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To Analyze The Positivity Rate of Mycobacterium Tuberculosis PCR In Patients With Chronic Meningitis

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I. INTRODUCTION

The most dangerous form of extrapulmonary tuberculosis is tuberculous meningitis (TBM), which occurs in 7-12% of tuberculous patients in developing countries (Tandon, 1978). TBM can occur at any age except in the newborn. Patients with TBM always have a focus of infection elsewhere, but one in four have no clinical or historical evidence of such an infection. Occasionally, the onset is much more rapid and may be mistaken for a subarachnoid hemorrhage (Braude, 1981). In spite of the availability of effective chemotherapy, the motality and morbidity of TBM remain high (Molavi and LeFrock, 1985). Tuberculosis (TB) is a major global problem and a public health issue of considerable magnitude. Approximately, eight million new cases of TB and three million deaths are reported annually (3). In recent times, there has been a resurgence of tuberculosis in both developing and developed countries; the incidence varies from 9 cases per 100,000 persons per year in the US to 110-165 cases per 100,000 persons in the developing countries of Asia and Africa (7,20,24). The attributing risk factors include the increasing prevalence of HIV infection, overcrowding in the urban population and in abnormal communities (such as prisons, concentration camps and refugee colonies), poor nutritional status, appearance of drug-resistant strains of tuberculosis and ineffective tuberculosis control programmes. TB is a chronic, systemic infectious disease caused by the *Mycobacterium tuberculosis* primarily manifesting as pulmonary Koch's. The inhaled bacilli can localize in alternate sites, leading to extrapulmonary TB (EPTB). Tuberculous involvement of the central nervous system (CNS) is an important and serious type of extra-pulmonary involvement (26). It has been estimated that approximately 10% of all patients with tuberculosis have CNS involvement (27). Fatality rates in developing countries have been reported to range from 44 to 69% (6,8,19). In fact, missed diagnosis and delayed treatment often results in serious longterm debilitating complications. Moreover, the clinical response to antituberculosis therapy in all forms of neuro-TB is excellent, provided the diagnosis is made early; before an irreversible neurological defect occurs (delay in diagnosis is directly related to neurologic sequelae in 20-25% of patients who do not receive early treatment). Clearly, prompt laboratory diagnosis is of vital importance. The great majority of patients with neuro-TB are diagnosed on the basis of clinical criteria, radiographic findings and laboratory investigation of the cerebrospinal fluid (CSF) (11). Acid-fast staining of CSF sediment is the most rapid method for detection of mycobacteria, but this method requires >104 cells ml–1 hence lacks sensitivity. Conventional methods like microscopy and culture, although considered as gold standards, are quite inadequate (12). The diagnostic reference standard, isolation of *Mycobacterium tuberculosis* from CSF samples, is insufficiently timely (it requires 2-6 weeks) to aid clinical judgment with respect to treatment and because of the paucibacillary state in the cerebrospinal fluid this method is insensitive if large amounts of CSF are not tested. PCR and molecular analysis techniques show promise as tools for rapid diagnosis of pulmonary, EPTB and CNS tuberculosis (1,3,11,12,17,18,23). However, the accuracy and reproducibility of these molecular analysis techniques for the detection of *M. tuberculosis* in CSF has not been clearly defined. Therefore, an in-house developed, *MPB64* gene targeted PCR was evaluated at our centre for rapid and specific diagnosis of CNS tuberculosis. Conventional bacteriology, such as direct microscopy and culture, are not sufficient for the diagnosis of TBM because there are too few bacilli in the cerebrospinal fluid (CFS) to be demonstrated by direct microscopy and on the other hand, successful culture identification of tubercle bacilli takes about 7 weeks. Fortunately, molecular techniques have been developed as sensitive and reliable diagnostic tools for the identification of tubercle bacilli. The most important advance in the usefulness of molecular methods, especially in diagnostic application, is the polymerase chain reaction (PCR), which has in many cases increased not only the speed of DNA-based assays, but

also greatly enhanced their sensitivity. In the study described here, PCR was performed to detect specific *M. tuberculosis* DNA in CSF specimens from highly probable TBM patients.

II. OBJECTIVES

The present investigation was done for the diagnosis of Mycobacterium tuberculosis in patients having a probability of Meningitis occurrence using polymerase chain reaction, of different CSF samples brought in our laboratory with the following objectives.

- ❖ Collection of various CSF samples from different hospitals of Delhi and NCR.
- ❖ Molecular Diagnosis

1. MTB Genomic DNA extraction.
2. Amplification of DNA obtained.

- ❖ To compare among the positivity of AFB/ZN Stain, Culture and PCR.

III. REVIEW OF LITERATURE

Mycobacterium tuberculosis

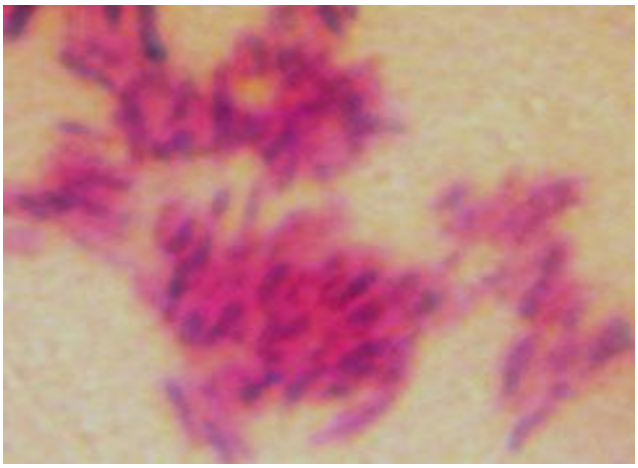


Fig 1: Mycobactrium tuberculosis

1.Historical Background

Mycobacterium tuberculosis is the causative agent of tuberculosis. It is a pathogenic bacterial species and belongs to genus Mycobacterium (1). Tuberculosis has been present in humans since ancient times. About tuberculosis, first reference in Asian civilization is found in the Vedas. Rigveda called this disease as yaksma while Atharveda called it as balasa. However, it is not clear yet that whether tuberculosis originated in cattle and then transferred to humans, or diverged from a common ancestor infecting a different species. Tuberculosis was considered as vampirism before the industrial revolution because if one member of a family died from it, then other members of family may also lose their health slowly (2). Mycobacterium tuberculosis was first discovered in 1882 by Robert Koch (Fig 1). For this he utilized a new staining method and applied it to the sputum of tuberculosis patients, revealing for the first time the causal agent of the disease, Mycobacterium tuberculosis or Koch's bacillus. Before tuberculosis, he discovered the Bacillus anthracis, the causative agent of anthrax. On 18 August 1881, while staining tuberculous material with methylene blue, he noticed oblong structures. To improve the contrast, he decide to add Bismarck Brown, after which the oblong structures were rendered bright and transparent. He improved the technique by varying the concentration of alkali in the staining solution until the ideal viewing conditions for the bacilli was achieved (3). He made his results public on 24 March 1882 at the Physiological Society of Berlin, in a famous lecture entitled Uber Tuberculose, which was published three weeks later (4). Since 1982, 24 March has been known as World Tuberculosis Day. He received the Nobel Prize in physiology or medicine in 1905 for this discovery. On 20th April 1882, Koch presented an article entitled Die Atiologie der Tuberculose in which he demonstrated that Mycobacterium was the single cause of tuberculosis in all of its forms (3).

Fig 2: German scientist- Robert Koch (5)

In 1890 Koch developed a purified protein derivative of the bacteria called tuberculin (6). It proved to be an ineffective means of immunization but in 1908, Charles Mantoux found it was an effective intradermic test for diagnosing tuberculosis (7). People infected with TB often have symptoms such as red, swollen eyes (which also creates a sensitivity to bright light), pale skin, extremely low body heat, a weak heart and coughing blood (8).

1.2 *M. tuberculosis* complex

The *Mycobacterium tuberculosis* complex consists of a highly related group of acid- alcohol-fast bacilli which are human and animal pathogens. It includes following classical species: *M. tuberculosis, Mycobacterium bovis and Mycobacterium microti.* There are *Mycobacterium microtii* and its BCG and *Mycobacterium peniipedi* species also which are included in *M. tuberculosis* complex (10). Other known pathogenic mycobacteria include *Mycobacterium leprae*, *Mycobacterium avium* and *M. kansasii*. The last two are part of the nontuberculous mycobacteria (NTM) group. Nontuberculous mycobacteria cause neither TB nor leprosy, but they do cause pulmonary diseases resembling TB (11).

1.3 Structure of MTB

Mycobacterium tuberculosis is a large nonmotile rod-shaped bacterium distantly related to the Actinomycetes (Fig 1). Many non pathogenic mycobacteria are components of the normal flora of humans, found most often in dry and oily locales. *Mycobacterium tuberculosis* is an obligate aerobe. Therfore, MTB complexes are always found in the well-aerated upper lobes of the lungs. The bacilli are 2-4 micrometers in length and 0.2-0.5 µm in width. *M. tuberculosis* is characterized by caseating granulomas containing Langhans giant cells, which have a "horseshoe" pattern of nuclei. The cell wall of mycobacterium has a waxy coat which is composed of mycolic acid and it makes the cell impermeable to gram stain (1). In smears chains of cells made from in vitro-grown colonies often form distinctive serpentine cords. This observation was first made by Robert Koch who associated cord factor with virulent strains of the bacterium. MTB can not be classified as either Gram-positive or Gram-negative because it does not have the chemical characteristics of either, although the bacteria do contain peptidoglycan (murein) in their cell wall. If a Gram stain is performed on MTB, it stains very weakly Gram-positive or not at all. *Mycobacterium* species, along with members of a related genus *Nocardia*, are classified as acid-fast bacteria due to their impermeability by certain dyes and stains. Despite this, once stained, acid-fast bacteria will retain dyes when heated and treated with acidified organic compounds.

1.4.1Cell Wall Structure

The cell wall structure of *Mycobacterium tuberculosis* is unique among prokaryotes, and it is a major determinant of virulence for the bacterium. The cell wall contains peptidoglycan and complex lipids. Over 60% of the mycobacterial cell wall is lipid and lipid fraction contains three major components, mycolic acids, cord factor, and wax-D (Fig.).

1. **Mycolic acid :** Mycolic acids are unique alpha-branched lipids found in cell walls of *Mycobacterium* and *Corynebacterium*. They make up 50% of the dry weight of the mycobacterial cell envelope. Mycolic

acids are strong hydrophobic molecules that forma lipid shell around the organism and affect permeability properties at the cell surface. Mycolic Acids are thought to be a significant determinant of virulence in MTB. Probably, they prevent attack of the mycobacteria by cationic proteins, lysozyme, and oxygen radicals in the phagocytic granule. They also protect extracellular mycobacteria from complement deposition in serum.

2. **Cord Factor :** Cord factor is responsible for the serpentine cording. Cord factor is toxicto mammalian cells and is also an inhibitor of PMN migration. Cord factor is most abundantly produced in virulent strains of MTB.
3. **Wax-D:** Wax-D in the cell envelope is the major component of Freund's complete adjuvant (CFA).

The peptidoglycan layer is linked to arabinogalactan (D-arabinose and D-galactose). The arabinogalactan is then linked to an outer membrane containing high-molecular weight mycolic acids. The arabinogalactan/mycolic acid layer is overlaid with a layer of polypeptides and mycolic acids consisting of free lipids, glycolipids, and peptidoglycolipids. Other glycolipids include lipoarabinomannan and phosphatidyl-inositol mannosides (PIM). Like the outer membrane of the gram-negative bacteria, porins are required to transport small hydrophilic molecules through the outer membrane of the acid-fast cell wall. The outer surface of the acid- fast cell wall is studded with surface proteins that differ with the strain and species of the bacterium. The periplasm is the gelatinous material between the peptidoglycan and the cytoplasmic membrane.

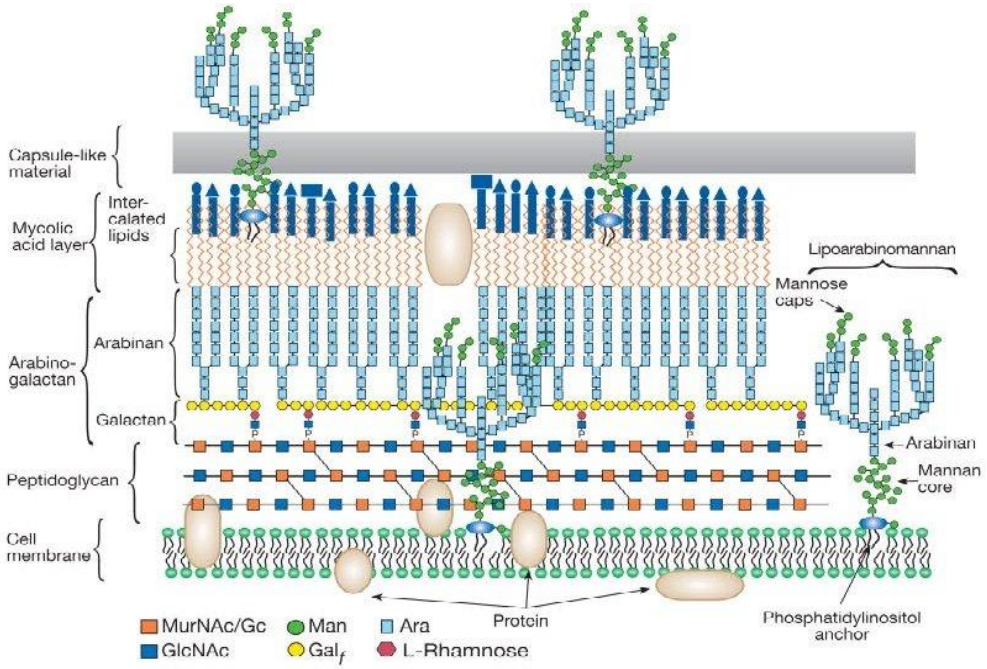


Fig 3: Mycobacterium tuberculosis cell wall structure (12)

The high concentration of lipids in the cellwall of Mycobacterium tuberculosis plays important role in providing it resistance against several factors such as impermeability to stains and dyes, resistance to many antibiotics, resistance to killing by acidic and alkaline compounds, resistance to osmotic lysis via complement deposition, resistance to lethal oxidations and survival inside of macrophages.

1.5Genome structure

The genome of MTB is 2.5 x 109 D, which has 4411529 base pairs along with 3924, predicted protein encoding genes (ORF) and G+C content of 65.6% (13). The genome is rich in replicative DNA, particularly insertion sequences, multigene families and duplicated house keeping genes. RV 1120 gene in MTB is a pseudo gene (14). Pseudo genes are genomic DNA sequences similar to normal genes but non-functional, they are regarded as definite relatives of functional genes. The complete genome sequence of the best-characterized strain of MTB, H37Rv, (Fig 4) has been determined and analyzed in order to improve our understanding of the biology of this slow-growing pathogen (15).

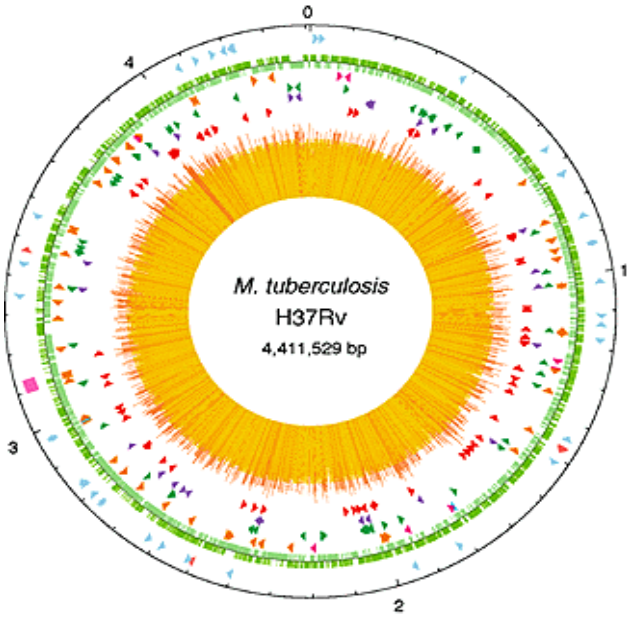


Fig 4: Mycobacterium tuberculosis genome structure (13)

1.6Physiology

M. tuberculosis is aerobic hence requires oxygen to grow (16). *M. tuberculosis* divides every 15–20 hours, which is extremely slow compared to other bacteria. It is a small bacilli that can withstand weak disinfectants and can survive in a dry state for weeks. Its unusual cell wall, rich in lipids (e.g., mycolic acid), is likely responsible for this resistance and is a key virulence factor (17). In the lungs, *M. tuberculosis* is taken up by alveolar macrophages, but they are unable to digest the bacterium. Its cell wall prevents the fusion of the phagosome with a lysosome. Specifically, *M. tuberculosis* blocks the bridging molecule, early endosomal auto-antigen 1 (EEA1); however, this blockade does not prevent fusion of vesicles filled with nutrients. Consequently, the bacteria multiply unchecked within the macrophage. The bacteria also carried the *UreC* gene, which prevents acidification of the phagosome (18). The bacteria also escape from macrophage killing by neutralizing reactive nitrogen intermediates. The ability to construct *M. tuberculosis* mutants and test individual gene products for specific functions has significantly advanced our understanding of the pathogenesis and virulence factors of *M. tuberculosis*. Many secreted and exported proteins are known to be important in pathogenesis.

1.7 Types of Tuberculosis

1.7.1Pulmonary Tuberculosis

Pulmonary tuberculosis is TB that affects the lungs, and represents about 85% of new cases diagnosed. It usually presents with a cough, which may or may not produce sputum. In time, more sputum is produced that is streaked with blood. The cough may be present for weeks or months and may be accompanied by chest pain and shortness of breath. Persons with pulmonary TB often run a low-grade fever and suffer from night-sweats. The patient often loses interest in food and may lose weight. If the infection allows air to escape from the lungs into the chest cavity (pneumothorax) or if fluid collects in the pleural space (pleural effusion), the patient may have difficulty breathing. The TB bacilli may travel from the lungs to lymph nodes in the sides and back of the neck. Infection in these areas can break through the skin and discharge pus (19).

Pulmonary Tuberculosis is divided into 5 different types of TB. These include**:-**

1. **Primary TB pneumonia:** It is an uncommon form of TB, which mostly occurs in patients with lower immunity, like children and the elderly. It presents itself in the form of pneumonia and is highly contagious.
2. **Laryngeal TB:** It affects the throat, in the vocal chord area. It is also contagious.
3. **Cavitary TB:** This form of tuberculosis has the classic symptoms associated with TB. It tends to form large cavities in the lungs. It is a highly contagious form of TB.

4. **Miliary TB:** This form often tends to affect the young, the elderly and anyone else who has a weak immune system. Victims are at the risk of contracting a very dangerous fever. It is characterized by the appearance of small granules in the lungs visible through a chest x-ray.
5. **TB Pleurisy:** It can develop shortly after catching the infection. This type of TB is characterized by shortness of breath, chest pain and fluid in the lungs.

1.7.2Extra pulmonary Tuberculosis

Although the lungs are the major site of damage caused by tuberculosis, many other organs and tissues in the body may also be affected. About 15% of newly diagnosed cases of TB are extra pulmonary, with a higher proportion of these being HIV-infected persons. The usual progression of the disease is to begin in the lungs and spread to locations outside the lungs (extra pulmonary sites). In some cases, however, the first sign of disease appears outside the lungs (20*)*. Extra pulmonary TB is divided into 7 different types. They include the following:-

1. **Adrenal Tuberculosis:** This form affects the adrenal gland; hence the hormone production is also affected. Patients suffering from this form of TB are known to experience fainting or weakness.
2. **Lymph node disease:** It is characterized by the enlargement of the lymph nodes. The nodes could also rupture through the skin.
3. **Osteal Tuberculosis:** This form of TB affects the bones. The affected area's bone tissue weakens, and it could cause the patient to fracture the affected area.
4. **TB Peritonitis:** Due to the TB, fluid gets collected in the outer lining of the intestine, causing the affected to experience pain in the abdomen.
5. **Renal TB:** It is characterized by the patient experiencing pyuria, which is the presence of white blood cells in the urine. It could end up affecting the reproductive organs and cause Epididymitis in men.
6. **TB Meningitis:** The symptoms for this include the patients displaying signs of being affected by a stroke or a brain tumor. It is extremely dangerous and could even prove to be fatal.
7. **TB Pericarditis:** This form of TB is characterized by an increase in the amount of fluid around the heart, and could also hamper its function (21).

Tuberculous meningitis

Tuberculosis is the world's leading cause of death from a single infectious agent. It is on the increase not only in underdeveloped countries but in the developed world too with a 30% increase in the number of cases in Switzerland in the four years up to 1990 and a 25% increase in Italy between 1988 and 1990 (32). In Britain no increase is reported yet but the decline noticed in the 1980s has now stopped; 7000 cases are reported each year. Multiple drug resistance, inadequate disease control programmes, and the advent of HIV infection all contribute to the current picture. In 1985, 5% of 4000 extrapulmonary cases of tuberculosis in the USA were due to tuberculous meningitis.2 Whereas non-osseous tuberculosis affecting the central nervous system may take a number of different forms including discrete large tuberculomata acting as space occupying lesions and more rarely myeloradiculopathy, tuberculous meningitis remains the most common threat to health (33). Outcome in tuberculous meningitis is strongly associated with the stage of disease at presentation. Disease staging was first proposed by the Medical Research Council in 19483: stage I=conscious, non-specific symptoms and no neurological signs; stage II=a degree of mental confusion and emerging neurological signs; stage III=these children are extremely ill with deepening coma often accompanied by the evolution of focal neurological signs (34). The incidence of residual neurological handicap or death rises steeply where appropriate treatment is not initiated until after the emergence of reduced conscious level and focal neurological signs. Recently, Humphries et al reported on outcome in 1990 Chinese children with tuberculous meningitis treated in Hong Kong between 1961 and 1984,4 and Schoeman reported on a prospective epidemiological study of 75 with tuberculous meningitis in Bloemfontein (35). Their findings correspond to those of many other authors. Complete recovery was the rule in stage I cases, but was seen in only about 20% in stage III. None died in stage I whereas up to 23% died in stage III. A third in stage III retained severe disability. Both studies showed that younger children were less likely to make a full recovery than older children (36). In the South African study 55% aged 12 months or less had a poor outcome compared with good recovery in all those aged 10 years or more. These findings serve to emphasise that early recognition of Tuberculous meningitis with early treatment, particularly in young children, is of paramount importance to optimum outcome. None the less, significant delays are still seen from time to time. In my experience, the delay seems to result not from clinicians failing to think of

Tuberculous meningitis but in their interpretation of investigations. Clinicians often prefer to interpret cerebrospinal fluid findings as representing a viral meningitis requiring no specific treatment. It may be they are reticent to commit themselves to 12 months of potentially toxic therapy (37). The resulting expectant policy brings appropriate intervention only after there has been a significant deterioration in the child's condition. This annotation should serve to evaluate and reemphasise the key factors in a child's presentation with Tuberculous meningitis and the therapeutic options.

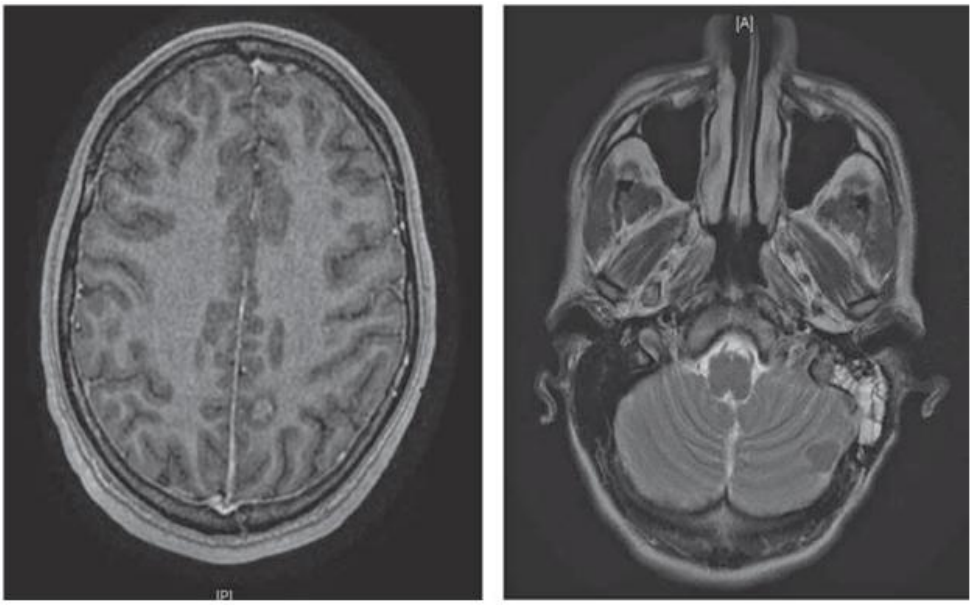


Fig 5: Magnetic resonance imaging showing (a) a cerebral tuberculoma in the left frontoparietal region and (b) a left cerebellar tuberculoma.

CSF: *Liquor cerebrospinalis*, is a clear, colorless bodily fluid, that occupies the subarachnoid space and the ventricular system around and inside the brain and spinal cord. In essence, the brain "floats" in it. The CSF occupies the space between the arachnoid mater (the middle layer of the brain cover, meninges), and the pia mater (the layer of the meninges closest to the brain). It constitutes the content of all intra-cerebral (inside the brain, cerebrum) ventricles, cisterns, and sulci (singular sulcus), as well as thecentral canal of the spinal cord. It acts as a "cushion" or buffer for the cortex, providing a basic mechanical and immunological protection to the brain inside the skull. It is produced in the choroid plexus (40, 41).

CSF is produced in the brain by modified ependymal cells in the choroid plexus (approx. 50-70%), and the remainder is formed around blood vessels and along ventricular walls. It circulates from the lateral ventricles to the foramen of Monro (Interventricular foramen), third ventricle, aqueduct of Sylvius (Cerebral aqueduct), fourth ventricle, foramina of Magendie (Median aperture) and foramina of Luschka (Lateral apertures); subarachnoid space over brain and spinal cord; reabsorption into venous sinus blood via arachnoid granulations (41,42,43). It had been thought that CSF returns to the vascular system by entering the dural venous sinuses via the arachnoid granulations (or villi). However, some[1]have suggested that CSF flow along the cranial nerves and spinal nerve roots allow it into the lymphatic channels; this flow may play a substantial role in CSF reabsorbtion, in particular in the neonate, in which arachnoid granulations are sparsely distributed. The flow of CSF to the nasal submucosal lymphatic channels through the cribriform plate seems to be specially important (44).
The CSF is produced at a rate of 500 ml/day. Since the brain can contain only 135 to 150 ml, large amounts are drained primarily into the blood througharachnoid granulations in the superior sagittal sinus. Thus the CSF turns over about 3.7 times a day. This continuous flow into the venous system dilutes the concentration of larger, lipoinsoluble molecules penetrating the brain and CSF (45).

The CSF contains approximately 0.3% plasma proteins, or approximately 15 to 40 mg/dL, depending on sampling site.[4] CSF pressure ranges from 80 to 100mmH_2O (780–980 Pa or 4.4–7.3 mmHg) in newborns, and <

200 mmH_20 (1.94 kPa) in normal children and adults, with most variations due to coughing or internal compression of jugular veins in the neck (46).
There are quantitative differences in the distributions of a number of proteins in the CSF. In general, globular proteins and albumin are in lower concentration in ventricular CSF compared to lumbar or cisternal fluid (47).
CSF serves four primary purposes:

1. Buoyancy: The actual mass of the human brain is about 1400 grams; however, the net weight of the brain suspended in the CSF is equivalent to a mass of 25 grams (44, 45). The brain therefore exists inneutral buoyancy, which allows the brain to maintain its density without being impaired by its ownweight, which would cut off blood supply and kill neurons in the lower sections without CSF (48).
2. Protection: CSF protects the brain tissue from injury when jolted or hit. In certain situations such asauto accidents or sports injuries, the CSF cannot protect the brain from forced contact with theskull case, causing hemorrhaging, brain damage, and sometimes death (49).
3. Chemical stability: CSF flows throughout the inner ventricular system in the brain and is absorbed back into the bloodstream, rinsing the metabolic waste from the central nervous system through theblood-brain barrier. This allows for homeostatic regulation of the distribution of neuroendocrine factors, to which slight changes can cause problems or damage to the nervous system. For example, high glycine concentration disrupts temperature and blood pressure control, and high CSF pH causes dizziness and syncope (50)
4. Prevention of brain ischemia: The prevention of brain ischemia is made by decreasing the amount of CSF in the limited space inside the skull. This decreases total intracranial pressure and facilitates blood perfusion (51).

CSF can be tested for the diagnosis of a variety of neurological diseases (52). It is usually obtained by a procedure called lumbar puncture. Removal of CSF during lumbar puncture can cause a severe headache after the fluid is removed, because the brain hangs on the vessels and nerve roots, and traction on them stimulates pain fibers. The pain can be relieved by intrathecal injection of sterile isotonic saline. Lumbar puncture is performed in an attempt to count the cells in the fluid and to detect the levels of protein and glucose. These parameters alone may be extremely beneficial in the diagnosis of subarachnoid hemorrhage and central nervous system infections (such as meningitis). Moreover, a CSF culture examination may yield the microorganism that has caused the infection. By using more sophisticated methods, such as the detection of the oligoclonal bands, an ongoing inflammatory condition (for example, Multiple Sclerosis) can be recognized. A beta-2 transferrin assay is highly specific and sensitive for the detection for, e.g., CSF leakage.

1.8 Stages of the Disease

The following stages that will be explained are for a MTB sensitive host. Only a small percent of MTB infections progress to disease and even a smaller percent progress all the way to stage 5.Disease progression depends on:

1. Strain of MTB.
2. Prior exposure
3. Vaccination
4. Infectious dose

Stage 1:

Droplet nuclei are inhaled. One droplet nuclei contains no more than 3 bacilli. Droplet nuclei are so small that they can remain air-borne for extended periods of time. The most effective (infective) droplet nuclei tend to have a diameter of 5 µm. Tuberculosis begins when droplet nuclei reach the alveoli. When a person inhales air that contains droplets, most of the larger droplets become lodged in the upper respiratory tract (the nose and throat), where infection is unlikely to develop. However, the smaller droplet nuclei may reach the small air sacs of the lung (the alveoli) where infection begins.

Stage 2: Begins 7-21 days after initial infection. MTB multiplies virtually unrestricted within un activated macrophages until the macrophages burst. Other macrophages begin to extra vasate from peripheral blood.

Stage 3:

At this stage lymphocytes begin to infiltrate. The lymphocytes, specifically T-cells, recognize processed and presented MTB antigen in context of MHC molecules. This results in T- cell activation and the liberation of cytokines including gamma interferon (IFN). The liberation of IFN causes in the activation of macrophages. These activated macrophages are now capable of destroying MTB. It is at this stage that the individual becomes tuberculin-positive. This positive tuberculin reaction is the result of the host developing a vigorous cell mediated immune (CMI) response. A CMI response must be mounted to control an MTB infection. An antibody mediated immune (AMI) will not aid in the control of a MTB infection because MTB is intracellular and is resistant to complement killing due to the high lipid concentration in its cell wall (22). Activated macrophages may release lytic enzymes and reactive intermediates that facilitate the development of immune pathology. Activated macrophages and T-cells also secrete cytokines that may also play a role in the development of immune pathology, including Interleukin 1 (IL-l), tumor necrosis factor (TNF), and gamma IFN. It is also at this stage that tubercle formation begins. The center of the tubercle is characterized by "caseation necrosis" meaning semi-solid or "cheesy" consistency. MTB cannot multiply within these tubercles because of the low pH and anoxic environment. MTB can
however persist within these tubercles for extended periods.

Stage:
4.Although many activated macrophages can be found surrounding the tubercles, many1. Exudative lesions result from the accumulation of PMN's around MTB. Here the bacteria replicate with virtually no resistance. This situation gives rise to the formation of a "soft tubercle".
2. Productive or granulomatous lesions occur when the host becomes hypersensitive to tuberculoproteins. This situation gives rise to the formation of a "hard tubercle".
Stage 5: For unknown reasons, the caseous centers of the tubercles liquefy. This liquid is very conducive to MTB growth and hence the organism begins to rapidly multiply extra cellularly. After time, the large antigen load causes the walls of nearby bronchi to become necrotic and rupture. This results in cavity formation. This also allows MTB to spill into other airways and rapidly spread to other parts of the lung (19).

1.12.1 Tuberculin skin test (Mantoux test).

The TB skin test may be used to find out if a person has TB infection. Skin test can be performed at any pathology laboratory. A small amount of testing fluid (called tuberculin or PPD) is injected just under the skin on the underside of the forearm. After 48 hours, skin test is read by the laboratory technician. There may be swelling where the tuberculin was injected. The technician will measure this swelling and tell reaction to the test is positive or negative. A positive reaction usually means that person is infected by someone with active TB disease. If person have recently spent time with and been exposed to someone with active TB disease, TB skin test reaction may not be positive yet. Then person may need a second skin test 8 to 10 weeks after the last time he spent time with the person. This is because it can take several weeks after infection for his immune system to react to the TB skin test. If reaction to the second test is negative, person probably do not have latent TB infection.

1.12.2 Acid fast staining

Ziehl-Neelsen stain is the acid-fast staining method developed for *Mycobacterium tuberculosis*. In this method the MTB smear is heat fixed, stained with carbol-fuchsin (a pink dye), and decolorized with acid-alcohol. The smear is counterstained with methylene-blue or certain other dyes. Acid-fast bacilli appear pink in a contrasting background. In order to detect *Mycobacterium tuberculosis* in a sputum sample, an excess of 10,000 organisms per ml of sputum are needed to visualize the bacilli with a 100X microscope objective (44). One acid-fast bacillus/slide is regarded as "suspicious" of an MTB infection.

1.12.3Loweenstein - Jensen medium

M. tuberculosis is grown on a selective medium known as Lowenstein-Jensen medium, which has traditionally been used for this purpose. However, this method is quite slow, as this organism requires 6–8 weeks to grow, which delays reporting of results.

1.12.4Radiometric Bactec 460 TB Method

Researchers and doctors are looking for the faster and more efficient systems for the diagnosis of infectious diseases in order to start treatment at the earliest for better patient management. Keeping this

concern of doctors in mind, BACTEC 460 TB SYSTEM, (Fig) has been introduced which is a rapid system for automated detection, drug susceptibility testing and differentiation of mycobacterium (61).

Fig 6: Bactec 460TB system (29)

1.12.5 Polymerase Chain Reaction

Thermal Cycler:

The thermal cycler is a laboratory apparatus used to amplify segments of DNA via the polymerase chain reaction (PCR) process. The device has a *thermal block* with holes where tubes holding the PCR reaction mixtures can be inserted. The cycler then raises and lowers the temperature of the block in discrete, pre-programmed steps.

The earliest thermal cyclers were designed for use with the Klenow fragment of DNA Polymerase I. Since this enzyme is destroyed during each heating step of the amplification process, new enzyme had to be added every cycle. This led to a cumbersome machine based on an automated pipettor, with open reaction tubes. Later, the PCR process was adapted to the use of thermostable DNA polymerase from *Thermus aquaticus*, which greatly simplified the design of the thermal cycler. While in some old machines the block is submerged in an oil bath to control temperature, in modern PCR machines a Peltier element is commonly used. Quality thermal cyclers often contain silver blocks to achieve fast temperature changes and uniform temperature throughout the block (56).

Modern thermal cyclers are equipped with a *heated lid*, a heated plate that presses against the lids of the reaction tubes. This prevents condensation of water from the reaction mixtures on the insides of the lids and makes it unnecessary to use PCR oil to cover the reaction mixture (57).Long car journeys can sometimes be a godsend. Driving along a monotonous stretch of dark road one April weekend in 1983, American chemist Kary Mullis was struck by an idea that was later to earn him the Nobel Prize: the principle of the polymerase chain reaction.Among the instruments and glassware of his laboratory Mullis might never have had the most momentous and far-reaching idea of his life (58). Within a few years PCR – short for 'polymerase chain reaction' – took the world's biological laboratories by storm. By the mid- 1980s the technique was used for the first time to diagnose a disease, when researchers identified the gene for sickle cell anemia. At about the same time the method was introduced into forensic medicine. The polymerase chain reaction reaped the highest scientific honour for its inventor in record time: In 1993, just ten years after his historical car journey, Kary Mullis received the Nobel Prize for Chemistry. The reason for this extraordinary success is that the technique provided a solution to one of the most pressing problems facing biology at the time – the replication of DNA.

Polymerase chain reaction is an alternative detection method which is specific, sensitive and quick, when bacteriological proof of diagnosis is lacking. Polymerase chain reaction (PCR) test has been found to be useful for rapid diagnosis of tuberculosis from a variety of clinical specimens (55). PCR for the detection of *Mycobacterium tuberculosis* in clinical specimens requires 1-2 days and has a specificity and sensitivity comparable to culture methods .

PCR (Polymerase Chain Reaction) Step: Mechanism

The Polymerase Chain Reaction (PCR) is a technique to amplify a single or few copies of a piece of DNA across several orders of magnitude, generating millions or more copies of a particular DNA sequence. The method relies on thermal cycling, consisting of cycles of repeated heating and cooling of the reaction for DNA melting

and enzymatic replication of the DNA. Primers (short DNA fragments) containing sequences complementary to the target region along with a DNA polymerase are key components to enable selective and repeated amplification

A basic PCR setup includes:

1. DNA template that contains the DNA region (target) to be amplified.
2. Two primers, which are complementary to the DNA regions at the 5' (five prime) or
3. 3' (three prime) ends of the DNA region.
4. Taq polymerase or another DNA polymerase with a temperature optimum at around 70°C.
5. Deoxynucleoside tri-phosphates (d NTPs; also commonly called deoxynucleotide tri- phosphates), the building blocks from which the DNA polymerases synthesizes a new DNA strand.
6. Buffer solution, providing a suitable chemical environment for optimum activity and stability of the DNA polymerase.
7. Divalent cations, magnesium or manganese ions; generally Mg2+ is used, but Mn2+ can be utilized for PCR-mediated DNA mutagenesis, as higher Mn2+ concentration increases the error rate during DNA synthesis.
8. Monovalent cation potassium ions.

The target gene is exponentially amplified using repeat cycles of three steps (Fig).

1) DNA Denaturation
2) Primer annealing
3) Extension of primer-DNA duplex

1. Initialization step: This step consists of heating the reaction to a temperature of 94-96°C (or 98°C if extremely thermo stable polymerases are used), which is held for 1-9 minutes. It is only required for DNA polymerases that require heat activation by hot-start PCR.

2. Denaturation step: This step is the first regular cycling event and consists of heating the reaction to 94-98°C for 20-30 seconds. It causes melting of DNA template and primers by disrupting the hydrogen bonds between complementary bases of the DNA strands, yielding single strands of DNA.

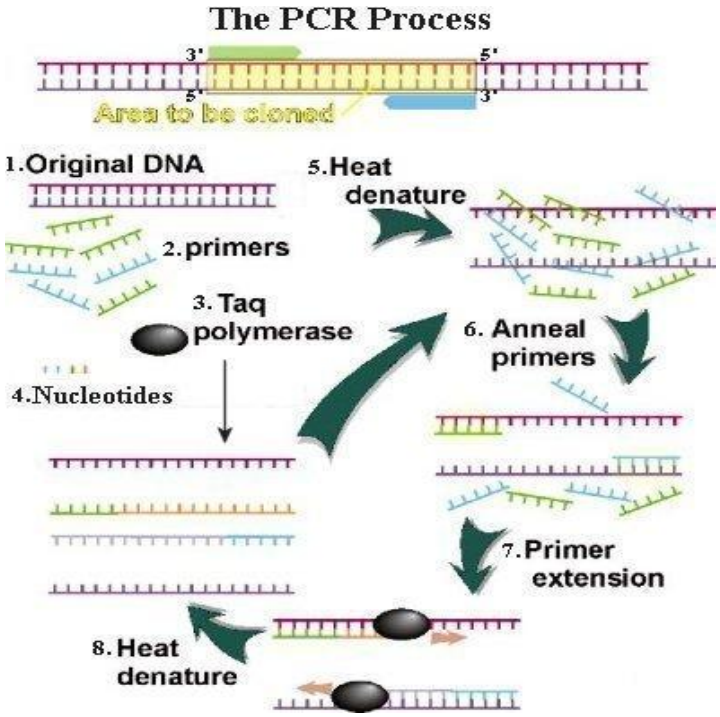


Fig 7: Diagrammatic representation showing polymerase chain reaction (51)

3. Annealing step: The reaction temperature is lowered to 50-65°C for 20-40 seconds allowing annealing of the primers to the single-stranded DNA template. Typically the annealing temperature is about 3-5 degrees Celsius below the Tm of the primers used. Stable DNA-DNA hydrogen bonds are only formed when the

primer sequence very closely matches the template sequence. The polymerase binds to the primer-template hybrid and begins DNA synthesis.

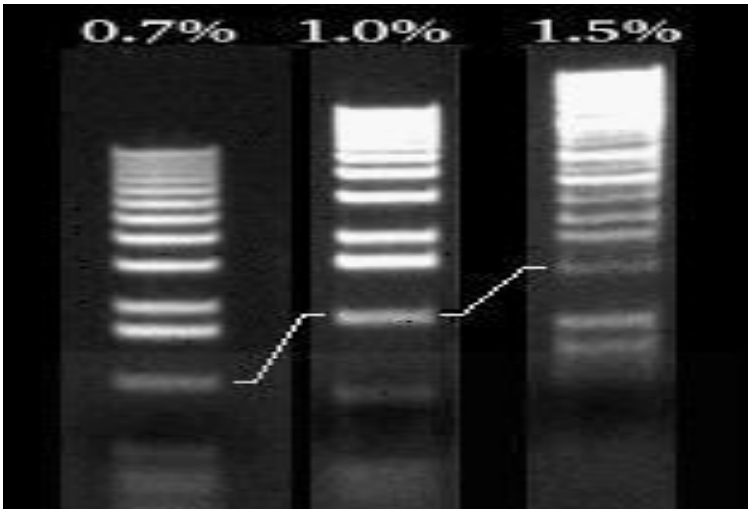
4. Extension/elongation step: The temperature at this step depends on the DNA polymerase used; Taq polymerase has its optimum activity temperature at 75-80°C and commonly a temperature of 72°C is used with this enzyme. At this step the DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand by adding dNTPs that are complementary to the template in 5' to 3' direction, condensing the 5'-phosphate group of the dNTPs with the 3'-hydroxyl group at the end of the nascent (extending) DNA strand. The extension time depends both on the DNA polymerase used and on the length of the DNA fragment to be amplified

5. Final elongation: This single step is occasionally performed at a temperature of 70-74°C for 5-15 minutes after the last PCR cycle to ensure that any remaining single-stranded DNA is fully extended.

To check whether the PCR generated the anticipated DNA fragment (also sometimes referred to as the amplimer or amplicon), agarose gel electrophoresis is employed for size separation of the PCR products. The size(s) of PCR products is determined by comparison with a DNA ladder (a molecular weight marker), which contains DNA fragments of known size, run on the gel alongside the PCR products.

Electrophoresis

Agarose gel electrophoresis followed by Ethidium Bromide staining represents the most common way to analyze PCR products. A 1.5% agarose gel is adequate for the analysis of PCR products from 150 to 1000 bp. DNA markers of different size ranges are available commercially. The development of capillary electrophoresis has significantly increased the throughput and resolution of PCR product analysis. The improved resolution of capillary electrophoresis has allowed the discrimination of a single nucleotide difference in size.



II. MATERIALS AND METHODS

2.1 Sample Collection

CSF Samples were collected from various NCR regions hospitals and collection centers and then they were carried out to molecular testing laboratory for further processing and analysis. Samples are usually stored in vacutainer which is basically at 4°C and then they are transported. Samples are tabulated according to its site of action. These samples are tested just as a reference for the lab study, not for any survey and any in-disciplinary to the company on our behalf. The procedures followed for the analyses of various samples were as provided below:

After the initial observations of some of the critical processes/tests the rest of the samples were preserved in the deep freezer at -20° Celsius till further use.

2.2.Step by step processing/ testing of samples

2.2.1. Decontamination

Decontamination is the first and foremost step before samples go to different bench for further processing. The purpose of decontamination is to kill all other bacteria except required species i.e. *M. tuberculosis.*

2.2.1.1. Reagents used for Decontamination

Table 2: Reagents used for Decontamination

S .No	Reagent Name	Concentration
1.	NALC (N-acetyl-L-cysteine)	0.5 % (0.1 gm per sample)
2.	NaOH (Sodium Hydroxide	4.0 % (4 gm in 100 ml distilled water)
3.	Trisodium Citrate	2.9 % (2.9 gm in 100 ml)
4.	Sterile Phosphate Buffer	pH-6.8

Solution A: Disodium Hydrogen Phosphate (Na_2HPO_4)-Mol. Wt- 141.96 gm/mol. Dissolve 4.735 gm in 500 ml of DDW (mix with magnetic stirrer).
Solution B: Mono potassium Dihydrogen Phosphate(KH2PO4)- Mol. Wt- 136.09 gm/mol. Dissolve 4.535 gm in 500 ml of DDW (mix with magnetic stirrer). The solution A and B were mixed with the help of magnetic stirrer and pH was adjusted to pH 6.8-7.0. The prepared solution was autoclaved at 121°C/15 psi/ 15 min.

2.2.1.2 Equipments and materials used for Decontamination
Centrifuge (5000g rotor for 15ml centrifuge tube) - Refrigerated, Vertical Biosafety subunit (class-II), Conical Centrifuge tubes (15ml), Aerosol barrier tips (20, 200, 1000 µl), Measuring cylinder, Latex gloves, Bleach (0.5%) sodium hypochlorite.
2.2.1.3 Procedure:

1. Transfer the clinical samples in 15 ml centrifuge tubes (TARSON/FALCON).
2. Centrifuge at 4500 rpm for 20 minutes at 4°C (Plastocraft/Remi Centrifuge).
3. Discard the supernatant till 2 ml of the sample is left in the tube.
4. To this add equal volume of digestant/decontaminant solution (sodium citrate (NaOH) into centrifuge tubes.

1. The contents are mixed by inversion and vortexing for 2 minutes exactly.
2. Incubate at room temperature for 20 minutes.
3. Vortex again for 15-30 seconds.
4. Add sterile Phosphate Buffer (pH 6.8) into the tube till 14 ml mark to neutralize the samples.
5. Vortex again for 15-30 seconds.
6. Centrifuge at 4500 rpm for 20 min at 4oC.
7. Discard supernatant from the top keeping 3ml into the bottom of centrifuge tube.
8. Vortex briefly for 5-10 seconds.

a. Aliquot in different micro centrifuge tubes as follow-200 µl for lysis.1 ml for culture.1.5 ml for sample storage.

2.2.2. **DNA Isolation**
After decontamination samples are taken for isolation and identification of bacterial DNA in samples provided for testing. This basically includes isolation of DNA followed by PCR set up using primer against MPB 64 gene**.**

2.2.2.1 Reagents for DNA isolation

1. AuPrepTM GENbt DNA extraction kit
2. Ethanol (Mol. Bio. Grade)
3. Autoclaved ultra pure water (MQ waterwith 18 mega ohms resistance)
4. Proteinase K (supplied with the AuPrepTM GENbt kit. Dissolved into 1ml milli Q water and mixed by overvortexing).
5. Wash buffer (60 ml of 98% ethanol is added to the wash buffer provided with the kit to prepare working solution)
6. Extraction buffer

2.2.2.2 Equipments and accessories for DNA isolation

1. Biosafety cabinet (Class 2)
2. Centrifuge (10,000 g, rotor for 50 ml, 1.5 ml and 0.2 ml tubes), preferably refrigerated.
3. Aerosol barrier tips (20 µl, 200 µl and 1000 µl).
4. Pippetes (20 µl, 200 µl and 1000 µl).
5. Latex gloves
6. Micro centrifuge tubes(1.5 ml)

2.2.2.3 Procedure for DNA isolation (By AuPrepTM GENbt kit) Component of DNA isolation kit

All contents of the kit except Proteinase K are stable at room temperature (20-25oC) for one year.

(a) Spin columns

(b) Collection tubes

(c) Proteinase K

(d) Extraction buffer, Lysis buffer, Wash buffer

Preparation of working solution of Proteinase K and wash buffer

1. Proteinase K (10 mg): MQ water to lyophilized 10 mg proteinase k to get a final concentration of 10 mg/ml.
2. Wash buffer (15 ml): Prior to use, add 60 ml of 98% of ethyl alcohol.

Procedure of DNA isolation

1. Proceeded DNA isolation from 200 µl decontaminated and aliquoted clinical sample in 1.5 ml microcentrifuge tube.
2. After each 4 specimen sample put 1 negative control. In the tube of negative control added 200 µl phosphate buffer at the place of specimen.
3. Added 20 µl of 10mg/ml concentrated proteinase k in to the sample.
4. Added 200 µl of extraction buffer for fluids and negative control samples or 200 µl of lysis buffer for tissue samples (provided with kit) to each microcentrifuge tube individually.
5. Mixed well by vortexing for 10-15 sec.
6. Incubated all standard samples at 60oC heating block for 20 minutes for fluid and 60 minutes for tissue samples.
7. Vortexed well for 10-15 sec.
8. Then transferred them to 95°C heating block for 10 minutes.
9. Removed the specimen from 95°C and add 210 µl of 98% chilled ethanol (Mol. Grade) to each specimen.
10. Vortexed for 10-15 sec.
11. Transferred the contents to pre-arranged mini columns in collection tubes.
12. Centrifuged at 10,000 rpm for 3 minutes at 4°C (Plastocraft/Remi centrifuge).
13. Transferred the mini columns to fresh collection tubes. Added 500 µl already reconstituted wash buffer.

14. Centrifuged at 10,000 rpm for 3 minutes at 4°C.
15. Transferred the columns to fresh collection tubes. Added 500 µl already reconstituted wash buffer.
16. Centrifuged at 10,000 rpm for 3 minutes at 4°C. Decanted the supernatant followed by adding 500 µl of wash buffer again into mini columns.

17. Transferred the columns to autoclaved microcentrifuge tubes.
18. Added 65 µl of pre heated at 95°C (on heating block) MQ water to each column.
19. Centrifuged at 14,500 rpm for 5 minutes at 4°C. Discarded the columns from the microcentrifuge tubes and collect the DNA.
20. Added 25 µl of prepared DNA template in PCR master mix aliquoted into PCR tube for amplification.

2.2.3DNA Amplification

2.2.3.1 Chemical/Reagents Required for DNA amplification

1. Isolated DNA followed by SOP # 1002.
2. PCR Buffer (10X) (Larova Cat # Taq 500).
3. dNTP's (Larova Cat # DNTP100).
4. MgCl2 (Larova Cat # Taq 500).
5. Forward Primer (P1) (25 mM) (Life Technology Cat # E 06325).
6. Reverse Primer (P2) (25 mM) (Life Technology Cat # E 06326).
7. Taq Pol (5 Unit/ml) (Larova Cat # Taq 500).
8. 100bp DNA Ladder (Bangalore GENEI Cat # MBD13J).
9. Tris Base (Life Technology Cat # 1000 LT).
10. EDTA (Amresco Cat # 9572E).
11. Agarose (Amresco Cat # GR 100-LELT).
12. Bromophenol Blue (Amresco Cat # 0312).
13. Ethidium Bromide (Amresco Cat # 0532).
14. Gel loading dye.
15. 1X TAE Buffer.
16. Milli Q Water.

2.2.3.2 Procedure for making MTB Master Mix (MTB MMX):

1. Carried all the reagents for MTB master mix preparation into AREA 1, in a cool box. Always wear sterile powder free gloves while preparing the master mix as powder can adversely affect the activity of Taq polymerase.
2. Thawed all the reagents before use.
3. Mixed all the reagents well before aliquoting; otherwise changes in the concentrations will disturb the accuracy of quantity. Companies provide Taq Polymerase with its specific buffer and MgCl2. (*It is important to use Taq Polymerase, Buffer and MgCl2 of the same lot no., for the optimum activity of Taq polymerase*).
4. Before proceeding to make the MTB MMX, ensure that all the working reagents (like dilution of dNTP's, MgCl2, and preparation of primer solution from the stock solution) are available/made before hand.

(A) Different volume of ingredients in MTB MMX for certain number of PCR reactions.

Table 3: Ingredients in MTB MMX

S. No	Reagents (with working concentrations)	Final con. in 50 µl vol.	Final vol. for one PCR R/c	Final vol. for
1	Buffer (10x)	1x	5µl	250µl
2	dNTP (2mM)	200mM	5µl	250µl
3	MgCl2 (25mM)	2.0 mM	4µl	200µl
4	P1 (25mM)	0.5mM	1µl	50µl
5	P2 (25mM)	0.5mM	1µl	50µl
6	MQ Water	-	8.75µl	437.5µl
7	Taq Pol (5 Unit/ml)	1.25 unit	0.25µl	12.5µl
	Total Volume	-	25µl	1250µl

Reaction is performed in total of 50 µl for a single sample in PCR (25 µl DNA + 25 µl PCR MMX). All the components are mixed carefully in a 2 ml sterile screw cap tube. Vortex the tube gently and spin it briefly to accumulate the entire MMX in bottom of tube. Aliquot 25 µl of Master Mix into PCR tubes and store in –20 º C refrigerators up to 1 year but repeated freezing and thawing should be avoided.

B) Set up of PCR reaction:

PCR setup has to be performed with following the steps given below;

1. Take 25microl Master Mix aliquot PCR tubes and allow it to thaw completely if stored in – 20° C.
2. Mix with gentle finger tapping and spine shortly to settling down MMX to the bottom of PCR tube.
3. Mark on the tube based on sequence of DNA sample and positive control.
4. Add 25 µl of isolated DNA template and negative control to the MMX. The final volume would become 50µl for reaction.
5. Pipette up and down to mix DNA template with MMX.
6. Spin briefly for a second in spin win to bring down reaction mix to the bottom of PCR tube.
7. Switch on Thermal cycler for five minutes before start reaction for auto calibration.
8. Keep all tubes into the thermal cycler block.
9. Set the program for amplification as follows for 40 cycles.

Table 4: Sequence of temperature in PCR set up

Temperature °(C)	Time (in min)	Cycles	Activity
94° C	6	1	Initial Denaturation
94° C	1		Denaturation
55° C	1.5	40	Annealing
72° C	1.5		Elongation
72° C	7	1	Final elongation
4 ° C			Holding

10. After completion of amplification keep PCR amplified product in refrigerator at 4oC until the detection of amplified product.

2.2.4Electrophoresis and Detection Of Amplified Product

Electrophoresed and resolve the amplified product on 1.6 % agarose gel to detect the band of interest. Follow the steps mentioned below for electrophoresis.

Preparation of 1.6% Agarose Gel

1. Sealed the Gel casting tray with tape and placed on a horizontal surface. The well forming comb has to be positioned parallel to the tray.
2. Measure 125 ml 1X TAE Buffer with the help of measuring cylinder of 250ml capacity and pour into 250ml conical flask.
3. Weigh 2.0 gram agarose on electronic balance and add into conical flask containing 125 ml of 1X TAE buffer.
4. Keep in microwave oven to boil till the complete dissolution of agarose added to the flask.
5. Take out the conical flask and allow it to cool down up to the 40-50oC then add 5.5µl of Ethidium-bromide (Conc. 0.5mg/ml) and mix thoroughly.
6. Pour boiled agarose into sealed casting tray without creating any air bubbles then allow it to solidify at room temperature.
7. Pour 40-50ml 1X TAE buffer on the solidified gel and pull out comb upwardly without disturbing wells.
8. Place the gel in electrophoresis tank and fill the tank with 1X TAE buffer to cover the gels to a depth of 1mm.
9. 20 µl of each reaction mixture was poured in the wells through micropipette.
10. Tank was closed and attached by electrode wires to the power supply and run for hours at 100-150 V.
11. Bands were visualized by UV lamp (ULTRA-LUM) and were photographed under UV- light in 'Syngene' multigenious bioimaging system.

Preparation of Gel Loading Buffer

To make 20 ml gel loading buffer, weigh 8 grams sucrose (40%, w/v), 50 mg Bromophenol blue (0.25% w/v) and dissolve in 15 ml Milli Q water. Make up the volume 20 ml with Milli Q water with the help of 25 ml measuring cylinder.

Preparation of DNA Ladder

Readymade - Prepare as recommended by the commercial supplier.

1. A known molecular weight marker (100bp DNA Ladder) should be loaded in the first well.
2. 20 µl of PCR product is loaded into each well along with 4 µl of gel loading buffer.
3. Positive control and a negative control are loaded along with the samples.
4. Electrophoresis is carried out at 100-150 Volt (5 to 8 V/cm for 20 cm gel) until the bands in the molecular weight marker are resolved. Then the gel is examined under UV light (302nm) on ULTRA LUM Electronic UV transilluminator gel documentation system for the presence of 240 base pair PCR product and photographed.

Precautions
1. Ethidium Bromide (EtBr) is a powerful mutagen and toxic. Wear gloves when working and a mask when weighing the powder.
2. UV light is harmful to eyes so UV protection shield should be used always.

.**Equipment: BACTEC 460 TB SYSTEM**

III. MATERIALS

Middlebrook 7H 12(bacte 12 b)
It is a liquid medium, 4 ml quantity in a 20 ml glass vial sealed with rubber septum and crimp containS:
7H9 Broth, 0.47 % w/v Casein hydrolasate, 0.10 % w/v Bovine Serum albumin,0.50 % w/v 14C – substrate, 4.0 µCi, Deionised Water 4.0 ml.
Final pH = 6.8 ± 0.2

Panta
Lyophilized mixture of five antimicrobials:
Polymixin B =10000 units, Ampotericin=100.0 µg, Nalidix acid=4000 µg, Trimithoprim=1000 µg, Azlocillin=1000 µg.

Reconstitution fluid
Distilled water containing growth promoting substances POES (Poly Ethylene Styrate) Alcohol Swabs.
Procedure:
1. Pulmonary as well as extra pulmonary specimens were processed for recovery of mycobacterium.
2. Sputa and other mucoid specimens which might contain other bacteria were processed for digestion decontamination.
(a) Digested or decontaminate with NaOH-N-Acetyl-L-Cysteine for 15-20 min in a 50 ml centrifuge tube.
(b) Added phosphate (pH 6.8) up to 50 ml ring and mix.
(c) Centrifuged at 3000 x g for 15-20 minutes.
(d) Discarded the supernatant.
(e) Re-suspended the sediment with 1-2 ml phosphate buffer and mix
(f) Used this concentrate to make 2 smears and to inoculate 12 B and other media
3. Aseptically collected specimen may be inoculated directly. If the quantity was large, the samples were concentrated by centrifugation. Mucoid specimens were liquefied before centrifugation.
4. Supplemented 12 B medium with antimicrobial PANTA supplement prior to inoculation. The vials were tested on the BACTEC 460 TB system prior to incubation to purge with 5-10 % CO2 .
5. Incubated inoculated media at 37 ±1°C
6. Tested 12B vials every 2-3 days for the first 2-3 weeks and weekly thereafter for a total of 6 weeks.
7. A GI 200 or more was considered as presumptive positive. Tested the vial thereafter.
8. A sudden increase in GI or presence of turbidity indicated contamination. The presence of Mycobacterium was confirmed by making smear or sub culturing on a blood agar plate.
9. Reported a positive culture when a AFB smear was positive.

2.4 Afb Smear Microscopy: (A) Zn Staning:
ZN staining is done to stain the acid fast bacili present in the specimens and to differentiate them from other microorganisms (73).
Reagents/Materials needed: Microscopic slides Micropipette and tips Bunsen burner
Carbol fuschin dye, 20 % H2SO4, Malachite green (counter stain) and Tap water

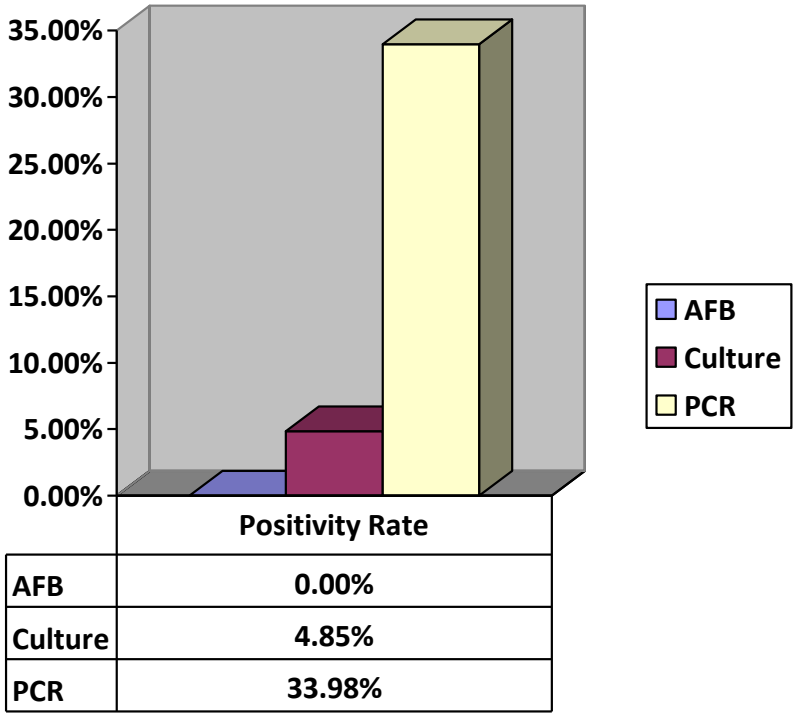
Procedure for ZN staining:
1. The smears of the specimens were prepared by putting a few drops on the slide and spreading the sample on to the surface. The slides were kept for air drying.
2. The slide was flamed to heat fix them.
3. The entire slide was flooded with hot carbol fuchsin for 5-6 minutes. Also ensured that enough stain was added to keep the slides covered throughout the entire staining step.
4. The slides were rinsed with tap water.
5. The slides were flooded with 20 % H_2SO_4 for 10-12 minutes and allowed to decolorize.
6. The slides were thoroughly rinsed with the water and then drained any excess from the slides.
7. Flooded the slides with the counter stains (Malachite green). Kept the counter stain on slide for 2 minutes.
8. Rinsed the slides thoroughly with tap water.
9. The slides were observed under compound microscope under oil immersion lens.

MICROSCOPY: Equipments and materials: A compound microscope, ZN stained slides
Immersion oil (cedar wood oil), Xylene.

Procedure for microscopy :
1. Examined carbol fuchsin stained smears with a 100x oil immersion objective.
2. Included a known positive slide and a known negative slide with each day's work. The positive control ensures the staining capability of the solutions and of the staining procedure. The negative control confirms the acid fast contaminents are not present in the stains or in other solutions.
3. Made a series of systematic sweeps over the length of the smear. After examining a microscopic field , moved the slide longitudinally so that the neighboring fieldb to the right could be examined. Searched each field thoroughly.
4. Examined a minimum of 100 fields before the smear was reported negative. In a smear of 1.5cm x 1.5 cm the number of microscopic fields in one length of the slide corresponded to around 100. If the smear was moderately or heavily positive fewer fields were examined and a report of positive was made even though the entire smear had not been examined.
5. At the end of examination, took the slide from the microscopic stage, checked the identification number and noted the result. Dipped the slide into xylene to remove the immersion oil and placed it in a box for examined slides.
6. Before examining the next slide, wiped the immersion lens with a piece of lens tissue paper

Results

Specimen	No. tested	Positive by:		
	AFB	Culture	PCR	
CSF from susceptible patients	103	0 (4.85%)	5 (33.98%)	35



	Positivity Rate
AFB	0.00%
Culture	4.85%
PCR	33.98%

Fig 9: Positivity of different diagnostic tools for Tuberculous meningitis

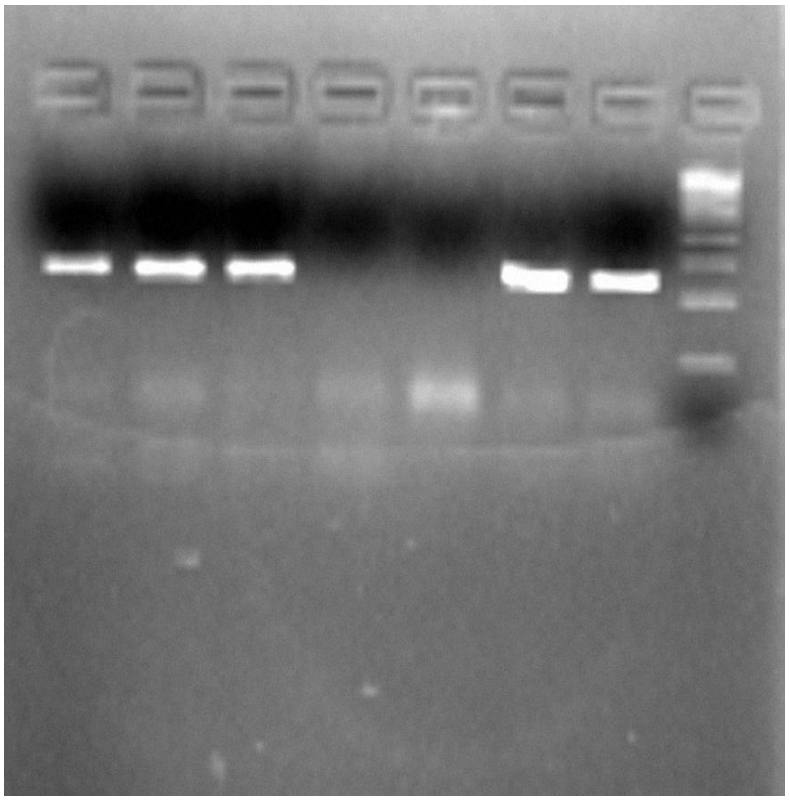


Fig: MPB64 gene-targeted PCR for detection of Mycobacterium tuberculosis

The data of clinical samples in the month of February and March 2011 was used for the present study. In this study, a total number of 103 CSF samples from different patients with some probabilities of TBM were taken for the detection of Mycobacterium tuberculosis pathogen. For this all the criteria were used viz., AFB/ZN Stain, Culture by using BacTec and PCR. Among these criteria least positive was the ZN staining but the most positive reports were detected by PCR which comes in the picture as a potential tool to detect the MTB in the various cases of Neurological TB and meningitis.

IV. DISCUSSION

Meningitis is an extremely severe and life-threatening infection that necessitates immediate diagnosis and prompt therapy. Bacterial meningitis is endemic in India and sporadic cases occur all over the year. Tuberculous meningitis is the second most frequently encountered infectious meningitis in developing countries with the highest mortality rate among the other causes of meningitis (72). This study showed that among the CSF samples of 103 cases with probability of meningitis, 34% of cases were positive results for MTB in PCR, 5 % of cases were positive results for *M. tuberculosis* by culture on LJ medi and none of cases were positive for ZN stain. This study shows that the efficiency of PCR is significantly higher than microscopy and culture for the early diagnosis of TBM. It is also important to remember that the volume of samples used for PCR is much less than that used for culture. This may affect the sensitivity of PCR, especially in samples with low bacterial counts, which is often the case in CSF samples**.** Thus a negative-PCR result can never rule out tuberculous meningitis (73).

Since there are normally few bacilli in CSF specimens from TBM patients to be demonstrated by direct microscopy, and culture examination of CSF specimens from TBM patients takes several weeks, PCR will prove useful as an efficient technique for the rapid diagnosis of TBM, even though conventional diagnostic methods are less expensive. PCR can specifically identify *M. tuberculosis* in a clinical specimen within 7-8 hours. The use of PCR thus saves valuable time in the early identification of mycobacteria and implementing effective treatment (74).

The repetitive nature of the target sequence amplified by the PCR described here probably contributes to the high sensitivity, and fewer than 10 bacilli can be detected by application of this PCR. The above-mentioned detection limit of this PCR, combined with the use of the best method of DNA extraction from clinical specimens, provide a powerful tool for the specific and rapid diagnosis of paucibacillary situations.

The low number of bacteria in CSF leads to poor sensitivity of acid fast staining and culture **(75)**. Microscopy is fast and inexpensive, but the sensitivity is merely 0 – 20 %. Culture on broth media had somewhat shortened the delay in diagnosis occurring with conventional culture media, but it often takes 5 – 12 days. The detection of antibody in CSF is rapid, but this approach is not standardized and lacks both sensitivity and specificity **(78)**.The main limitation of our study is that there is no diagnostic test to serve as an adequate gold standard to evaluate PCR in diagnosis of tuberculous men meningitis. Culture has low sensitivity, clinical assessment may be subjective, and CT-scanning and magnetic resonance imaging are expensive and uncertain. PCR still has problems with sensitivity and specificity. The increased sensitivity may come at the expense of decreased specificity **(80)**. There is a need for a more rapid and reliable method for the early laboratory diagnosis of Tuberculous meningitis, where the clinical presentation may be obscure and the clinical course and severity may call for immediate action (75). Several nucleic acid amplification (NAA) methods had been introduced for specific detection and identification of *M. tuberculosis* in respiratory specimens. They were not approved for use with non respiratory specimens (for instance, CSF) but, they are often applied to such specimens **(76)**. NAA assays are more sensitive than smears in the cases of extrapulmonary tuberculosis e.g. tuberculous meningitis. A positive CSF-NAA assay can be considered presumptively to represent a case of tuberculous meningitis (**78**). PCR is more sensitive and rapid than the culture and smear-staining methods in the diagnosis of extrapulmonary tuberculosis (80).

V. CONCLUSION

Results of PCR are available with speed comparable to microscopy; sensitivity is higher than both microscopy and culture and the direct identification of the organism, as belonging to the *M.tuberculosis* complex is possible. To further enhance the sensitivity of PCR, alternative procedures like double repetitive-element PCR (DRE-PCR) using hot-*Taq* should be employed (81). However, over-reliance on PCR should be avoided, as premature cessation of treatment will have serious consequence in patients with TBM, in whom PCR is negative. Hence, a combination of clinical criteria and PCR is needed for the final outcome to address the disease. In

conclusion, the result of our study suggest that, given the specificity, sensitivity and rapidity of the PCR described here, it can be applied as a reliable method for the diagnosis of difficult cases of tuberculosis, such as TBM.

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LIST OF ABBREVIATIONS:

%	: Percentage.
µg	: Microgram.
µl	: Microlitre.
bp	: Base pairs.
CSV	: Cerobro Spinal Fluid.
DNA	: Deoxyribonucleic Acid.
Ds	: Double strand.
e.g.	: Example Gratia.(Latin: For example)
EDTA	: Ethylene Diamine Tetra Acetic Acid.
Fig	: Figure.
g	: Gram.
HIV	: Human Immunodeficiency Virus.
TBM	: Tuberculous meningitis
IC	: Internal Control.
kb	: Kilo base.
l	:Litre..
M	: Molar.
min	: Minutes.
ml	: Millilitre.
nm	: Nanometer.
ºC	: Degree Centigrade.
PBS	: Phosphate Buffer Solution.
PCR	: Polymerase Chain Reaction.
pH	: Potential Hydrogen.
rpm	: Revolution per minute.

TAE : Tris Acetate EDTA.
TBE : Tris Borate EDTA.
TM : Trade Mark.
V : Voltage.
ZR : Zymo Research.

DECLARATION

I do hereby declare that the thesis entitled "**To analyze the positivity rate of Mycobacterium tuberculosis PCR in patients with chronic meningitis***"* submitted *to* 'Biotech Consortium India Limited' in partial fulfillment of the "Biotech Industrial Training Programme" is a faithful record of project work carried by Anil Kumar, under the guidance and supervision of Dr. Yogesh Kumar Singh and Mr. Mukesh Kumar

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