2. REVIEW OF LITERATURE

2.1 TUBERCULOSIS

Tuberculosis (TB) has been called by several names like Consumption, King’s evil, lupus vulgaris, captain of death, phthisis and white plague. Hippocrates (400 B.C.) first wrote about in ‘Of the Epidemics’ and later Claudius Galen demonstrated it during the Roman empire (Mathema et al., 2006). TB continues to wreak havoc in developing countries. Developed countries have kept TB under control with new technology and drugs and antibiotics for over 40 years now. However, the process of developing new antibiotics is really slow in the case of TB [Bedaquiline approved in December 2012 by United States Food and Drug Administration (US-FDA), after rifampicin was introduced in 1960s] (WHO, 2017). Moreover, the TB bacteria have evolved to develop new mechanism of drug resistance against the antibiotics of the prevailing treatment regimen. MDR and XDR strains of TB are now a very real and major threat to the world (Ratledge et al., 1999).

The dual curse of TB among Human Immuno-deficiency Virus (HIV) infected patients with a proportional increase in smear negative pulmonary disease has frequently atypical presentation, extrapulmonary involvement and results in increased mortality. The diagnosis and treatment of of TB is particularly challenging in patients undergoing Antiretroviral Therapy (ART) when their HIV infection is advanced (Wood et al., 2007). The magnitude of the problem in conjunction with high Acquired Immune Deficiency Syndrome (AIDS) comborbidity came to light in 2006 when Gandhi et al., (2006) reported an outbreak of XDR-TB in HIV-I and TB-coinfected individuals in a rural area in KwaZuluNatal (KZN), South Africa (Smith et al., 2003; Grobusch et al., 2010).

2.2 HISTORY

Tuberculosis claims the largest number of lives due to a single infectious disease (WHO, 2017b) and continues to do so since ancient history of humankind. It has followed the typical trend of an infectious disease of resurgence and decline but along a very long period of time, hard to explain for epidemic cycles. TB was called “Captain Among these Men of Death” as it resulted in an outbreak in North America and Europe and killed thousands of people in the 18th and 19th centuries.
First efforts to understand pathogenesis of TB were made by Theophile Laennec in 19th century. Then, in 1865 Jean Antoine Villemin showed how TB is transmitted (Koch, 1882). In 1859 a first of its kind sanatorium was opened by Hermen Brehmer where rest, a rich diet and exercises were advised to patients for the treatment of TB. 24th March, 1882 was a momentous day in history of TB as Robert Koch demonstrated *M. tuberculosis* as the causative organism of TB in front of the Berlin Physiological Society. The tuberculin skin test was developed by a pediatrician from Vienna, Clemens von Pirquet. An extensive study demonstrating its use in detecting latent TB in asymptomatic children was published two years later. The test was improved by the work of Charles Mantoux and Florence Siebert by introduction of cannulated needles and syringes in 1908 and development of Purified Protein Derivative (PPD) by 1930s. As the understanding of the disease and its causes increased, public health measures were also taken to stop TB from spreading. BCG vaccination was employed to stop TB from spreading during World War I. The modern era of TB care and control was ushered in by discovery of streptomycin and isoniazid in 1944 and 1952 respectively (Hayman, 1984).

It is hypothesized that genus Mycobacterium originated 150 million years ago. New age tools of genetic analysis and whole genome sequencing of several *M. tuberculosis* strains and a low mutation rate have helped to estimate its time of origin. There was evidence to suggest that *M. tuberculosis* was present 3 million years ago and it might have infected early hominids at the time. One theory suggests that all the members of MTBC (*M. tuberculosis*, *M. africanum*, *M. canettii* and *M. bovis*) originated 35,000 - 15,000 years ago from a common African ancestor (Kapur et al., 1994). Modern strains of *M. tuberculosis* appear to have originated from a common ancestor about 20,000-15,000 years ago (Brosch et al., 2002). The mutation rate analysis of *M. tuberculosis* predicts that the diversity among the present strains has originated only between 250 to 1,000 years ago (Sreevatsan et al., 1997).

**2.3 M. tuberculosis COMPLEX (MTBC)**

Bacteria which have more than 99.9% nucleotide identity and identical 16S rRNA sequence are classified as members of MTBC. *Mycobacterium cannetti*, *M. africanum*, *M. pinnipedii*, *M. caprae*, *M. microti*, *M. bovis*, *M. bovis* BCG and *M. tuberculosis* are members of this complex. All these members differ in their host range, epidemiology, clinical presentation, in human beings and laboratory phenotype, but little is known about these differences. It has been shown
that genomic deletions are important for the evolution of MTBC members. *M. cannetti* is the most ancestral MTBC member. Human is the natural host of *M. cannetti, M. tuberculosis, M. africanum* type II; while pinnipeds, goats, voles and cows are natural hosts of *M. pinnipedii, M. caprae, M. microti* and *M. bovis* respectively (Mostowy et al., 2005). Members of the MTBC share a lot of similarity in their genomes (Sreevatsan et al., 1997), but vary in their transmission ability (Valway et al., 1998), disease manifestation (Dorman et al., 2004), immunological responses (Manca et al., 2004; Reed et al., 2004; Chacon-Salinas et al., 2005), replication rate (Zhang et al., 1999) and possibly the frequency of drug resistance (Glynn et al., 2002) and ability to evade vaccination (Abebe et al., 2006). The understanding of MTBC evolution and genetics was broadened after the whole genome sequencing of *M. tuberculosis* H37Rv (Cole et al., 1998; McEvoy et al., 2007) and few *M. bovis* strains (Rothschild et al., 2001; Garnier et al., 2003). Various genetic resources have been used to study MTBC members- fingerprinting patter, sequencing, Single Nucleotide Polymorphisms (SNPs) and large sequence polymorphisms (Mostowy et al., 2005). The region between the 16S rRNA and 23S rRNA is a highly polymorphic 275bp-ITS region. The sequence analysis shows that it is completely conserved in members of MTBC (Mathema et al., 2006).

### 2.4 ORIGIN AND EVOLUTION

TB was suspected to have originated by a zoonoic infection from animals to humans. Sequencing and spoligotyping analysis data from a 17,000 years old sample of the metacarpal of an extinct long horned bison suggests that the disease was widespread in the Pleistocene age and reached North America around 20,000 years ago (Rothschild et al., 2001). Earlier it was believed that the present day *M. bovis* was the evolutionary precursor of MTBC. However, genomic analysis of members of MTBC shows otherwise, it suggests a stepwise accumulation of deletions. The genetic make-up of different MTC isolates studied is a mixture of both modern MTBC and present day *M. bovis*. It is a consequence of the build-up of irreversible genetic changes in the precursor strains. The distribution of variable regions in the MTB genome seconds this observation (Daniel, 2006). *M. tuberculosis* strains can be divided into ancestral and modern based on *M. tuberculosis* specific Deletion 1(TbD1). The strains lacking this are called modern MTBC strains and responsible for major epidemic outbreaks of Haarlem, Beijing and Africa clusters (Brosch et al., 2002). Moreover, it can also be said that the diversion of *M. africanum,
M. microti and M. bovis lineage took place prior to TbD1 deletion. The divergence was an outcome of the Region of Difference 9 (RD 9) and some other deletions in the genome of the precursor organism. This is contrary to the theory that M. tuberculosis causing infection in humans evolved from M. bovis, its bovine counterpart. However, M. canettii and progenitor M. tuberculosis strains do not lack any of these deletions seemingly suggesting that the common ancestor before the M. africanum-M. bovis lineage diversion happened was already a human pathogen (Brosch et al., 2002).

2.5 TRANSMISSION AND PATHOGENESIS

TB spreads from patients of active PTB disease through infective aerosols expelled by coughing, sneezing, speaking or spitting. About 40,000 aerosol droplets each 0.5 to 5µm in diameter can be expelled from a single sneeze. Since the infectious dose of tuberculosis is very low (inhaling even 10 bacteria are enough to cause the disease) a single droplet is capable of causing TB (CDC, 2013). People in close proximity, and perpetual and extended contact with diseased and infectious individuals are at a greater risk of catching infection with approximately 22% infection rate. An active PTB patient lacking any proper treatment and medication can infect about 15 healthy people in a year. People living in high TB prevalence areas or high risk crowded localities, or those who inject drugs using common needles and syringes are especially susceptible to catching TB infection. People belonging to economically weaker sections of the society and high risk racial or ethnic minority populations who are unable to avail access to quality medical facility are also predisposed to TB infection. Health care workers and immune-compromised patients (HIV/AIDS patients or people on immunosuppressant drugs) are also prone to TB infection. Consuming an infected cows’ milk or meat can also result in acquiring TB infection through the gastrointestinal tract. However, this method of disease transmission has now been eliminated by pasteurization of milk (Churchyard et al., 2017).

Disease can be transmitted from an active PTB patient to healthy individuals and the factors responsible for transmission include: infection carrying aerosols released by a carrier, the amount of ventilation in close confined spaces, virulence of M. tuberculosis strain, frequency and time span of exposure. The spread of disease can be stopped only by segregating infected patients and providing effective Anti-Tuberculous Therapy (ATT) (Churchyard et al., 2017). Primary tuberculosis is the first infection caused as a result of the inhaled mycobacteria in a non-
immunised individual. The TB bacteria inhaled with the infective aerosols are delivered to the alveoli in the middle or lower lobe of the lungs where they are engulfed by the alveolar macrophages (Brosch et al., 2009). This process is started by the contact of bacteria with CRP and mannose present on the macrophages (Schlesinger et al., 1993). The bacteria or other pathogen phagocytosed by the macrophage are initially stored in a phagosome. Phagosome fuses with a lysosome to form a phago-lysosome which puts the pathogens under stress in the form of toxic peptides, lysosomal enzymes, reactive oxygen intermediates and acidic pH (Nathan et al., 1991). This fusion is inhibited by the mycobacteria by an unknown mechanism. Cell mediated hypersensitivity develops two to eight weeks after primary infection. The place of infection is infiltrated by lymphocytes, monocytes and neutrophils (vanCrevel et al., 2002) which release lymphokines and interleukins. However, none of these cells are able to kill the bacteria effectively (Fenton et al., 1996). Monocytes attracted to these released chemicals convert to macrophages and then in turn to histiocytes with a specific function to form granulomas. Typically a granuloma is composed of Langerhans cells (macrophages fuse to form large multinucleated cells), epithelioid cells and bordered by fibroblasts and lymphocytes (Dannenberg et al., 1994). Increased hypersensitivity develops and dying macrophages release reactive oxygen and nitrogen species. As a result, centre of granuloma becomes necrotic which can be seen as a residual lesion on the chest radiograph. Inspite of the increased lysozyme production bacteria can survive in macrophage for several years within these cells but their proliferation and dissemination is restricted. This enclosed infection is called latent TB. The combination of a calcified peripheral lung lesion and calcified hilar lymph node is known as a Ghon complex. If the host generates effective macrophages and Cell Mediated Immunity (CMI), the infection will lead to tubercle formation and control. Mostly there is a phase of dormancy before the pathogen multiplies vigorously causing the disease.

The bacteria dispersed by the bursting of alveolar macrophages are ingested by blood macrophages. Lymphatic channels also carry few such macrophages to the lymph nodes. It is important to control infection at this level otherwise the bacteria reach the blood circulation and spread extensively to different tissues and organs including liver, spleen, kidney, bone, brain, meninges and apices or other parts of the lungs. Such a widespread diffusion of bacteria can have lethal consequences in case of children and immunocompromised people as it results in milliary or meningial TB. There is minor inflammation in the seeded tissues but the lesions at the site of primary infection and hilar lymph nodes can be observed radiologically (Dannenberg et al., 1994; Smith, 2003). Not much is known about how the *M. tuberculosis* survives in the lung
environment due to which there has been no effective strategy to combat chronic and acute infections. From the chemically infected mouse models it is still uncertain if the bacteria are constantly viable and not multiplying or multiplying and dying at the same rate (Smith, 2003; Flynn et al., 1998). Biochemical evidence suggests that *M. tuberculosis* metabolism shifts from carbohydrate utilizing, aerobic mode to anaerobic and lipid-utilising mode (Segal et al., 1956; 1957). The importance of this change in intermediary metabolism of mycobacteria still needs to be explored.

### 2.6 *M. tuberculosis* GENOME ORGANISATION

The *M. tuberculosis* genome (Figure 1) consists of 44,11,532 bp with a guanine-cytosine content (GC-content) of 65.6%. It contains approximately 4000 genes which can be grouped into 11 classes based on their broad functional characteristics (Cole et al., 1998). However, only 52% of these genes have been assigned specific functions. Around 200 genes (or 6% of the total) code for enzymes of fatty acid metabolism; half of them for β-oxidation of fatty acids. It is unique as no other organism has been reported to have such a large number of lipid degrading functions. It can be related to its ability to survive in host tissues where fat is more abundant as energy source. Other genes in the genome include 207 genes encoding information pathways, 517 encoding cell wall structures and processes, 50 genes for stable RNAs, and 188 genes encoding regulatory proteins. Besides these, 91 genes are involved in virulence and detoxification and 877 genes are involved in intermediary metabolism and respiration. PE and PPE gene families consisting of 100 and 67 members each (about 8% of the genome) are also present. It has a conserved ~100 and 180 amino acid residue long N-terminal domain with Pro-Glu (PE) or Pro-Pro-Glu (PPE) motifs at 8-9 or 8-10 positions respectively. These are part of the acidic glycine-rich proteins based on multiple copies of polymorphic repetitive sequences referred to as Polymorphic GC-rich sequence (PGRS) in the PE and Major Polymorphic Tandem Repeat (MPTR) in the PPE gene families. Although it is too early to speculate their functions, they might result in antigenic variations (Cole et al., 1998).

It has been shown that over 50% of the *M. tuberculosis* genome has resulted from domain shuffling events or gene duplication and 3.4% (137 genes) is made up of insertion sequences. There are 56 copies of IS elements out of which IS6110 belonging to the IS3 family is found in the maximum number and maintains the plasticity of the genome (Cole et al., 1998; Stevenson et al., 1976).
Figure 1. Circular map of chromosome of *M. tuberculosis* H37Rv (Smith, 2003)  
Note: The scale of the *M. tuberculosis* genome is in Mega-base pairs. The replication origin is denoted by 0. First circle [blue triangles (tRNA), pink triangles (other RNA), pink cubes (direct repeats)]; Second circle [dark green and light green are coding sequences in clockwise and anticlockwise direction respectively]; Third circle [repetitive DNA: orange triangles (insertion sequences), dark pink triangles (13E12 REP family), blue (prophage)]; Fourth circle [green (PPE family members)]; Fifth circle [purple (PE family members excluding PGRS)]; Sixth circle [Dark red (PGRS sequences)]; Centre [G + C content]
2.7. BACTERIOLOGY OF M. tuberculosis

*M. tuberculosis* is a slim, non-motile, non-spore forming, acid-alcohol fast bacteria. It has a generation time of 14-15 hours or 0.07 doubling per hour and undergoes dormancy for prolonged periods of time. It grows at 37ºC and requires either enriched or complex media for growth. Growth of the bacilli is enhanced at 5-10% Carbon Dioxide (CO₂) and pH-6.5-6.8. Surfactants like Tween 80 when added to *M. tuberculosis* culture media wet the surface and result in a more dispersed and rapid growth (Stevenson *et al.*, 2007). *M. tuberculosis* takes around 3-6 weeks’ time to culture and appears as dry, rough and buff coloured colonies on the classic Lowenstein-Jensen (LJ) media which also contains dyes to inhibit the growth of non-mycobacterial contaminants (Ryan *et al.*, 2004).

The MTB cell wall contains N-glycolymuramic acid peptidoglycan in place of N-acetylmuramic acid found in Gram positive bacteria. Other important components of the cell wall are mycolic acids- which are long chain fatty acids and make up 60% of the total cell wall mass and Lipoarabinomannan (LAM) which is analogous to lipopolysaccharide of Gram negative bacteria. Mycosides and sulfolipids are also present which give a hydrophobic nature to the cell wall and forms a permeability barrier similar to Gram-negative bacteria. Porin molecules form aqueous channels for the absorption of hydrophilic nutrients. Owing to its hydrophobic surface, *M. tuberculosis* is specifically resistant to drying, dessication and a majority of disinfectants. However, it is sensitive to heat (65ºC for 15 minutes) and ultraviolet (UV) irradiation. A number proteins and polysaccharides comprise the antigenic make-up of *M. tuberculosis*. A Purified Protein Derivative (PPD) of the secreted antigen tuberculin is used to check hypersensitivity by skin testing (Ratledge *et al.*, 1999; Ryan *et al.*, 2004).

2.8. EPIDEMIOLOGY

2.8.1 Global Scenario

WHO TB Report being published since 1997 provides updated data and analysis related to TB in a single document. It also enumerates the efforts made to control the disease all over the world and the developments related to its prevention, diagnosis and cure. According to the WHO TB Report, 2017 (WHO, 2017a), the TB epidemic is larger than it was estimated (Figure 2). About 10.4 million incident TB cases were reported in 2016 of which 1 million were children and 6.2
and 3.2 million were males and females, respectively. Hence, 90% cases were in adults and 6.9% among children. The male:female ratio for global notifications was 1.7:1. There were approximately 1.7 million deaths due to TB in 2016, and an additional 0.4 million deaths due to TB-HIV. Although the number of TB deaths has fallen by a 37% over the period of 2000 and 2015, it remains one of the leading causes of deaths worldwide. Around 10% of total TB cases were those of HIV-TB. The emergence of MDR-TB is major challenge in the control of the disease. India, China and the Russian Federation contribute about half of the 6,00,000 MDR TB cases reported in 2016. Seven countries (China, Niger, Indonesia, India, Philippines, South Africa and Pakistan) account for 64% TB burden of the world. Global control of TB depends on prevention and cure in these countries. (WHO, 2017a;b).

Figure 2. TB incidence rates as estimated by WHO in Global TB report (WHO, 2017b)

2.8.2 Indian Scenario

India ranks among the top ten in all the three lists of TB high burden countries by WHO: a) Thirty high TB burden countries; b) Thirty high HIV/TB burden countries and c) Thirty high MDR-TB burden countries (Figure 3). The WHO estimates about 2.2 billion incident TB cases to be in India out of the global 9.6 billion. This shows the number of new active TB cases reported this year whereas TB prevalence or percentage of the total population suffering from TB is about 2.5 billion. About 40% of the total population of the country is infected with TB but have latent
infection rather than active disease (WHO, 2017b). Revised National Tuberculosis Control Program (RNTCP) showed the two third of population in India carries latent TB infection. In addition to this, HIV accounts for 0.36% of total population. HIV increases the risk of latent TB progressing to active TB (Ryan et al., 2004). In India, this condition increases the risk of mortality in case of HIV infected patients.

The weather conditions, congestion and amount of exposure to infected aerosols affect the transmission of TB. This infection can be prevented by separating active TB patients from healthy individual and by giving anti-tubercular therapy.

The risk of healthy person catching infection from infected patient is 22%. The immune-compromised patients, drug abusers, TB endemic area population, diabetic patients are at higher risk of TB infection (Churchyard et al., 2017; Stevenson et al., 2007). Unfortunately, Indian population carries all the above risk factors.

![WHO based TB profile of India](WHO, 2017b)

Despite a 91% Directly Observed Treatment Strategy (DOTS) coverage and 86% treatment success, 4.8 lakh people died of TB last year. India witnessed catastrophic rise in the number of HIV cases which far exceeded South Africa in prevalence. India might be at the verge of a major TB outbreak due to the HIV-TB alliance aggrevated by the aggressive Beijing strains and 1.1 lakh HIV-TB cases. Considering the large diabetic population in the country, the combination of TB and diabetes is expected to become a major public health challenge with serious implications.
The only good news are the high cure rates under DOTS and controlled MDR rates as compared to those in former USSR countries and Africa. (WHO, 2017a)

2.9 TB DIAGNOSIS

To control a disease like TB which has reached epidemic proportions and the threat of eminent outbreak looms large, proper laboratory systems for diagnosis are as important as an effective treatment regimen (like DOTS). Accurate and rapid diagnosis of active TB is imperative for reducing the burden of tuberculosis. Almost half of the 5.2 million patients with pulmonary tuberculosis reported in 2016 (43%), were diagnosed using clinical criteria alone (symptom history or chest X-ray) (WHO, 2017b), highlighting the need for increased availability of diagnostics in resource limited settings. The lack of bacteriological confirmation in a majority of cases results in delayed or misdiagnosis of the disease which contributes to deteriorating the health of the patient. Mycobacteria are obligately aerobic, non-motile, rod-shaped bacilli. Members of the genus Mycobacterium owe a number of their characteristics to the unique structural and chemical composition of their cell walls. They contains a higher content of complex lipids (>60% as compared to about 5% and 20% in gram-positive and gram-negative organisms respectively). Mycobacteria also contain including long chain (C60-C90) fatty acids called mycolic acids (Asselineau et al., 2002; Mahapatra et al., 2007). These make the cell wall extremely hydrophobic and protect from desiccation, killing by disinfectants, staining with basic aniline dyes, and invasion by drugs used to treat other bacterial infections. All these factors necessitate special laboratory considerations while staining and culturing the mycobacteria (Caulfield et al., 2016).

2.9.1 Clinical, Laboratory and Radiological Diagnosis of TB

Lack of fast and reliable diagnostic methods for TB results in 2 to 3 weeks delay in the management of active TB patients. There is a huge time lag between an initial suspicion and starting antituberculous chemotherapy (Houk et al., 1968). Longer delays are often accompanied by declining health of patients and can also result in fatal cases of TB. Physical examination of patients often shows normal or misleading results and is less helpful in diagnosis. However, it is important in EPTB, where cardiologists could check the patient for pericarditis or spinal cord
compression in case of spinal TB (Banner, 1979). Signs such as lymphopenia, C-reactive protein (CRP), leucocytosis, and anaemia could indicate but cannot be used to confirm TB (Trinker et al., 1996)

Chest X-ray is used for diagnosis in active TB, as primary infection often results in typical features on the radiograph. However, it can present residual signs of any previous TB infection. Hence, we cannot rely on it for conclusive diagnosis of TB and microbiological confirmation is essential. For more sensitive diagnosis High-Resolution Computed Tomography (HRCT) thorax, is better in comparison to chest x-ray (Verma et al., 2002). Due to lack of simple, accurate and specific test for diagnosis, there is an unmet need for efficient diagnostic tests for TB.

2.9.2 Acid Fast Staining

Mycobacteria contain complex fatty acids in their cell walls and hence are hydrophobic in nature. These are impenetrable to aniline dyes such as crystal violet. Hence, mycobacteria are either not visible with the Gram stain or appear as “ghosts” when direct specimens are stained (Trifiro et al., 1990). On the contrary, mycolic acids of the mycobacterial cell wall form stable complexes with aryl-methane dyes. In Ziehl-Nelson (Z-N) staining carbol fuschin is used to stain mycobacteria on heat fixed slides and methylene blue is used as a counter stain (Ziehl, 1882; Neelsen, 1883). These cell wall-dye complexes cannot be broken even by mineral acids and hence they are called “acid-fast bacilli” or “AFB” (Figure 4). Fluorescence staining using a mixture of auramine-O and rhodamine-B dyes has several advantages over detection with regular dyes (Hanscheid et al., 2007). Fluorescent dyes bind to the nucleic acids within acid-fast organisms and are more sensitive and allows for more rapid reading of slides (Kommareddi et al., 1984). Hence Z-N staining light microscopy is gradually phasing out worldwide on WHO directions in favor of auramine-rhodamine staining (Kommareddi et al., 1984). Although fluorescent Light-Emitting Diode (LED) microscopes are expensive and out of reach for resource-limited settings. In 2014, only 7% of laboratories had the capability of performing fluorescent AFB smears (WHO, 2017b).
The clinical sensitivity of sputum smear microscopy is dependent on many factors like the burden of mycobacteria, the type of AFB stain used, and experience of the laboratory technician. Overall sensitivity of Acid Fast Bacilli (AFB) varies from 22-80% while the positive predictive value for mycobacteria is >95% (Lipsky et al., 1984). However, there are limitations of the technique. Acid-fast stains cannot differentiate between mycobacteria species. Sensitivity is also dependent largely on the bacterial load in the sample. Atleast $10^3$-$10^5$ bacteria/ml must be present for positive smear result. Such high bacterial load is indicative of a severe infection and hence sputum smear microscopy is a rapid and reliable test for screening highly infective PTB patients by an inexpensive technique. The Centre for Disease Control and Prevention (CDC) recommends that 3 sputum samples be submitted for smear examination atleast one of which is an early morning sample, hence necessitates repeated hospital visits (CLSI, 2008). The results of AFB staining are reported within 24 hours of specimen collection (ATS, 1999). Positive results have a high predictive value, but negative results cannot rule out TB disease. Therefore,
culturing of decontaminated sputum samples for MTBC growth is important, given the low sensitivity of sputum smear AFB.

2.9.3 Mycobacterial Culture

Definitive diagnosis of TB can be established by culturing sample specimens. It helps to check the viability and specificity of the bacteria. However, the generation time of mycobacteria is longer compared to other bacteria (18-24 hours for mycobacteria and 20 minutes for *Escherichia coli*). Hence their growth is extremely slow and takes 2-6 weeks’ time to show positive results on culture plates. Cultures are generally kept for at least 8 weeks before being discarded and reported as negative (Simner *et al.*, 2016) and approximately in 10–20% of total cases the bacteria either do not grow or gets contaminated (Anderson *et al.*, 2000). Still, culture is about 100-times more sensitive than AFB smear, as it requires only 10–100CFU/mL of specimen for reliable growth. However, culturing of bacteria requires more technical expertise and is costly as compared to microscopy. Air containing 5–10% CO₂ and temperature of 35–37°C are ideal conditions for mycobacterial growth and incubation (Ryan *et al.*, 2004). After culture growth, next step is the identification of bacteria. *M. tuberculosis* has a characteristic colony morphology which helps in its identification. Mycobacterial colonies have a very dry surface with rough texture and crème colour. Hence these are often described as “rough and buff.” All members of the *M. tuberculosis* complex are non-pigmented, so the presence of any pigment indicates Non-tuberculous Mycobacteria (NTM) growth (Caulfield *et al.*, 2016).

Any respiratory sample needs to be decontaminated before culturing. The most common method of decontamination is the N-acetyl L-cysteine (NACL) sodium hydroxide (NaOH) method (CLSI, 1990). NACL helps to breakdown the mucus present in sputum to expose the mycobacteria and NaOH kills any contaminating bacteria while mycobacteria are left viable for culture. There are many different media for mycobacterial growth and three major groups are: (a) egg based media, (b) agar based media and (c) liquid media.

Egg based solid media are more sensitive than agar based media and hence are used for primary isolation of *M. tuberculosis* from clinical samples for example LJ, Petragnani or American
Tradeau Society (ATS). LJ media is the most commonly used egg based media containing egg proteins, potato flour, salts and glycerol and supports good growth of MTBC (Robbe-Austerman et al., 2013). On the other hand, agar based media are transparent and chemically defined for faster mycobacterial growth. For example, there is visible MTBC colony growth in Middlebrook 7H10 or 7H11 agars, in only 10–12 days as compared to 18–24 days with LJ media (Pfyffer et al., 2015). Likewise, L-J cultures are preserved for 6-8 weeks before being discarded and reported as negative (Simner et al., 2016) whereas agar-based media is unstable and starts deteriorating. The exposure to excessive heat or light leads to the release of toxic formaldehyde which inhibits the growth of mycobacteria.

Mycobacteria grow faster in broth than on solid media and which helps in better patient management (Moreira et al., 2015). The three FDA-cleared commercial platforms for the semi-automated broth-based culture of mycobacteria are:

(a) BACTEC MGIT 960 system (Becton Dickinson Microbiology Systems),

(b) Versa TREK system (Trek Diagnostic Systems) and

(c) MB/BacT Alert 3D (bio Mérieux)

The MGIT system (Figure 5) is named so because it uses Mycobacterial Growth Indicator Tubes. Each MGIT tube contains a modified Middlebrook 7H9 broth and a fluorescent indicator that is quenched by the presence of oxygen within the tube. When the growth of mycobacteria in the medium consumes all the oxygen over time, the fluorescent indicator gives a positive signal once a certain growth threshold has been met. The instrument continuously monitors tube fluorescence which lets the technical staff to recognise positive tubes and identify the growth organism. A meta-analysis of published studies found the MGIT system to have 81.5% analytical sensitivity (and 99.6% specificity) compared to 67% sensitivity for L-J solid media (Cruciani et al., 2004). The Versa TREK system detects the growth of inoculated specimens by measuring pressure changes in the bottle head space above the broth medium. Finally, the MB/BacTAlert3D system uses a colorimetric carbon dioxide sensor for growth. All three systems use Middlebrook broth and have comparable levels of performance for the culture of mycobacteria (Falconi et al., 2008; Gravet et al., 2011; Caulfield et al., 2016).
2.9.4 Tuberculin Skin Test

In routine, Latent TB infection (LTBI) is diagnosed by Tuberculin Skin Test (TST). A combination of antigens shared by several mycobacteria called the Purified Protein Derivative (PPD) is used. A type-IV hypersensitivity reaction to injected PPD is detected. Patients need to visit the hospital twice- initially for PPD injection by needle and next visit to read and interpret the result based on skin reaction after 48–72 hours. This test is economical as it is carried out without any sophisticated instrument. However, there are also some drawbacks of TST. It requires two visits to the hospital which might be a problem for people residing in remote areas. It occurs mostly with the urban HIV population who do not return for a second visit (Chaisson et al., 1996). Also the inoculation of PPD is painful and results in inflammation at the site of injection (Nardell et al., 2004) which is not acceptable to some people. The test might be difficult to perform for people with skin problems. The procedure of the test is not fixed as the amount of PPD injected and criteria for reading the results differ from place to place. Consequently, weak and strong dose of PPD result in false negative and positive results respectively (Pouchot et al., 1997; Stuart et al., 2000; ). The method of inoculating PPD doses
can also cause variation in results (Comstock *et al*., 2009). Since there is no fixed rule on interpreting TST results, 70 different cut-offs are used for positivity of TST. The results are classified on the basis of 5, 10 or 15 mm of induration (ATS, 2000). Operator variability can lead to false TST results (Pouchot *et al*., 1997). There could be digit preference while rounding of TST inflamed area and interpretation bias (Bearman *et al*., 1964). A reason for false-positive TST results could also be the composition of PPD. It is a poorly defined mixture of mycobacterial antigens. As all different mycobacterial species share some antigens false positivity could result from BCG vaccination by live attenuated *M. bovis* (Fine *et al*., 1999; Floyd *et al*., 2002; Huebner *et al*., 1993; Kwamanga *et al*., 1995). BCG vaccination can affect TST results for up to 15 years although in a small and inconsistent manner (Wang *et al*., 2002; ATS, 2000). *M. tuberculosis* specific PPD and sensitins can be used to solve the specificity problem in TST and patients of *M. tuberculosis* can be easily differentiated from *M. avium* complex (Andersen *et al*., 2000; von Reyn *et al*., 1994; 1998). In spite of that TST if repeated a number of times on the same patient can lead to an immune response and thus false positive results (Menzies *et al*., 1999). False negative results could be because of several reasons such as immune-suppression after organ transplantation, or in case of HIV-TB disease (Chin *et al*., 1996; Barnes *et al*., 2001). Different strategies are being devised to overcome the drawbacks of TST. Some countries have altogether stopped using TST. Others use it to identify relatively newer infected cases and give prophylactic isoniazid therapy in Netherlands and United States.

### 2.9.5 Interferon Gamma Release Assay

Interferon-Gamma Release Assays (IGRA) are blood based assays to check the presence of immune response from past infection. For IGRA blood sample of the patient is incubated with *M. tuberculosis* complex specific antigens but different from the ones present in BCG vaccination strains. These are either Early Secretory Antigen Target 6 (ESAT-6) and Culture Filtrate Protein 10 (CFP-10) or in some cases TB7.7 antigen. Effector or memory T-cells from the blood react with antigens and produce interferon as an indication of active TB (Figure 6) (Lein *et al*., 1997). However there is a difference in response to effector and memory T-cells. Effector T-cells previously exposed to TB antigens in-vivo produce interferon whereas the response of memory T-cells is slow as they produce cytokines (interleukins) first during the assay (Mack *et al*., 2009). There are two types of tests which either measure the interferon-
gamma produced quantitatively using ELISA method [(QuantiFERON-TB Gold In-Tube (Cellestis Ltd, Australia) (Cellestis.com) (Quantiferon test)) or T-SPOT.TB assay (Oxford Immunotec, UK) which measures the number of T-cells producing interferon gamma using Enzyme Linked Immune-Sorbent Spot Assay [ELISPOT].

Figure 6. Interferon-gamma release assay (Mahmoudi et al., 2017)

IGRA were meant to replace TST and have succeeded up to some extent. Just one hospital visit is required for drawing blood and the assay can be carried out. Secondly, the maximum time required for IGRA is 16-20 hours and the reading of results is automated and accurate. BCG vaccination does not bear any affect on the results of IGRA as is the case with TST. Antigens are not administered to the patient and hence booster effect does not occur. Individuals constantly exposed to TB can be screened easily by this method. LTBI which could not be detected by TST like in immunocompromised individuals and HIV positive patients are diagnosed with IGRA. (Chapman et al., 2002) There are also a few drawbacks of IGRA. Some people might not be comfortable in giving blood sample for the test. Secondly, there is a time constraint associated with processing the blood which should be completed within 12 hours after collection. Technical training of the staff is also required to perform procedures like isolation of lymphocytes and monocytes (Barnes et al., 2001). The Quantiferon test which can detect more than one antigen simultaneously (Desem et al., 1998; Mazurek et al., 2001; 2003. It can also distinguish between M. tuberculosis and M. avium intracellulare complex infection (Fietta et al., 2003; Pottumarthy
et al., 1999). It does not boost anamnestic immune responses (Mazurek et al., 2003). The problem with Quantiferon as with TST is the possibility of false positivity in people who have undergone BCG vaccination and its inability to distinguish between *M. tuberculosis* and atypical mycobacteria (Arend et al., 2001; Brock et al., 2001; van Pinxteren et al., 2000) RD-1 region is the region in *M. tuberculosis* and *M. bovis* genome but absent in *M. bovis* BCG (Lalvani et al., 2001; Harboe et al., 1996; Pollock et al., 1997). ESAT-6 and CFP-10, antigens present in the RD-1 region of *M. tuberculosis* genome are used to design tests which do not interfere with BCG vaccination results (Liu et al., 2004). CFP-10 and ESAT-6 have a conserved mRNA and might these interact to perform the same function of identifying *M. tuberculosis* (Berthet et al., 1998). A mixture of both these proteins has been shown to have better efficiency than TST and recognize only those interferons which are produced in response to activation by them only (Caulfield et al., 2016).

### 2.9.6 TB Diagnosis Directly from Clinical Samples

The long generation time of *M. tuberculosis* demands methods of diagnosis which are independent of culturing. Hence the focus has shifted to methods to detect *M. tuberculosis* directly from clinical samples.

#### 2.9.6.1 Phage Based Diagnostic Tests

Phage based tests are easy to perform, rapid and economical tests. Phages which infect mycobacteria are called mycobacteriophages. These mycobacteriophages are used to infect the bacteria present in the samples. If present, the phages get ingested by the mycobacteria in the sample and multiply within them (Figure 7). The viable phages present in the sample and not ingested by the mycobacteria (as in case of a negative sample) are killed by the addition of a viricidal substance. Now the phages trapped in the mycobacteria are amplified either by infecting a *M. smegmatis* culture or using a luciferase assay. In the PhaB method, the phages are amplified biologically by infecting a *M. smegmatis* culture (Eltringham et al., 1999; Wilson et al., 1997). If present in the sample now, the phages infect the *M. smegmatis* and produce plaque. This result could also be converted into a numerical signal and quantitated to obtain the number of mycobacteria originally present in the sample. Another method to detect the infecting mycobacteriophages is by using a reporter phage which has luciferase activity. A positive light
signal is detected or quantitated using a polaroid film box (Riska et al., 1999). Light signal is not observed if there are no mycobacteria in the sample or have been killed during antibiotic sensitivity checking (Banaiee et al., 2001). Hence, either of these methods could be used for TB diagnosis or checking antibiotic resistance (Eltringham et al., 1999).

Figure 7. Detection of *M. tuberculosis* by PhaB assay (Hazbon et al., 2004)

Note: A) Clinical sample after decontamination is mixed with mycobacteriophage. Bacterial cells present in a positive clinical sample are infected by the phage. B) A viricidal substance (ferrous ammonium sulphate) is added to kill all the non-infecting phages. C) Phage replication results in lysis of the bacteria. D) Release of infecting phages in the solution. E) These phages are used to further infect sensor cells (which could be a *M. smegmatis* culture). F) Plaque formation is
observed. G) Plaque formation is not observed if the sample is originally negative for mycobacteria.

2.9.6.2 Nucleic Acid Amplification Tests

Nucleic acid amplification tests (NAATs) are methods to identify microorganisms present even small amounts of DNA or RNA by repeated amplification of specific gene or repeat sequences in the genome. There is no amplification if the original clinical sample does not contain the causative organism for the disease. A number of techniques could be used to amplify DNA namely polymerase chain reaction (PCR) or Ligase Chain Reaction (LCR) in which a genetic probe is amplified. However, PCR is the most commonly used method of all. Agarose gel electrophoresis is used to analyse the amplified products of the PCR with the help of a molecular weight marker. Other methods used to identify the amplified products are DNA sequencing or recombination with a nucleic acid probe which can be detected by colorimetric ELISA or fluorescence (Louie et al., 2000). Molecular amplification using a genetic oligonucleotide for recombination (probe) is a multi-step process. First of all amplification of the target DNA takes place and then DNA probe hybridizes with the target element like the IS6110. This hybridization produces a detectable fluorescent or colorimetric signal which could be measured qualitatively or quantitatively. Such a technique could be used in different ways to either to differentiate between MTBC and atypical mycobacteria or to determine the genes involved in drug resistance mechanisms. The sensitivity and specificity of PCR methods have been studied extensively. Inspite of the fact that the specificity of PCR assays is high, their sensitivity is said to be less than culture. Sensitivity of PCR in smear positive and smear negative but culture positive samples is expected to be 90-100% and 60-70% respectively (Drobniewski et al., 2003).

One of the commercially available kit tests is the Roche Amplicor® Mycobacterium tuberculosis test (ATS, 1997). This test targets the 16S rRNA gene for amplification and the PCR product is detected colorimetrically. The Gen-Probe Amplified Mycobacterium tuberculosis Direct Test (MTD ®),(CDC, 1996) which is an isothermal method of amplification targets the rRNA in place of DNA. The BD ProbeTec also uses semi-automated real time amplification system with two primers and a fluorescently labeled probe (McHugh et al., 2004). However, commercial kits
are expensive and their procurement is not feasible for routine testing in resource limited countries. Hence in-house PCR assays are used. IS6110 is a repeat element present in multiple copies in the MTBC genome. Mostly PCR tests exploit this sequence for amplification (Eisenach et al., 1990; 1991; Kocagoz et al., 1993; Nolte et al., 1993). It can result in false positive results also as some atypical mycobacteria are reported to contain IS6110 (Kent et al., 1995) and some strains lacking it might give false negative results (Radhakrishna et al., 2001; El Dawi et al., 2004). Other genes which have been studied for diagnosis by PCR are rpoB (Mokaddas et al., 2007), hsp65 (Pao et al., 1990; Walker et al., 1992; Bhattacharya et al., 2003; Schewe et al., 2005; Singh et al., 2012), 16S rRNA (Miller et al., 1994), 32-kDa (Soini et al., 1992; 1996), 38kDa (Sjobring et al., 1990; Miyazaki et al., 1993; Yuen et al., 1993), devR (Chakravorty et al., 2005) IS986 (Anderson et al., 1993; Kolk et al., 1992; Hermans et al., 1990), IS6110 (Forbes et al., 1993; Claridge et al., 1993; Hashimoto et al., 1995; Thierry et al., 1995; Noordhoek et al., 1995; Cohen et al., 1998; Gunisha et al., 2001), mpt40 (Marchetti et al., 1998; Portillo et al., 1991), mpt64 (Mitarai et al., 1995; Therese et al., 2005), etc. Using PCR is advantageous as it greatly decreases the time required for diagnosis. It can be performed within 3-6 hours of sample collection and has good sensitivity and specificity. Secondly, clinical samples need not be processed much for PCR as it can be performed even with a crude extract. This is helpful in situations when culturing of the sample is difficult as in the case of paucibacillary infections or resource limited settings (Jatana et al., 2003). Still PCR is not used for routine diagnosis in developing countries because of the high expenses involved in sophisticated instrument, laboratory and technical training of the staff (Kivihya-Ndugga et al., 2003). Given the high sensitivity of PCR, a number of false positive results might arise from cross contamination in laboratory (Barnes et al., 1997).

2.9.6.2.1 Cepheid Xpert MTB/RIF Test

The GeneXpert MTB/RIF is a diagnostic method based on a fully automated DNA extraction, amplification and detection system. It was developed by Cepheid Inc., USA), Foundation for Innovative Diagnostics (FIND) and US National Institute of Health (NIH). It is based on a single
use cartridge system to detect MTBC organisms (Figure 8) from clinical samples and can also detect mutations in the \textit{rpoB} gene for rifampicin resistance. 96% of rifampicin resistance can be attributed to mutations in 81bp region of the \textit{rpoB} gene. This is an important indicator of MDR-TB since most of these could also be expected to be resistant to isoniazid. Since it a fully automated procedure which takes place in a sealed cartridge, a number of precautions and necessary biosafety laboratory and infrastructure can be avoided and minimally skilled staff is required. The liquefied sputum sample is inactivated to kill any mycobacteria present in the sample. It is then placed in the cartridge and sealed and inserted in the machine for analysis. In 2 hours the machine gives the results for nested real time PCR of the sample. Just an initial introduction and training of the staff to use the instrument is required. The machine is capable of processing from 16-48 tests at the same time. The 48-test module is called the infinity and can process up to 2074 samples in a day (WHO, 2017c).

![Figure 8. The Cepheid Xpert® system and MTB/RIF test cartridge for diagnosis of \textit{M. tuberculosis} infection and rifampicin resistance (Caulfield et al., 2016)](image)

FIND and other government agencies have undertaken a number of evaluation studies of the GeneXpert® MTB/RIF. The results for sensitivity in diagnosis and drug resistance detection are encouraging. High sensitivity in smear positive samples and around 72% in smear negative
culture positive samples makes GeneXpert an outstanding method for screening the suspected patients. On further evaluation, the sensitivity increased to 90.2% on repeating it thrice (Boehme et al., 2011). A majority of existing PCR systems perform well only in smear positive samples. The reagents required for DNA extraction from the sample, its amplification and detection are all stored in the cartridge and have a shelf life of 18 months and can be stored at up to 28°C. Recent guidelines from CDC recommend that NAA testing be performed on a minimum of one clinical sample (preferably the first) from each PTB suspect (Ling et al., 2008). It is recommended by WHO that the use of Xpert MTB/RIF be expanded to district levels and below and not limited to central/reference laboratories. It is mostly recommended for MDR-TB suspects and in case of TB-HIV co-infection. Encouraging results have been observed in preliminary tests using extrapulmonary samples (Vadwai et al., 2011; Hilleman, et al., 2011). Overall, strengths of the XpertMTB/RIF assay include good sensitivity and specificity for respiratory specimens, a rapid 2-hour TAT, the ability to detect *M. tuberculosis* complex and rifampin resistance directly from specimens, and the use of a closed PCR system with a low risk of cross-contamination. The limitations of GeneXpert are related to its high cost and continuous power supply demand. It is a fully automated instrument and the cost of the instrument and cartridges is very expensive. Secondly, the cartridges and the machine itself needs proper storage conditions (at optimum temperature) and connectivity with a computer and continuous power supply are absolute essentials. All these conditions are hard to fulfill in rural areas and peripheral laboratories where even the disposal of used material would be difficult (Trebcq et al., 2011) GeneXpert® is a cost-effective method of TB diagnosis, but cost-effectiveness will clearly depend on the context and setting (Vassall et al., 2011).

2.9.6.2.2 Line Probe Assay (LPA)

It is a technique for identification of mycobacteria that make use of hybridization-based probes. This technology utilizes nitrocellulose membrane strips embedded with genus- and species-specific probes. DNA from smear positive samples directly or lysed culture isolates hybridizes to probes and produces colorimetric bands when complementary DNA is present to allow for
species identification (Figure 9). The commercially available line probe assays include the GenoType MTBDRplus assay (Hain Lifescience, Germany) and the Inno-LiPA Rif. TB line probe assay (Innogenetics, Belgium). Test can only be performed in a Bio Safety Level (BSL)-2 (with sputum samples) and BSL-3 (with MTBC cultures) laboratory with special rooms for DNA extraction and amplification. Such facilities are only found in reference level laboratories or hospitals. Only a day or two are required for MTBC detection and isoniazid or rifampicin resistance by LPA. On the contrary drug sensitivity testing using culture might take a few weeks. Inno-LiPA test analyses the mutation of rpoB gene (for rifampicin resistance) which are found in 90% of MDR-TB cases. This means that the results are in coherence with GeneXpert MTB/RIF and can be suspected for MDR. But GenoType MTBDRplus assay tests for katG and inhA (isoniazid resistance) gene mutations (WHO, 2017c).

Figure 9. Line probe assay (schematic representation) (Caulfield et al., 2016)

Note: The GenoType MTBC test (Hain Lifescience) utilizes several embedded DNA probes to detect and differentiate members of the M. tuberculosis complex.

For LPA, sputum sample or TB culture is processed for DNA extraction and amplified by PCR method. The amplified product is allowed to hybridize with sequence-specific oligonucleotide
on nitrocellulose strip. MTBC members are identified by colorimetric labeling of the obtained hybrids. The status of a mutated or wild type gene is ascertained by comparing to a chart of a specific pattern of stripes showing specific alleles. It takes only 4-5 hours for observing the results of a smear positive sample (visually or with the help of an instrument). Whereas DST with the help of culture method takes at least 60 days. LPA have been approved and recommended by WHO for MDR-TB diagnosis (WHO, 2008). A pre-requisite for carrying out LPA is a smear positive sample or any sample which has bacterial load sufficient for DNA amplification. Hence, LPA results are not reliable for paucibacillary samples or samples from which good quality DNA for PCR cannot be prepared. Analysis in China, India and South Africa shows that LPA costs about ~$10 per test in these countries which is way less than DST by culture method.

2.10 LOOP-MEDIATED ISOTHERMAL AMPLIFICATION (LAMP)

Loop mediated isothermal amplification was developed by Tsugonori Notomi and co-workers (Eiken Chemical Co. Ltd., Japan) in 2000 (Notomi et al., 2000; Nagamine et al., 2001; 2002).

2.10.1 Principle

LAMP assay is a DNA synthesis method performed with Bst DNA polymerase and set of inner / outer primers. It relies on strand displacement activity of Bst DNA polymerase.

In this method, inner primers hybridize to the target DNA and form first strand. This strand is displaced by the outer primer (present in low concentration). As a result, a part of Forward Inner Primer (FIP) attains a loop structure. Hence, a process is started in which Backward Inner Primer (BIP) anneals with complementary strand that results in formation of a dumbbell shaped structure which is formed by synthesis of strand followed by displacement with inner primers. This structure acts as a template for products in a LAMP reaction. Four or six highly specific designed primers are known to hybridize with six or eight distinct sequence sites of the template DNA respectively; therefore the amplification of the target DNA is highly specific (Eiken Chemical Co., Japan) (Notomi et al., 2000).
2.10.2 Parameters of LAMP Primer Designing

For amplification of LAMP products highly sensitive and specific primer set need to be designed (Primer Explorer, Net Laboratory, Japan). Secondary structure formation, GC-content and primer length are important factors to be kept in mind while designing primers. There are three pairs of primers for LAMP amplification namely: two outer, inner and loop primers each. In non-cycling steps, the strand displacement is caused by outer primers called the Forward outer primer (F3) and Backward outer primer (B3). A loop is formed with the sense and antisense sequence of forward and Backward Inner Primers (FIP and BIP). In addition to this, Forward Loop Primer (FLP) and Backward Loop Primers (BLP) are used to increase the rate of amplification (Virocon, 2008).

The eight regions of these primers are complementary to F3c, F2c, F1c, FLP (3’-end) and B1, B2, B3, BLP (5’-end) regions of the target gene (Figure 10A). FIP consist of F2 and F1c region at 3’-end and 5’-ends respectively. Similarly BIP contains B2 region (3’-end) and B1c region (5’-end). Backward outer primer (B3) consists of B3 region that is complementary to the B3c region. The FLP and BLP primers consist of sequence complementary to regions between F1 & F2 and B1 & B2 respectively. LAMP primers have same properties like ideal set of primers in regard to GC-content, secondary structure formation, melting temperature and site specificity. The distance between 5’-end of F2 and B2 is considered to be 120-180bp, and the distance between F2 and F3 as well as B2 and B3 is 0-20bp. The distance for loop forming regions (5’ of F2 to 3’ of F1, 5’ of B2 to 3’ of B1) is 40-60bp. The stability of primer ends should be established based on the dG calculated on the 6 bp from the following end regions should be less than -4kcal/mol, 5’-end of F1c/B1c and 3’-end of F2/B2 as well as F3/B3 (Notomi et al., 2000).

2.10.3 Mechanism of LAMP Reaction

The mechanism of LAMP reaction involves non-cyclic and cyclic steps (Figure 10).
2.10.3.1 Non-Cyclic Steps

At 65°C LAMP primers anneal to the target DNA and Bst DNA polymerase results in strand displacement and DNA synthesis. Single stranded (ss) DNA is formed. The complementary DNA strand is formed starting from the 3’ end of the F2 region of the FIP. Same process takes place when F3 primer anneals to the F3c region. Double stranded DNA is only formed by F3 anneals to target DNA. This displaces the complementary ss DNA formed from FIP linking to target DNA. The ssDNA forms stem-loop structure because of its self-complementarity. Further this acts as template for strand displacement and DNA synthesis for other primers- BIP and B3. Hence BIP initiates strand formation from 3’-end. This results in opening of the loop to a linear structure. Again, the B3 anneals to the single stranded outer portion of BIP. DNA synthesis starts here from the B3 primer and previously annealed DNA is released as a single strand. Double stranded DNA is produced through the processes described above. The resulting strand with BIP-linked strand at one end and stem loop structure at the other serves as an initiation point for the cyclic steps of LAMP reaction.

2.10.3.2 Cycling Amplification Steps

The stem loop structure results in self-primed DNA synthesis. This newly synthesised strand is displaced by the FIP annealing to ssDNA in stem loop and synthesis of a fresh DNA strand starts. This released single strand again forms a stem loop structure resulting from B1 and B1c complementarity. FIP-linked complementary strand is released. The released single strand then forms a dumbbell-like structure as both ends have complementary F1 - F1c and B1c - B1 regions, respectively. The process starts all over again with self-primed DNA synthesis BIP anneals to the B2c region and primes strand displacement DNA synthesis, releasing the B1-primed DNA strand (Notomi et al., 2000).
Figure 10. Schematic representation of the mechanism of LAMP A) Non-cyclic and B) Cyclic Amplification steps (Parida et al., 2008)
2.10.4 Bst DNA Polymerase

LAMP reaction requires Bst DNA polymerase large fragment which is a 67kDa protein from *Bacillus stearothermophilus* with a high strand displacement activity that omits the requirement of denaturation step and denatured DNA template. It lacks an exonuclease activity. Its function lies between thermophilic and mesophilic polymerase. The optimum temperature range (60-65°C) is higher in comparison to DNA polymerase I, Large (Klenow) fragment and lower as compared to Vent DNA Polymerase. This wide range results in designing useful sequencing strategies. As reported in literature, Bst DNA polymerase has 100% activity at 60-65°C. That’s why most of the workers have reported the reaction temperature between the range 60-65°C.

2.10.5 LAMP- An Economical Amplification Technique

LAMP is an isothermal DNA amplification reaction which only needs a regular laboratory water-bath or a heating block to maintain a constant temperature for the assay. Hence sophisticated and expensive instruments like thermocycler are not needed at all. The duration of the assay ranges from 15-90 minutes. After the assay is over, the enzyme is inactivated at 80°C for 2-10 minutes to terminate the reaction. Another advantage over PCR is its tolerance to culture media and biological substances (heme, PBS, MEM, saline, serum, plasma, etc.) which inhibit PCR (Kaneko *et al*., 2007). A study also reports that DNA isolation can be omitted in LAMP assay.

2.10.6 Monitoring LAMP Reaction

2.10.6.1 Real-Time Monitoring: The real time monitoring of LAMP amplification product can be done through spectrophotometric analysis with the help of LoopAmp real-time turbidimeter (LA-200, Termecs, Japan) that records the turbidity in the form of optical density (OD) at 400nm at every 6 seconds (Pandey *et al*., 2008; Parida *et al*., 2004; 2006). The turbidimeter is relatively inexpensive as compared to real time PCR machine.

2.10.6.2 Agarose Gel Electrophoresis: The LAMP amplification products can be detected qualitatively by agarose gel electrophoresis. The DNA is stained by incorporation of ethidium bromide in the gel and visualised using a gel-doc or UV-transilluminator. Positive LAMP reaction produces a characteristic ladder-like pattern of multiple bands on agarose gel indicative
of stem loop DNA with inverted repeats of target sequence (Enosawa et al., 2003; Saito et al., 2013; Solimon et al., 2005; Paris et al., 2007).

2.10.6.3 Naked Eye Visualisation

a) Turbidity: Turbidity is a unique phenomenon associated with LAMP, owing to its high DNA amplification efficiency. When the amplified DNA conc. exceeds 4µg the resulting pyrophosphate ion concentration exceeds 0.5mM. The LAMP reaction produce DNA yield of more than 10µg/25µl, this pyrophosphate ion reacts with magnesium in the buffer forming a precipitate of magnesium pyrophosphate. While in PCR the DNA yield of 0.210µg/25µl and the resulting pyrophosphate ion approximates 0.02mM. This pyrophosphate amount may also get hydrolysed to phosphate due to high temperature >94ºC. The generation of magnesium pyrophosphate precipitate has been confirmed by IR spectrum of isolated precipitate (Mori et al., 2001; 2006). When the precipitate dissolved with hydrochloric acid was treated with titan yellow, its colour changed from yellow to red. Moreover, this red colour was similar to that of commercially available magnesium pyrophosphate with the same weight when compared with qualitative analysis. This result qualitatively indicates that the main cationic component in the precipitate was composed of magnesium ion. Furthermore, when the LAMP reaction was conducted with the presence of Tth- pyrophosphatase, enzyme that hydrolyses pyrophosphate ion to orthophosphate ion, no precipitate was produced while DNA amplification occurred. So, the production of precipitate based on LAMP reaction can be represented by the following reaction:

\[(\text{DNA})_{n-1} + d\text{NTP} \rightarrow (\text{DNA})_n + \text{P}_2\text{O}_7^{-4} \quad (1)\]

\[\text{P}_2\text{O}_7^{-4} + 2\text{Mg}^{2+} \rightarrow \text{Mg}_2\text{P}_2\text{O}_7 \quad (2)\]

b) Using SYBR Green I: SYBR Green I is an asymmetrical cyanine dye that binds to double stranded (ds)DNA with great specificity. The LAMP amplicons can be visualised by addition of diluted SYBR Green I after the assay is over. In case of a positive reaction, the original orange colour of the dye changes to green and but shows no change in case of a negative reaction (Iwamoto et al., 2003; Aryan et al., 2010)

c) Using a Fluorescent Metal Indicator Calcein: A simple visual detection system has been developed in which a metal fluorescent indicator, calcein is added to the reaction mixture. This
indicator forms complexes with divalent metallic ions, such as magnesium and calcium. In LAMP, a large amount of DNA is synthesised, yielding a large pyrophosphate ion by-product from deoxyribonucleotide triphosphates (dNTPs). The calcein in the reaction mixture initially combines with manganous ion (Mn$^{2+}$) so as to remain quenched. When the amplification reaction proceeds, manganous ion is deprived of calcein by the generated pyrophosphate ion, which results in emission of fluorescence. And the free calcein is apt to combine with (Mg$^{2+}$) in the reaction mixture, so that it strengthens the fluorescence emission. This detection is simpler, safe and improved as compared to visual detection of magnesium pyrophosphate precipitate and SYBR Green I. The white precipitate is rather faint for visual detection of Magnesium pyrophosphate precipitate and opening the tube to add SYBR Green I involves a high risk of workspace with amplicons. Since the large amounts of products are a repeat of the same sequence, the highly sensitive LAMP reaction may lead to incorrect result upon contamination of even a small quantity of amplicons (Tomita et al., 2008; Zhu et al., 2009).

2.10.7 Specificity, Sensitivity and Applicability of LAMP

LAMP is a sensitive method which can amplify a few copies of DNA to $10^9$ copies in less than an hour and under isothermal conditions (Notomi et al., 2000). The use of six primers for LAMP ensures high specificity for target amplification. Hence, target selectivity is higher than that of other amplification methods like PCR and Strand Displacement Assay (SDA) (Aryan et al., 2010).

LAMP is a promising method for rapid diagnosis in cases of infections. LAMP has a wide range of applicability in the detection of micro-organisms. At present time, all over the world studies have been developed to recognise infectious agent by LAMP technique. It has been used to identify pathogenic agents in dentistry (Yoshida et al., 2005), food-borne pathogens (Zende et al., 2017), veterinary sciences (Rekha et al., 2015), etc. Although most of the research are about virus detection such as tomato yellow leaf curl-virus (Fukuta et al., 2003), Varicella roster virus (Okamoto et al., 2004), West-Nile virus (Parida et al., 2004), Newcastle disease virus (Pham et al., 2005), Respiratory syncitial virus (Ushio et al., 2005), Herpes simplex virus (Enomoto et al., 2005), Human papilloma virus (Hagiwara et al., 2007), Hepatitis B virus (Moslemi et al., 2009), influenza (Ito et al., 2006; Kubo et al., 2010), Epstein barr virus (Iwata et al., 2006), Ebola virus (Kurosaki et al., 2007) and HIV (Curtis et al., 2009), but also there are many investigations that
approved the detection of bacteria and parasites like *M. tuberculosis* (Geojith et al., 2011; Mitarai et al., 2011; Kaewphinit et al., 2013), *M. bovis* (Zhu et al., 2009; Bi et al., 2012), *M. intracellulare* (Iwamoto et al., 2003), *M. avium subsp. paratuberculosis* (Enosawa et al., 2003), environmental mycobacteria (Adhikari et al., 2009), *M. pneumonia* (Saito et al., 2005), *Plasmodium falciparum* (Poon et al., 2006), *Cryptosporidium oocysts* (Karanis et al., 2007), *Enterococcus faecalis* (Kato et al., 2007), *Trypanosoma brucei rhodesiens* (Njiru et al., 2008), Filarial parasites (Aonuma et al., 2009), *Leptospira*, Toxoplasma gondii (Zhang et al., 2009) by LAMP.

### 2.10.8 LAMP for *M. tuberculosis*

LAMP was shown to be more sensitive than conventional PCR, nested PCR for *M. avium* subsp. *paratuberculosis* (Enosawa et al., 2003) and similar sensitivity to Amplicor assay for MTBC, *M. avium* and *M. intracellulare* (Iwamoto et al., 2003), Tuberculin Skin Test, nested PCR for MTBC (Aryan et al., 2010). Further, sensitivity of MTB LAMP in culture positive samples was 100% and the specificity in culture negative was 94.25% (WHO, 2016). The positive and negative predictive values of MTB LAMP were 94.1 and 100% respectively (Boehme et al., 2007). In another study, the sensitivity of LAMP in smear-negative, culture-positive specimen was 48.8%. The specificity in culture negative samples was 99% (Pandey et al., 2008). Table 2 summarises the results of studies for evaluation of LAMP for TB diagnosis.

### Table 1. Performance of LAMP assay using different DNA targets in previous studies

<table>
<thead>
<tr>
<th>Study</th>
<th>Country</th>
<th>Facility</th>
<th>Target</th>
<th>Sensitivity (% CI**)</th>
<th>Specificity (% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Present study</td>
<td>India</td>
<td>Tertiary care centre</td>
<td>In-house <em>mpt64</em></td>
<td>1.00 [0.96, 1.00]</td>
<td>1.00 [0.90, 0.99]</td>
</tr>
<tr>
<td>Aryan (2013)</td>
<td>Iran</td>
<td>University Hospital</td>
<td>In-house IS6110</td>
<td>0.89 [0.80, 0.95]</td>
<td>0.89 [0.71, 0.98]</td>
</tr>
<tr>
<td>Boehme (2007)</td>
<td>Peru, Bangladesh, Tanzania</td>
<td>Centres</td>
<td>In-house <em>gyrB</em></td>
<td>0.88 [0.83, 0.92]</td>
<td>0.99 [0.98, 1.00]</td>
</tr>
<tr>
<td>Dolker (2012)</td>
<td>India</td>
<td>TB hospital</td>
<td>In-house IS6110</td>
<td>0.97 [0.94, 0.99]</td>
<td>0.60 [0.47, 0.72]</td>
</tr>
<tr>
<td>Fujisaki</td>
<td>Japan</td>
<td>University</td>
<td>In-house IS6110</td>
<td>1.00 [0.48, 1.00]</td>
<td>1.00 [0.48, 1.00]</td>
</tr>
<tr>
<td>Year</td>
<td>Country</td>
<td>Location</td>
<td>Method</td>
<td>Rifampicin Sensitivity</td>
<td>Ethambutol Sensitivity</td>
</tr>
<tr>
<td>--------</td>
<td>---------</td>
<td>---------------------------</td>
<td>-------------------</td>
<td>------------------------</td>
<td>------------------------</td>
</tr>
<tr>
<td>2004</td>
<td>George</td>
<td>India College hospital</td>
<td>In-house rimM</td>
<td>0.79 [0.64, 0.91]</td>
<td>0.94 [0.79, 0.99]</td>
</tr>
<tr>
<td>2011</td>
<td>Hong</td>
<td>China TB hospital</td>
<td>In-house esat-6, mpt40</td>
<td>0.92 [0.64, 1.00]</td>
<td>0.85 [0.66, 0.96]</td>
</tr>
<tr>
<td>2011</td>
<td>Iwamoto</td>
<td>Japan Community hospitals</td>
<td>*Loopamp MTBC</td>
<td>0.90 [0.68, 0.99]</td>
<td>0.96 [0.85, 0.99]</td>
</tr>
<tr>
<td>2012</td>
<td>Kaewphinit</td>
<td>Thailand TB laboratory</td>
<td>In-house IS6110</td>
<td>0.99 [0.94, 1.00]</td>
<td>1.00 [0.63, 1.00]</td>
</tr>
<tr>
<td>2011</td>
<td>Kohan</td>
<td>Iran TB centre</td>
<td>In-house IS6110</td>
<td>1.00 [0.94, 1.00]</td>
<td>0.96 [0.88, 0.99]</td>
</tr>
<tr>
<td>2011</td>
<td>Lee</td>
<td>Taiwan University hospital</td>
<td>In-house 16S rDNA</td>
<td>0.94 [0.80, 0.99]</td>
<td>0.94 [0.88, 0.98]</td>
</tr>
<tr>
<td>2011</td>
<td>Mitarai</td>
<td>Japan TB hospital</td>
<td>*Loopamp MTBC</td>
<td>0.86 [0.80, 0.90]</td>
<td>0.93 [0.86, 0.97]</td>
</tr>
<tr>
<td>2015</td>
<td>Moon</td>
<td>Korea University hospital</td>
<td>In-house hspX</td>
<td>0.91 [0.77, 0.98]</td>
<td>0.95 [0.92, 0.97]</td>
</tr>
<tr>
<td>2013</td>
<td>Nimesh</td>
<td>India Hospital</td>
<td>In-house sdaA</td>
<td>1.00 [0.81, 1.00]</td>
<td>0.97 [0.94, 0.99]</td>
</tr>
<tr>
<td>2014</td>
<td>Ou</td>
<td>China Microscopy centres</td>
<td>*Loopamp MTBC</td>
<td>0.71 [0.66, 0.75]</td>
<td>0.98 [0.97, 0.99]</td>
</tr>
<tr>
<td>2015</td>
<td>Rafati</td>
<td>Iran NA</td>
<td>In-house 16S rDNA</td>
<td>0.90 [0.55, 1.00]</td>
<td>0.95 [0.83, 0.99]</td>
</tr>
<tr>
<td>2013</td>
<td>Saito</td>
<td>Japan NA</td>
<td>*Loopamp MTBC</td>
<td>0.84 [0.64, 0.95]</td>
<td>1.00 [0.97, 1.00]</td>
</tr>
<tr>
<td>2013</td>
<td>Sethi</td>
<td>India Chest clinic</td>
<td>In-house 16S rRNA</td>
<td>0.95 [0.87, 0.99]</td>
<td>0.48 [0.28, 0.69]</td>
</tr>
<tr>
<td>2011</td>
<td>Watari</td>
<td>Japan NA</td>
<td>*Loopamp MTBC</td>
<td>0.88 [0.47, 1.00]</td>
<td>1.00 [0.83, 1.00]</td>
</tr>
</tbody>
</table>

Note: *Loopamp MTBC-Loopamp MTBC detection kit targeting gyrB DNA and IS6110 DNA manufactured by Eiken Chemical Co., Ltd (Togichi, Japan)

**CI-Confidence Interval
Literature review reveals that LAMP is a very promising technique as it is able to identify *M. tuberculosis* DNA with sensitivity more than or equal to PCR because of the higher efficiency amplification and in-turn the large amount of DNA produced (compared to PCR) as well as its immunity to inhibitors and the high specificity because of multiple primers targeting a region (Tomita et al., 2008). These when combined with the simplicity of the procedure, less time for result, low-cost and absence of sophisticated equipment, makes LAMP suitable for developing countries with high TB burden.