

ANALYSIS OF DIAGNOSTIC METHODS AND THEIR SENSITIVITY TEST FOR *MYCOBACTERIUM TUBERCULOSIS*

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ABSTRACT

Tuberculosis (TB) is one of the major causes of death in the world which is caused by *Mycobacterium tuberculosis*. The rate of mortality is high if it is conjugated with HIV. The multiple drug resistant strains make it difficult to diagnose and treat the disease. It can be easily spread around the world so there is a need to evolve new diagnostic methods that are rapid, cost effective and easy to use. Many methods have been used since years for diagnosis of disease. Some of the conventional methods are smear microscopy, chest radiography, cultivation of mycobacterium. But, in recent years many new molecular and advanced techniques have been used that are more specific and sensitive. These techniques are less time consuming and gives very few false positive results thereby, making diagnosis cost effective. In this article, some of the conventional and new advanced molecular diagnosis methods have been reviewed and there sensitivity and specificity has been taken into consideration.

Keywords: Diagnosis, tuberculosis, sensitivity.

INTRODUCTION:

Tuberculosis is the leading cause of death in the world which is caused by the infectious bacteria *Mycobacterium tuberculosis*. It mainly affects lungs but can affect other parts also like spine, kidney, brain, heart. It is ranked along with HIV in leading cause of deaths. Worldwide, 9.6 million people are estimated to have fallen ill with TB in 2014: 5.4 million men, 3.2 million women and 1.0 million children.

The person infected with HIV has high risk of TB. The emergence of drug resistant species has made it more difficult to diagnose and cure TB. Even today in India, two deaths occur every three minutes from TB (WHO Report, 2015). One woman on every 2.4 men is diagnosed with tuberculosis in India. India carries the burden of 30% tuberculosis population of the world (McArthur *et al.*, 2016). The prevalence of Diabetes Mellitus in tuberculosis patients is high in South Eastern Amhara region (Workneh *et al.*, 2016). Currently, more than 50 diagnostic methods are in development stage for the diagnosis of tuberculosis (Lienhardt *et al.*, 2016).

General Characteristics of *mycobacterium tuberculosis*:

Mycobacterium tuberculosis is a large rod shaped non motile bacterium which is distantly related to the Actinomycetes. The length of these rods is 2-4 micrometers and width is 0.2-0.5 um. It is obligate aerobic in nature. For this reason, tuberculosis is mainly found in lungs lobes where full aeration is

provided. The bacterium has a slow generation time of around 15-20 hours.

It cannot be classified as either gram positive or gram negative as it does not show any of the characteristics. But it contain murein in the cell wall so stains very weakly or not all with gram staining (cells referred to as "ghosts"). These are stained by carbol fuchsin stain and are referred to as acid fast bacilli.

Cell Wall Structure

The cell wall structure of *Mycobacterium tuberculosis* is a major determinant of virulence for the bacterium. The cell wall complex contains peptidoglycan. More than 60% of the mycobacterial cell wall is made up of lipid. The lipid portion of cell wall comprises of three major components, wax-D, mycolic acids and cord factors.

Mycolic acids are alpha-branched lipids that are present in cell walls of *Mycobacterium*. They make up 50% of the dry weight of the mycobacterial cell envelope is made by the mycolic acids. Mycolic Acids plays a significant role in virulence of mycobacterium. They prevent attack of the mycobacterium by cationic proteins, lysozyme, and oxygen radicals in the phagocytic granule. Cord factor is an inhibitor of PMN migration. It is toxic to mammals and produced in large amount in virulent strains of *Mycobacterium tuberculosis*. Wax-D is the major component of Freund's complete adjuvant (CFA).

The high concentration of lipids in the cell wall provides specialised and unique properties to bacterium. Some of them are;

- High concentration leads to provide resistance to many antibiotics.

- It leads to complement deposition which provides resistance to osmotic lysis.
- Provides survival in macrophages.
- provides resistance against acidic and alkaline compounds

Table : 1 Stages of Infection of *Mycobacterium tuberculosis*

First stage	<p style="text-align: center;">Airborne droplets</p> <p style="text-align: center;">↓</p> <p style="text-align: center;">Reaches alveoli</p> <p style="text-align: center;">↓</p> <p style="text-align: center;">Ingested by alveolar macrophages, where some of the bacteria are indigested but due to large amount they can reproduce in macrophages</p>
Second stage (After about a week)	Reproduction of bacteria exponentially
Third stage (After 2-3 weeks)	<p>Ghon focus is produced which is a round complex of TB bacilli and infected macrophages in the middle and healthy macrophages in the surrounding.</p> <p>Mostly infected people do not show any symptoms or signs of active disease.</p> <p>This is a latent stage and it can be survived for many years without being contagious known as latent stage.</p>
Fourth stage	<p>Bacteria get activated and form a cavity in the tissue where immune system cannot reach and then quickly spread and develops signs and symptoms like coughing.</p> <p>It is the highly contagious stage</p>

Reference from Issar Smith, 2003

DIAGNOSIS OF TUBERCULOSIS:

The rapid and early diagnosis of disease is required to prevent the further infection and spread of disease.

Smear microscopy:

In most of the high burden resource poor countries, this method is used in which acid fast bacilli are detected in smear using a light microscope. The smears are stained with Ziehl-Neelsen (ZN) stain. But this technique is not so reliable. So in this regard fluorescence microscopy can be used against conventional light microscopy. The sensitivity was 52 to 97% as compared to light microscope with sensitivity of 32 to 94% (Parsons *et al.*, 2011). One of the major advantage of fluorescence microscopy over ZN stain is that it is user friendly and the results are unaffected by HIV status of patient (Ndugga *et al.*, 2003).

Sputum culture:

It is the most common method of diagnosis. In this method the sputum is cultured and then the growth of bacteria is observed. But it takes long time of around 2-4 weeks. Sputum Induction is a new approach which has a high yield in pulmonary tuberculosis. Most of the patients in pleural tuberculosis do not produce spontaneous sputum so this method can be used for effective diagnosis (Conde *et al.*, 2003). Sputum induction gives best results when used with smear microscopy and can detect the patients who have negative spontaneous smear microscopy. (Hepple *et al.*, 2012)

According to a study by Won-Jung Koh *et al.* a new approach was found which can minimize the time for tuberculosis detection on culture and drug susceptibility testing (DST). This is known as MGIT 960 method which decreases the turnaround time of DST to 27 days as compared to absolute concentration method which takes around 70 days (Koh *et al.*, 2012). According to a study it was suggested that, with respect to the cost effectiveness in India, MGIT culture is more

preferred over serological tests as it gives less false positive results (Dowdy *et al.*, 2011). Sputum induction can be easily and safely performed in children and infants and is more sensitive in both HIV infected and non-infected individuals (Hesseling *et al.*, 2002)

An assay known as microscopic observation broth-drug susceptibility assay (MODS) was used which has enhanced sensitivity of around 92% as well as uses less time for the detection of growth of mycobacterium in liquid culture (Caviedes *et al.*, 2000).

Tuberculin skin test:

The Tuberculosis Skin Test (TST) has been in use for the diagnosis of tuberculosis infection since 1910. TST is a protein-purified derivative (PPD) method which results from a culture filtrate of tubercle bacilli containing over 200 antigens common both in bacilli Chalmette-Guerin vaccine (BCG) and in most non tuberculosis bacteria. Therefore, the specificity and accuracy of this test is low. Furthermore, it takes around 48-72 hours to read TST after initial administration. It can produce false results as error can be made in performing and reading of results.

A new advancement in this process has gained importance. The MPB64 skin patch test discriminates between active tuberculosis and PPD positive healthy controls and shows very high sensitivity of 98% with specificity 99% when tested in Japan. In Manila, Philippines it showed 100% specificity and 88% sensitivity (Drobniewski *et al.*, 2003)

Detection of Lipoarabinomannan in Urine for Diagnosis of HIV-Associated TB:

Lipoarabinomannan (LAM) is a heat-stable glycolipid constituent in the cell wall of *Mycobacterium tuberculosis*. When mycobacterium releases this antigen it enters the circulation and then gets filtered in renal tubes, so easily detectable in urine of patients. It can be detected by simple sandwich ELISA using polyclonal antibodies. It could be used in cases where patient is infected with HIV and it can prove a powerful tool for diagnosis of tuberculosis in HIV patients (Achkar *et al.*, 2011).

Fluorescence-activated cell sorting:

This technique can be used in suspects with negative AFB sputum smears. In this method BAL cells or sputum cells are immune phenotyped. But the frequency of region of difference-1 *M. Tuberculosis* specific T cells is too low in the sputum to be used as stimulants for flow cytometry cultures and other immune based assays (Lange and Mori, 2010)

Polymerase Chain Reaction (PCR):

It was the widely used technique for the detection of tuberculosis. It is a rapid method for the diagnosis of disease. The technique is more sensitive than the other previously used methods. Out of the 144 patients that were tested it gave 80% positive result but smear examination gave only 25% accuracy (Cheng *et al.*, 2004). In this approach, an experiment was carried out with PCR which was specific for *devR* and IS 6110 sequences. The sensitivity was 87.5% for the combined PCR results (Chakravorty *et al.*, 2005)

The problem arises when the extrapulmonary tuberculosis is to be detected as it contains many inhibitors which interfere in amplification. So, proper treatment prior to reaction is necessary. So Cobas Amplicor MTB assay was used which eliminated all unwanted inhibitors (Bouakline *et al.*, 2003)

The PCR was used to detect mycobacteria in urine sample using two methods- Sechi method and Githui method. But these methods were not that much sensitive that can be used for routine clinical diagnosis. The sensitivity of Sechi method was 28.6% and that of Githui method was 55.6% (Kafwabulula *et al.*, 2002).

The PCR was conjugated with smear microscopy to diagnose the disease in smear-negative results. This proved better technique by detecting two targets *devR* and IS6110 sequences in the sample. These can prove a cost effective process for rapid diagnosis of smear-negative tuberculosis (Haldar *et al.*, 2007).

PCR requires proper treatment of sample prior reaction. So a new battery operated bead beating system is developed which can mechanically lyse the pathogen. This device is known as OmniLyse device developed by Claremont BioSolutions. It takes less than 10 minutes for the lysis of the pathogen. It improves the safety of lysis of mycobacteria before molecular diagnosis (Ferguson *et al.*, 2016)

Table 2: Comparative analysis between PCR and conventional techniques

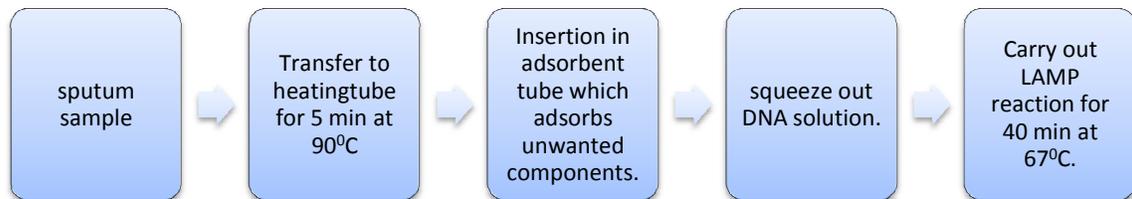
	Results	Culture positive
Smear positive but TB PCR negative	1	1
Both smear and TB PCR positive	34	34
Smear negative but TB PCR positive	83	51
Histology positive but TB PCR negative	8	8
Both smear and TB PCR negative	29	18

Reference from Cheng *et al.*, 2004

Table 3: Comparison of histopathologic diagnosis, TB PCR and AFB staining

Histopathologic diagnosis	AFB staining	TB PCR
Chronic granulomatus infection	36 negative	17 negative
	17 positive	36 positive
Chronic inflammation	28 negative	18 negative
	0 positive	10 positive

Fig1: Sequential Steps in LAMP reaction:



Reference from Boehme *et al.*(2011) and Mitarai *et al.*(2011)

Recently, a new technique of end point detection has been used which used calcein for the visual

Reference from Park *et al.*, 2003

Phenotypic detection:

It is a method in which *lux* gene is incorporated in the phage which codes for luciferase. Then the phage allows the detection of mycobacteriophage by emitting light and thud allows the indirect detection of viable *Mycobacterium tuberculosis*. But this is quite expensive (Drobniewski *et al.*, 2003).

Molecular Typing Techniques:

Restriction fragment Length Polymorphism (RFLP) is the most commonly used technique based on insertion sequence *IS6110*. It has been used to study molecular evolution of strains, to identify laboratory cross-contaminations, for monitoring transmissions. (Palomino JC, 2005)

Loop mediated isothermal amplification (LAMP):

It is the reliable, cheap and effective technique for the diagnosis of tuberculosis. It is a gene amplification technique which uses a constant temperature and a single enzyme for the detection. It can amplify the DNA even if it is present in very small quantity. (Tomita *et al.*, 2008).It yields results in 1 hour only. It uses *Bst* polymerase which has polymerase activity as well as strand displacement activity. It has two inner primers (FIP and BIP) and two outer primers (F3, B3) which recognise six distinct regions in the target DNA.

This reaction causes the production of insoluble magnesium pyrophosphate during amplification which appears as turbidity (Mori and Notomi, 2009).

detection of reaction.It binds to the manganese ions and produces fluorescence by quenching effect. This made it simple and cost effective that can be

used in resource limited developing countries.

The primer designing is very simple as it is done by primer design support software (<http://primerexplorer.jp/e/>). It automatically designs candid primer sets specific to target sequence. (Notomi *et al.*, 2015)

The sensitivity of LAMP was increased by using the repetitive Insertion Sequence IS6110 as a target gene. This assay has 50 times more sensitivity than the *gyrB* as a target gene (Aryan *et al.*, 2010). The combination of smear and LAMP is very sensitive and can be used as in-rule test combination (George *et al.*, 2011). LAMP has shown 100% sensitivity for culture positive and sputum positive results (Pandey *et al.*, 2008).

The PURE-LAMP has very high sensitivity and specificity which makes it economic, cost effective and rapid method for the diagnosis of tuberculosis (Ou *et al.*, 2014).

LAMP can be used for the RNA as well. Reverse transcriptase is added to the buffer and then the reaction is carried out (RT-LAMP). (Mori and Notomi, 2010)

The LAMP can be used for various applications like genetic testing, point of care testing, rapid testing of food products. But the major disadvantage is the risk of production of secondary products may be due to the high efficiency of the reaction. So it is recommended to prevent unwanted manipulations in master mix and reaction tubes and detection should be done carefully and separately. (Aryan *et al.*, 2010)

In recent years, a new advancement in this technique is use of capillary tubes to run the reaction known as Microfluidic LAMP. The glass capillaries are placed in between the glass slides and the sample is injected in the capillaries. (Raftai and Gill, 2015)

***Mycobacterium tuberculosis* resistance to rifampicin (MTB-RIF) Assay:**

In recent years, the direct nucleic acid amplification techniques have become a potential tool for the diagnosis of disease. In this approach, new technique in this field is evaluated which is real-time automated integrated system, the GeneXpert system using the Xpert MTB/RIF assay (GX). In this technique the sample is firstly treated with NaOH and isopropanol to make it inactive and then subjected to MTB/RIF cartridge and put in Xpert instrument. It leads to the DNA extraction and then amplification of 192-bp segment of the *rpoB* gene.

The detection is done by the hybridisation of amplified product with the overlapping probes which consist of core regions of *rpoB* gene. GX has shown very high sensitivity for the detection of mutations and rifampicin resistance with 100% specificity (Moure *et al.*, 2011).

The microscopic observation drug susceptibility (MODS) is a faster method which provides Drug Susceptibility Testing (DST). This technique provides diagnosis as well as detects resistance against rifampicin and isoniazid simultaneously (Kirwan *et al.*, 2016).

In a study by Surendra Sharma *et al.* it was observed that the Xpert assay detected 71% of the ‘‘confirmed TB’’ cases where culture and response to anti-TB treatment were positive (Sharma *et al.*, 2014). The pooled specificity of Xpert was 98% (Walusimbi *et al.*, 2013).

The sensitivity for isoniazid resistance improves to 90% with the advanced MTB-DRplus version of the assay (Ling *et al.*, 2008).

The comparison was made between two real time PCR based kits that are Cobas TaqMan MTB and Xpert MTB/RIF assay kit. The sensitivity of Cobas TaqMan was less as compared to Xpert MTB/RIF assay kit. The Xpert MTB/RIF was 100% sensitive as compared to Cobas TaqMan MTB which was 98% sensitive (Causse *et al.*, 2011).

The high cost of Xpert and lack of impact on TB morbidity/mortality is a major issue (Naidoo *et al.*, 2016). But the sensitivity and specificity of the test make it cost effective as there is no need to do multiple tests for diagnosis and there is reduced number of false positive results (Vassall *et al.*, 2011).

Interferon- γ release assay (IGRA):

IGRAs are the alternatives of tuberculin skin tests and more sensitive and less time consuming (Santin *et al.*, 2012). These are blood tests which measure *ex vivo* release of interferon- γ by T lymphocytes which are stimulated by antigens specific for *Mycobacterium tuberculosis*. These antigens are not found on BCG or most non tuberculosis mycobacterium so these assays are more specific tests (Starke *et al.*, 2014). Interferon γ release assays are useful for children diagnosis as it indicates recent exposure which can later form active tuberculosis (Rhwald *et al.*, 2012).

Early Secretory Antigen Target 6 (ESAT-6) and Culture Filtrate Protein 10 (CFP-10) are the antigens that are highly specific for active tuberculosis and can be used as a diagnostic reagent (Pinxteren *et al.*, 2000). The Quantiferon- TB Gold test was conducted in which the whole blood stimulated with either of the *M. tuberculosis*-specific antigens Early Secretory Antigen Target 6 (ESAT-6) or Culture Filtrate Protein 10 (CFP-10) was taken. The result was considered positive when the amount of interferon- γ in the sample wells is greater than or equal to 0.35 IU/ml. It was shown that the test has around 89% sensitivity. But it is not yet used in routine clinical laboratories (Ferrara *et al.*, 2005). These tests can prove beneficial for the diagnosis of active pulmonary tuberculosis where there is low prevalence of latent tuberculosis (Kang *et al.*, 2007).

In a study by Nathan Shaviya *et al.* it was suggested that the IFN- γ levels and the ratios of

IFN- γ to IL-10 and IFN- γ to adiponectin are associated with low body weight, body mass index, hip girth, diastolic pressure and plateletcrit suggesting mild-inflammation during early stage infection using newly infected TB patients. (Shaviya *et al.*, 2015)

A new version in this field is T-SPOT assay which is enzyme linked immunosorbent spot (ELISPOT) assay. In this method the peripheral blood mononuclear cells are incubated with two antigens and then the results are detected as the number of interferon- γ producing T-cells that produces spot. If the number of spots exceeds the threshold, i.e. usually 8, after subtracting from the number of spots in negative control then the sample is considered positive.

These tests are costly then the other tests but economical as they generate very few false positive results and requires only a single visit of the individual. (Starke *et al.*, 2014)

The most recent approach in this direction is the use of electrochemical immunosensor which is nanoparticle based. In this technique, the interferon γ is captured by the magnetic nanoparticles and labelled with gold nanoparticles. It is then subjected to another anti interferon γ antibody and multiple cadmium sulfide nanoparticles form a complex. Then the electrochemical signals are recorded. It takes only around 1 hour as compared to ELISA which takes around double time. (Wang *et al.*, 2016)

CONCLUSION:

In recent years, the diagnostic techniques have been advanced and became more reliable. The new approaches are used for making diagnosis more sensitive, economic and safe. But most of the advanced techniques are confined to the developed countries and are still not prevalent in developing countries. There is a need to make them available

to the whole world so that this disease can be diagnosed at early stage and can be declined from the world. Tuberculosis can be eradicated by mutual efforts, proper planning, effective diagnosis, proper funding and continuous efforts in the field of research and development.

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