsic factors, *e.g.*, insults to the immune system and the nutritional and physiological state of the host (1, 2).

In addition, the pathogen may play a role in disease progression since some *M. tuberculosis* strains are reportedly more virulent than others, as defined by increased transmissibility as well as being associated

with higher morbidity and mortality in infected individuals. Despite the widespread use of an attenuated live vaccine and several antibiotics, there is more TB than ever before, requiring new vaccines and drugs and more specific and rapid diagnostics (1, 3).

## Microbiological Prosperities of *M. tuberculosis*

The tubercle bacilli are non-spore-forming, non-motile, pleomorphic, weakly gram-positive curved rods 2–4µm long. They may appear beaded or clumped in stained clinical specimens or culture media. They are obligate aerobes that grow in synthetic media containing glycerol as the carbon source and ammonium salts as the nitrogen source (4). These Mycobacteria grow best at 37–41°C, produce niacin, and lack pigmentation. A lipid-rich cell wall accounts for resistance to the bactericidal actions of antibody and complement. A hallmark of all Mycobacteria is acid fastness the capacity to form stable Mycolate complexes with Arylmethane dyes such as crystal violet, Carbol-fuchsin, Auramine, and Rhodamine. Once stained, they resist de-coloration with ethanol and hydrochloric or other acids (4).

Mycobacteria grow slowly, their generation time being 12–24 hr. Isolation from clinical

specimens on solid synthetic media usually takes 3–6 wk, and drug susceptibility testing requires an additional 4 wk. Growth can be detected in 1–3 wk in selective liquid medium using Radiolabeled nutrients, and drug susceptibilities can be determined in an additional 3–5 days. Once mycobacterial growth is detected, the species of Mycobacteria present can be determined within hours using High Pressure Liquid Chromatography analysis, as each species has a unique fingerprint of Mycolic Acids (4).

#### **Biochemical Properties of *M. tuberculosis*.**

The cell wall of the bacteria is composed of two segments, upper and lower. Beyond the membrane is peptidoglycan (PG) in covalent attachment to arabinogalactan (AG), which in turn is attached to the Mycolic Acids with their long meromycolate and short  $\alpha$ -chains. This is termed the cell wall core - the mycolyl arabinogalactan–peptidoglycan (mAGP) complex (4).

The upper segment is composed of free lipids, some with longer Fatty Acids complementing the shorter  $\alpha$ -chains, and some with shorter Fatty Acids complementing the longer chains. Interspersed somehow are the cell wall proteins, the phosphatidylinositol

mannosides (PIMs), the phthiocerol containing lipids, lipomannan (LM), and lipoarabinomannan (LAM)(4,5). These lipid, protein and carbohydrate complexes can be considered as parts of signaling, effector molecules in the disease process and the insoluble core is essential for the viability of the cell and should be addressed in the context of new drug development (5).

#### **Mycolic Acids**

Mycolic Acids have been one of the defining features of bacteria such as *M.tuberculosis* and *M .leprae*, belonging to the Actinomycetales order (6). Chemically, they are long chain high molecular weight  $\alpha$  - alkyl- $\beta$ -hydroxy Fatty Acids consisting of an  $\alpha$ -meromycolate moiety, with carbon chain lengths of up to C<sub>56</sub> and a long saturated  $\alpha$  - branch of C<sub>20</sub> to C<sub>24</sub> (5,7). These compounds, which form the principal components of mycobacterial envelopes, confer unique properties to these organisms, such as unusually low permeability and consequent resistance to common antibiotics (7). They are also known to exhibit adjuvant immunological properties (6).

*Mycobacterium tuberculosis* produces three main types of Mycolic Acids: Alpha-, Methoxy-, and Keto-. Alpha-Mycolic Acids comprise at least 70% of the Mycolic Acids

present in the organism and contain several cyclosporine rings. Methoxy- Mycolic Acids, which contain several methoxy groups, comprise between 10% and 15% of the Mycolic Acids in the organism. The remaining 10% to 15% of the Mycolic Acids are keto- Mycolic Acids, which contain several ketone groups (6,8). The presence of Mycolic Acids gives *M. tuberculosis* many characteristics that defy medical treatment. They lend the organism increased resistance to chemical damage and dehydration, and prevent the effective activity of hydrophobic antibiotics. In addition, the mycolic acids allow the bacterium to grow readily inside macrophages, effectively hiding it from the host's Immune Systems. Mycolate biosynthesis is crucial for survival and pathogenesis of *M. tuberculosis* (8).

Mycolic Acids and its pathogenicity arise from strategies that have developed to survive in host cells, including the ability to colonize macrophages, and to remain quiescent and then become active decades later. *M. tuberculosis* also has an unusual cell wall containing mycolipids with Fatty Acid chain length of sixty or more carbon atoms (9).

**Lipoarabinomannan (LAM)** is a complex glycolipid consisting of repeating

Saccharide units of arabinose and mannose linked to a phosphatidylinositol moiety. This Phospholipid moiety, with palmitate and tuberculostearate(methyloctadecanoate) as the major acyl groups, apparently attaches LAM to the cytoplasmic membrane of the bacilli (10). Purified LAM from virulent and attenuated strains of Mycobacteria may differ structurally, and these differences may contribute to their varying abilities to stimulate cytokine production in mononuclear cell cultures (11).

LAM is a glycol-conjugate and one of the virulence factor associated with *M. tuberculosis*. LAM inhibits T cell proliferation and bactericidal activities of macrophages(12). In addition, LAM eliminates cytotoxic oxygen-free radicals produced by macrophages and inhibits the activity of protein kinase C and also blocks the activation of gamma interferon at transcriptional level. LAM release TNF may be responsible for the characteristics of tuberculosis, such as, loss in weight, fever, and cytokine-mediated necrosis (12, 13).

**Arabinogalactan (AG)** is an essential cell wall component. It provides a molecular framework serving to connect peptidoglycan to the outer Mycolic Acid layer. The biosynthesis of the arabinan domains of AG

and LAM occurs via a combination of membrane bound arabinofuranosyltransferases, all of which utilize decaprenol-1-monophosphorabinose as a substrate (14).

### **Advancement in the Diagnosis of *M. tuberculosis***

The need for a more accurate, inexpensive tuberculosis diagnostic test is applicable in tuberculosis and HIV endemic areas is greater nowadays than ever before, and will be crucial for achieving global tuberculosis control(14). Several modeling studies suggest that new diagnostics for tuberculosis disease and MDR tuberculosis could have an important effect within populations, especially in disease endemic countries, although improving population health and health services, and economic growth, might be as important (15).

Cytokine interferon gamma (IFN- $\gamma$ ) plays an important role in the protective immune response against TB is indicated by the susceptibility of mice and humans with IFN- $\gamma$  signaling pathway deficiencies to TB disease (13). Its detection in isolation however is not a sufficient indicator of a protective immune phenotype as those with latent infection and a positive IFN- $\gamma$  release

assay status can progress to active disease and IFN- $\gamma$  secretion can also be detected in samples from patients with active disease (16).

Lack of rapid and accurate diagnosis and case detection are major obstacles to TB control. TB diagnosis, even today, continues to rely heavily on tools such as direct smear microscopy, solid culture, chest radiography, and tuberculin skin testing tools that often performs poorly, and require infrastructure frequently unavailable in the periphery of the health system where patients first seek care (16, 17). The limitations of the existing diagnostics toolbox have been exposed by HIV epidemic, and by the emergence of MDR-TB and XDR-TB. Diagnostic delays and health system failures often result in missed or late diagnoses, with serious consequences for TB patients (17).

### **Diagnostic tests for latent TB infections (LTBI)**

There are currently two diagnostic methods that support the diagnosis of LTBI: the tuberculin skin test (TST) and Interferon-Gamma Release Assays (IGRAs). Both tests are immunological methods that detect an immune response to antigens and

consequently do not allow a direct measure of persistent infection (16).

The in vivo TST is based on the intracutaneous injection of *M. tuberculosis* antigens and subsequent identification of an immune reaction at the site of injection. A limitation of the TST is that the complex mixture of different antigens used are not specific for *M. tuberculosis*, and therefore local immunologic activity at the site of the antigen deposition does not differentiate between an existing immune response elicited by either, previous Bacillus BCG vaccination, exposure to Nontuberculous mycobacterial (NTM), or *M. tuberculosis* infections(15,16).

IGRAs are more recent in vitro assays that detect the presence of cellular immune responses towards *M. tuberculosis* specific antigens. These include the Early Secretory Antigenic Target-6 (ESAT-6), Culture Filtrate Protein 10 (CFP-10), and the TB7.7 antigens (17). In contrast to the TST, the antigens in IGRAs are absent in most of NTM, as well as from BCG strains. Although IGRAs cannot distinguish between active TB and LTBI, IGRA-results are not confounded by BCG vaccination and less likely to be confounded by exposure to NTM (16, 17).

## **Tuberculin Skin Testing (TST)**

In 1891, Robert Koch discovered that components of *M. tuberculosis* in a concentrated liquid culture medium, subsequently named "old tuberculin" (OT), were capable of eliciting a skin reaction when injected subcutaneously into patients with tuberculosis. In 1932, Seibert and Munday purified this product by ammonium sulfate precipitation to produce an active protein fraction known as tuberculin purified protein derivative (PPD) (16).TST is most widely used in screening for LTBI. The test is of limited value in the diagnosis of active tuberculosis because of its relatively low sensitivity and specificity and its inability to discriminate between latent infection and active disease (17).

False negative reactions are common in immunosuppressed patients and in those with overwhelming tuberculosis. False positive reactions may be caused by infections with nontuberculous Mycobacterial and by Bacillus BCG vaccination (18).The greatest limitation of PPD is its lack of mycobacterial species specificity, a property due to the large number of proteins in this product that are highly conserved in the various species. In addition, subjectivity of the skin-reaction

interpretation, deterioration of the product, and batch-to-batch variations limit the usefulness of PPD (19).

### **Interferon-Gamma Release Assays (IGRA's)**

Upon infection with *M. tuberculosis*, the different subsets of immune cells involved in the immune response directed against the bacilli do not fully eradicate the bacilli, but rather contain the infection. Macrophages play an important role in the first line of defense against pathogen infection through their ability to ingest and subsequently kill pathogens. However, having developed immune escape mechanisms, *M. tuberculosis* bacilli have the ability to persist within macrophages, averting the attack by these host cells (19).

The cytokine interferon-gamma (IFN- $\gamma$ ) is produced by different cells of the immune system: CD<sub>4</sub> T-cells, CD<sub>8</sub> T-cells and Natural Killer cells (19). This cytokine is considered to play an important role in the elimination of *M. tuberculosis* by activating the production of reactive oxygen and nitrogen intermediates in macrophages, which in turn are involved in the destruction of bacterial pathogens (20). T-cells specifically recognizing *M. tuberculosis*

antigens, particularly CD<sub>4</sub> T-cells, produce IFN- $\gamma$  essential for the activation of *M. tuberculosis* infected Macrophages which, upon activation, can target *M. tuberculosis* bacilli and control their growth (19, 20).

In general, infection with *M. tuberculosis* triggers a complex immune response that, in most individuals, leads to the containment of the infection and the establishment of a pool of long-lasting memory T-cells specifically directed against *M. tuberculosis* antigens (19). IGRAs are blood-based tests assessing the presence of effector and memory immune responses directed against the *M. tuberculosis* antigens ESAT-6, CFP-10 and, in one of the available tests, the TB7.7 antigen (20). The IGRAs have been shown to predominantly measure the presence of *M. tuberculosis* -specific effector memory T-cells, the presence of which are considered indicative of previous in vivo exposure to the bacilli. Blood samples might also contain central-memory T-cells specific to the *M. tuberculosis* antigens and thus be measured in the assays. The latter is however seen as less likely, as this subset of cells react more slowly to antigen exposure and is considered to first release other cytokines during the time-span of the assays like interleukin-2 (20, 21).



The IGRAs measure the presence of an adaptive immune response to *M. tuberculosis* antigens, and are thus only an indirect measure of *M. tuberculosis* exposure. IGRAs have been developed for the identification of an immune response to *M. tuberculosis*-specific antigens, considered to be a correlate of *M. tuberculosis* infection, and are licensed for the use on blood specimens (21).

There are two types of commercially available IGRAs:

**A. The Quantiferon-TB Gold In-Tube assay (QFT-GIT)**, which has replaced the Quantiferon-TB Gold assay, detects the level of IFN- $\gamma$  produced in response to the *M. tuberculosis* antigens Early Secretory Antigenic Target-6 (ESAT-6), Culture Filtrate Protein 10 (CFP-10), and TB7.7, and uses the Enzyme-Linked Immunosorbent Assay (ELISA) detection method. This is an indirect measure of the presence of *M. tuberculosis* specific T-cells(21,22).

**B. The T-SPOT.TB** measures the number of IFN- $\gamma$  producing T-cells in response to the *M. tuberculosis* antigens ESAT- 6 and CFP-10, and is based on the enzyme-linked immunosorbent spot (ELISPOT) assay(21,22).

IGRAs are performed on fresh blood specimens. The QFT-GIT is performed by drawing 1 ml of blood into one of each of the three manufacturerprecoated, heparinized tubes. Within 16 hours of blood collection, the tubes must be incubated for another 16 to 24 hours at 37 °C(22). The plasma is harvested after centrifugation to assess the concentration of IFN- $\gamma$  by ELISA test. Results are interpreted according to the manufacturer's recommendations (19, 21, and 23).

#### **Biomarkers for the detection of *M. tuberculosis***

Microscopy and culture are still the mainstay of laboratory diagnosis of TB, and there is an urgent need for better diagnostic tools, especially in high-burden countries (23). An ideal diagnostic test would be sensitive and specific for active pulmonary TB, as well as rapid, cost-effective, non-invasive, and suitable for use in developing countries (24). One of the major research areas for tuberculosis (TB) focuses not only on diagnostics but also on biomarkers that can provide prognostic data about the disease course and response to treatment (25).

#### **Diagnosis of active TB**

TB diagnosis in most endemic countries still relies mainly on direct sputum smear microscopy, and the diagnostic services for drug-resistant TB (DR-TB) are based on complex technologies that require sophisticated, bio-safe laboratories with highly trained staff (22). In 2010, WHO endorsed a new automated real-time nucleic acid amplification technology (NAAT) for rapid and simultaneous detection of TB and Rifampicin resistance that offers the prospects of drastically improving the diagnosis of active TB and MDR-TB (16). The key to the diagnosis of tuberculosis is a high index of suspicion. Diagnosis is not difficult with a high-risk patient e.g., a homeless alcoholic who presents with typical symptoms and a classic chest radiograph showing upper-lobe infiltrates with cavities (20). On the other hand, the diagnosis can easily be missed in an elderly nursing home resident or a teenager with a focal infiltrate (18).

### **AFB Microscopy**

A presumptive diagnosis is commonly based on the finding of AFB on microscopic examination of a diagnostic specimen, such as a smear of expectorated sputum or of tissue. Although rapid and inexpensive, AFB microscopy has relatively low

sensitivity (40–60%) in confirmed cases of pulmonary tuberculosis (23). Most modern laboratories processing large numbers of diagnostic specimens use Auramine-Rhodamine staining and fluorescence microscopy. The more traditional method light Microscopy of specimens stained with Kinyoun or Ziehl-Neelsen basic Fuchsin Dyes is satisfactory, although more time consuming (17). For patients with suspected pulmonary tuberculosis, three sputum specimens, preferably collected early in the morning, should be submitted to the laboratory for AFB smear and mycobacterial culture (21). If tissue is obtained, it is critical that the portion of the specimen intended for culture not be put in formaldehyde. The use of AFB microscopy on urine or gastric lavage fluid is limited by the presence of commensal Mycobacteria that can cause false-positive results (17, 18).

### **Mycobacterial Culture**

Definitive diagnosis depends on the isolation and identification of *M. tuberculosis* from a clinical specimen or the identification of specific sequences of DNA in a nucleic acid amplification test. Specimens may be inoculated onto egg- or agar-based medium and incubated at 37°C (22). Because most species of

Mycobacteria, including *M. tuberculosis*, grow slowly, 4–8 weeks may be required before growth is detected (15). In modern, well-equipped laboratories, the use of broth based culture for isolation and speciation by molecular methods or high-pressure liquid chromatography of Mycolic Acids has replaced isolation on solid media and identification by biochemical tests. These new methods have decreased the time required for bacteriologic confirmation to 2–3 weeks (18,20).

### **Molecular Diagnostic Techniques (DNA probe and PCR)**

Several test systems based on amplification of mycobacterial Nucleic Acid are available. These systems permit the diagnosis of tuberculosis in as little as several hours, with high specificity and sensitivity approaching that of culture (24). These tests are most useful for the rapid confirmation of tuberculosis in persons with AFB-positive specimens but also have utility for the diagnosis of AFB-negative pulmonary and extra pulmonary tuberculosis (17). They are the newest diagnostic techniques in the armamentarium of clinical microbiology laboratory and include techniques such as DNA Probe and the PCR. Ribosomal RNA (rRNA) is useful genetic target for the

identification of organisms, since it often contain signature sequences and is present in the cells and culture in high quantity secondary to the growth of the bacillus. There are various applications of these in the detection and identification of *M. tuberculosis*. These includes; culture confirmation of isolates recovered from clinical specimens using DNA probes, the identification of Mycobacteria through DNA sequencing, direct detection of *M. tuberculosis* in clinical specimen using nucleic acid amplification assays. For instance Polymerase Chain Reaction (PCR) and DNA fingerprinting are also important in strain typing of Mycobacterium species (26, 27).

### **Polymerase Chain Reaction (PCR)**

Molecular technique such as PCR permits the direct detection and identification of infectious agents in clinical specimens, saving days to weeks in diagnostic time (23). Its application to infectious disease caused by fastidious or slow growing microorganism, such as *M. tuberculosis*, has the potential to provide a truly rapid laboratory diagnosis with the attended improvement in patient management and reduction of medical costs (27).

## **DNA Fingerprinting**

DNA fingerprinting based on Restriction Fragment Length Polymorphism (RFLP) is the gold standard for strain typing in mycobacteriology (23). The insertion sequence IS6110 was specifically identified as the target of DNA probe to be used in fingerprint analysis. It requires a week for the assay to be performed and results evaluated (23). The limitations of genotyping by RFLP is that a large cell mass is required and the results are band-pattern, thereby difficult to convert into digital format making comparison difficult (26).

## **Nucleic Acid Amplification test (NAAT)**

Due to the limitation of conventional bacteriological diagnosis of TB, the NAAT has emerged as a potential alternative in improving the diagnosis and control of TB (28). The NAAT systems, with rapid turn-around times, facilitate testing and treatment initiation in the same visit and, therefore, loss to follow-up cases can be reduced (29). Most NAAT assays detect the mycobacterial insertion element IS6110 for the identification of the MTB complex organisms. NAAT detects MTB ribosomal RNA or DNA directly from sputum specimens, both the acid-fast bacilli (AFB) smear-positive and AFB smear-negative

(30). The NAAT showed very high sensitivity in sputum smear-positive patients and around 61 to 76% sensitivity in patients with smear-negative sputum. Now a days, Xpert/RIF MTB assay is one of the NAAT that is endorsed by WHO (31).

## **XPRT MTB/RIF ASSAY**

The Xpert MTB/RIF assay is a nucleic acid amplification-based test using a cartridge based on the GeneXpert Instrument System (32). The basis of the Xpert MTB/RIF assay is a real-time PCR that can be used to detect DNA sequences specific to the MTB in sputum samples (33) a single Xpert MTB/RIF test directly from sputum can detect 99% of smear-positive patients and more than 80% of smear-negative cases (34). The Xpert MTB/RIF assay detects rifampicin resistance by PCR amplification of the 81-bp fragment of the MTB *rpoB* gene and rifampicin resistant-associated mutations in less than 2 hours (35, 36).

## **WHOLE-GENOME SEQUENCING (WGS)**

Microbial genomics has allowed to improve in the investigation of the organisms genetic markers that may impact treatment and infection prognosis (37). WGS is becoming an affordable and accessible method that can

identify microevolution within MTB lineages as they are transmitted between hosts (38). There are two classes of sequencers: the first generation sequencer and the second generation (the next-generation sequencer [NGS]) (39). The first generation sequencer is relatively slow, but has a high throughput and low cost. The second generation has a lower throughput, higher cost and is able to sequence multiple genomes in less than a day (39,40). The WGS can detect various types of mutations better than the Xpert MTB assay and WGS could avoid false positives when a polymorphism in the rifampicin resistance determining region (RRDR) of *rpoB* is found. Because of the need to culture MTB WGS has not been used as a routine diagnostic tool for TB (40, 41).

## Conclusion

Tuberculosis remains a major killer of adults worldwide. HIV, other immunosuppressive conditions and MDR-TB have changed the face of TB and posed challenges for its management. Additionally, environmental factors like; nutrition, medical conditions, overcrowding, and the immune status of the individual play a major role for the occurrence and progress of the disease. TB treatment requires administration of many

drugs for a long period of time due to this, drug resistance becomes the major problem that urgently needs the attention of all professionals to create a new drugs. The diagnosis of tuberculosis is depends on the identification of *M. tuberculosis* in different samples, thus to alleviate the diagnostic problems scientists try to create new diagnostic methods like: NAAT, PCR, TST, IGRA's and so on. New diagnostic and treatment strategies, however, are being developed that may improve patient care and decrease the incidence of tuberculosis. Advancement in diagnosis immensely contribute for the development of effective treatment and control of the disease.

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