EVALUATION OF MICROSCOPY, CULTURE AND PCR ASSAY IN THE DIAGNOSIS OF EXTRAPULMONARY TUBERCULOSIS

BY

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MAY 2017

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I hereby declare that this dissertation entitled "**EVALUATION OF MICROSCOPY, CULTURE AND PCR ASSAY IN THE DIAGNOSIS OF EXTRAPULMONARY TUBERCULOSIS**" is a bonafide and genuine research work carried out by me under the direct guidance of **DR BEENA P M** Professor & HOD, Department Of Microbiology, Sri Devaraj Urs Medical College, Kolar.

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LIST OF ABBREVIATIONS

TB	Tuberculosis
HIV	Human immunodeficiency Virus
AIDS	Acquired immune deficiency syndrome
PTB	Pulmonary tuberculosis
EPTB	Extrapulmonary tuberculosis
M TB	Mycobacterium tuberculosis
TST	Tuberculin Skin Test
IGRA	Interferon Gamma Release Assay
NAAT	Nucleic Acid Amplification Test
AFB	Acid Fast Bacilli
ATT	Anti-Tuberculosis Treatment
NMT	Non Tuberculous mycobacterium
LJ	Lowenstein Jensens
PCR	Polymerase Chain Reaction
ZN	Ziehl Neelsen
PAS	Para-amino salicylic acid

MDR-TB	Multi Drug Resistant Tuberculosis
XDR-TB	Extensively Drug Resistant Tuberculosis
ADA	Adenosine Deaminase
Th 1	T-helper type 1
IFN-γ	Interferon- γ
IL-12	Interleukin 12
CNS	Central Nervous System
BBB	Blood Brain Barrier
TNF-α	Tumor Necrosis Factor- α
CSF	Cerebro Spinal Fluid
BCG	Bacillus Calmette-Guerin
GUTB	Genito-urinary Tuberculosis
LAM	Lipoarabinomannan
RNTCP	Revised National Tuberculosis Control Programme
AR staining	Auramine Rhodamine staining

ABSTRACT

Background: Mankind has seen changing face of tuberculosis (TB): from an incurable disease to the curable one. The emergence of HIV/AIDS and spread of drug resistant TB has increased the incidence of pulmonary and extrapulmonary tuberculosis (EPTB) in last couple of decades.

EPTB is defined as tuberculosis involving organs other than the lungs. Due its variety of presentation EPTB often poses a great difficulty in early diagnosis. The conventional diagnostic tools i.e. microscopy and LJ culture have low and varied sensitivity. Though culture on LJ media is considered gold standard, it requires up to 8 weeks of incubation for maximum sensitivity.

The newer and rapid diagnostic techniques i.e. liquid culture and PCR are promising options to overcome the limitations of conventional methods. Hence we conducted this study to evaluate the sensitivity, specificity and turnaround time of microscopy, culture and PCR and to evaluate the use of PCR in the early diagnosis of extrapulmonary tuberculosis.

Material and methods: The present study included 71 patients with strong clinical suspicion of extra-pulmonary tuberculosis attending OPD and admitted in R L Jalappa Hospital and Research Centre, Tamaka, Kolar from January 2015-August 2016. All cases already on Anti-tubercular Therapy or had been confirmed as having tuberculosis was excluded from study.

The clinical specimens included in this study were pus (15), endometrial biopsy (14), lymph node aspirate (10), peritoneal fluid (9), pleural fluid (7), tissue (5), CSF (5), synovial fluid (3), urine (3).These specimens were processed and evaluated by ZN staining, fluorescent microscopy, LJ culture, BacT ALERT culture and PCR.

Statistical analysis was done using 'SPSS 22' software and the sensitivities, specificities, positive predictive values, negative predictive values were calculated considering LJ culture as gold standard. Cohen's kappa value was also obtained to assess the reproducibility and level of agreement between the PCR assay and other diagnostic tests employed.

Results and Discussion: Of the 71 cases studied 40 were male (56%) and 31 were females (44%). The majority of the patients belonged to the age group of 31-40 years accounting for 23.9% followed by 21-30 years age group. The positivity rates by microscopy, LJ culture, BacT ALERT culture and PCR were 11.26%, 8.45%, 14.08% and 14.08% respectively.

The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) of both staining methods was 50%, 95.08%, 37.5% and 95.2% respectively showing there was no difference observed between the results obtained by ZN staining and fluorescent staining.

The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) of BacT ALERT culture was 83.3%, 92.3%, 50% and

98.4% respectively. The recovery rate was higher by BacT ALERT culture (90.9%) compared to LJ culture (63.63%). The mean turnaround time for culture positivity was 36.3 days with LJ culture and 14.6 days with BacT ALERT culture. The use of BacT ALERT culture has reduced the mean detection time by 2.5 times when compared to LJ culture.

The sensitivity, specificity, positive predictive value and negative predictive value of PCR assay was 66.66%, 90.76%, 40% and 96.72% respectively. In our study, the level agreement between BacT ALERT and PCR was ‘substantial’ with a kappa value of 0.767 whereas ‘moderate’ level of agreement between LJ culture and PCR with kappa value of 0.441.

PCR has high sensitivity, specificity, substantial level of agreement with BacT ALERT culture and shorter turnaround time. Therefore, use of PCR in combination with other diagnostic modalities is a useful tool to detect additional EPTB cases to be missed otherwise.

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INTRODUCTION

"A dread disease in which the struggle between soul and body is so gradual, quiet and solemn and the result so sure that day by day and grain by grain, the mortal part wastes and withers away. A disease… which sometimes moves in giant strides and sometimes at a tardy sluggish pace, but, slow or quick, is ever sure and certain… Charles Dickens: Nicholas Nickleby.

Till date the words of Charles Dickens are true. Tuberculosis; A scourge of the mankind from time immemorial, the dread disease was called consumption in Dickens time had a profound social and economical effect on human existence worldwide[1].

Mankind has seen changing face of tuberculosis (TB): from an incurable disease to the curable one. With the emergence of HIV/AIDS epidemic (1981); the cursed and deadly coinfection of HIV and TB resulted in a global resurgence of TB[2]. In the early 1990's, a drug resistant TB strain caused an outbreak in New York, killing 80% of infected patients[3]. The HIV and TB coinfection, and spread of drug resistant TB has worsened the scenario to an extent that, TB has been declared a global emergency in 1993 by WHO[2].

The tuberculosis has varied presentation and it is divided into Pulmonary TB (PTB) and extrapulmonary TB (EPTB) based on clinical manifestation[4]. EPTB is defined as TB involving organs other than the lungs (e.g. pleura, lymph nodes, abdomen, genitourinary tract, skin, joints and bones, or meninges). If a patient with

EPTB also has tubercular lesion in lung parenchyma, then the patient is categorized as pulmonary TB (e.g. military TB)[2]. If the patient suffers from intrathoracic mediastinal and/or hilar lymph node TB or TB plural effusion without radiographic abnormalities in the lung is categorized as EPTB[5].

WHO estimates shows that globally there were 9.3 million cases of TB in 2014, of which 80% were in 22 high burden countries, with India ranked as the highest burden country[6]. EPTB constitutes about 15-20% of all TB cases with HIV pandemic, the EPTB scenario is of further complicated, as EPTB constitutes more than 50% of all cases of TB in HIV positive patients[7].

Due its variety of presentation EPTB often poses a great difficulty in early diagnosis. It may present with constitutional symptoms such as fever, anorexia, weight loss, malaise and fatigue[8]. In India, the only presentation may be fever of unknown origin due to its remote infection site[9].

Different techniques used in the diagnosis of TB and EPTB are smear microscopy, culture, histopathology, tuberculin skin test (TST), interferon gamma release assay (IGRA), serological tests and nucleic acid amplification tests (NAAT). The usefulness, priority and scope of these techniques used in diagnosis vary with individual countries and on the resources available. In many developing countries, the only available diagnostic tool is direct smear microscopy for Acid Fat Bacilli (AFB)[7,9].

Among the available modalities TST, IGRA and serological tests are not recommended in diagnosis of TB or initiation of Anti Tuberculosis Treatment (ATT)[10].

Smear examination is reliable, reproducible, rapid, cost effective and useful in monitoring response to ATT but requires large number of AFB in sample (10^4/ml) for detection and technical expertise in interpreting the smears. Apart from these limitations, it has variable sensitivity which ranges from 10-37% and it cannot differentiate between Mycobacterium Tuberculosis (MTB) from Non- tuberculous mycobacterium (NTM). Culture on Lowenstein Jensen media (LJ media) is still considered gold standard test in the diagnosis of TB with variable sensitivity ranges from 12-80% in different body fluids. But it is labor intensive, it needs viable organisms in the sample and requires up to 8 weeks of incubation for maximum sensitivity.[11, 12, 13] This adversely affects the treatment plan by delaying it or subjecting patients to inappropriate empiric therapy[14].

More recently introduced liquid culture and molecular diagnostic technique like Polymerase Chain Reaction (PCR) has helped in early diagnosis as well as speciation of MTB isolates. Colorimetric liquid culture (Bac T/ ALERT), the most rapid culture technique requires nearly 13 days to show positivity and increases the case yield by 10% over solid media[9, 11].

PCR assays targeting various genes (65 k Da, 38 k Da) or Insertion sequence 6110 (IS 6110) are suitable method for sensitive and rapid detection of MTB complex with encouraging results. These newer and rapid diagnostic techniques including imaging modalities and ATT helps to overcome the challenges that lie in TB control[7,9].

OBJECTIVE

1. To evaluate the sensitivity, specificity and turnaround time of microscopy, culture and PCR in the diagnosis of Extrapulmonary tuberculosis.

2. To evaluate the use of PCR in the early diagnosis of Extrapulmonary tuberculosis.

REVIEW OF LITERATURE

History

TB or illness resembling TB have been described from different civilization since ancient times. The earliest such description can be found in Vedas, where TB was referred to as Yakshma meaning wasting disease. Greek, Chinese and Arabic literature also describes TB like disease[2].

Mycobacteria exists on earth since last 150 million years. A typical tubercular vertebral lesions were seen in mummies from the Egyptian pre-dynastic era and the Peruvian pre-Colombian era. The first weak evidence of TB in humans is from a bone lesion found in a 500 thousand year old skull in Turkey. Human TB detection using PCR sequencing in a Neolithic infant and women from 9 thousand year old settlement in the Eastern Mediterranean is an oldest strong evidence. Galen (131-201) first suspected that TB could be contagious. It took many centuries until Girolamo Fracastorius (1483-1553) showed that some diseases could be transmitted through 'particles' by direct or indirect contact between humans. Thomas Willis (1621-1675) first described miliary TB. Calmette extracted a protein (tuberculin) from large cultures of the bacillus and first used for therapy known as 'tuberculinisation', which failed as treatment for TB. The Tuberculin was also used for intradermal skin test which was described by Charles Mantoux in the diagnosis of TB. Later this intradermal skin test was named after Charles Mantoux and is known as Mantoux test[15].

Benjamin Marten (1690-1752) hypothesized that TB is caused by 'wonderfully minute living creatures' in his theory of 'contagious living fluid'. It was Jean Antoine Villemin (1827-1892), a French army doctor who successfully demonstrated the transmission of TB from humans to animals and from animals to animals. In 1834, Johann Lukas Schonlein proposed the name 'Tuberculosis' which is derived from Latin word 'tubercula' meaning 'a small lump' seen in all forms of the disease[15].

On 24th March 1882, Robert Koch announced in the meeting of the Berlin Society of Physiology that he had discovered causative agent responsible for pulmonary TB and named it 'tuberkel virus' in his paper published 2 weeks later. First innovative decision of staining tuberculosis bacilli and second innovative decision of culturing it on solidified cow or sheep serum gave Robert Koch the Nobel Prize of medicine in 1905. Leon Charles Albert Calmette (1863-1933) and Camille Guerin (1872-1961) developed vaccine against TB by sub-culturing *Mycobacterium bovis* for more than 200 times in the Guinea pig model between 1908-1921[2, 15].

The effective treatment for TB became a reality after the discovery of anti-tubercular drugs like Streptomycin, Para-amino salicylic acid (PAS) and isoniazid by the mid-1940s. By late 1970 it was believed that TB may no longer be a public health problem in the developed world. But the emergence of Acquired Immune Deficiency Syndrome (AIDS) in the early 1980s has ended this optimism and let to the resurgence of TB worldwide[2].

Toxonomy and Description of the Genus

Mycobacterium Tuberculosis belongs to

ORDER- Actinomycetales

CLASS- Actinomycetes

FAMILY- Mycobacteriaceae

GENUS- Mycobacterium

Genera closely related to *Mycobacterium* are Gordomia, Tsukamurella, Nocardia and Rhodococcus.

Silent features of *Mycobacterium* genus:-

Mycobacteria are aerobic, non-spore forming, non-motile, slightly curved or straight rods, 0.2 to 0.6 µm by 1 to 10 µm.

Colony morphology varies from species to species, ranging from smooth to rough and from non-pigmented to pigmented (carotenoid pigment).

Cell wall contains N-acetyl muramic acid.

High content of Mycolic acid (70-90 carbon atoms) renders acid fastness.

High G + C content of the DNA (61-71 mol %)

Slow generation time ranging from 20 hours to 36 hours for Mycobacterium tuberculosis[16,17].

Epidemiology

M. Tuberculosis Bacilli has infected nearly 1/3rd of the world's population with 10% lifetime risk of developing TB disease[18]. Globally 9.6 million cases of TB reported in 2014, of which 5.4 were million men, 3.2 million were women and 1 million were children, which accounts to 133 cases/1,00,000 population. Most of these cases occurred in Asia (58%) followed by Africa (28%). India has an incidence rate of 167 cases/1,00,000 population with maximum TB burden prevalence rate of 195 case/1,00,000 population[19].

Among 0.8 million new EPTB cases reported worldwide (2013), maximum cases were from India accounting for 0.35 million cases[19]. In India, according to Revised National Tuberculosis Control Programme (RNTCP) data, the prevalence of EPTB is 50% in HIV infected patients and 15-20% in non-HIV patients. The distribution of EPTB was in lymph node 47%, pleural cavity 30%, abdomen 10%, bones and joints 8%, CNS 2% and others 3%[20].

Between 2000 and 2014, TB treatment has saved 35 million lives among HIV negative people and 8.4 million lives among HIV positive people on Anti-Retroviral Therapy (ART), thus TB incidence has fallen by an average of 1.5% per year since 2000 and now 18% lower than the levels reported in 2000. In 22 high burden countries, India has managed to reduce the prevalence rate by 50% as set by Stop TB Partnership Programme[19].

Drug resistant TB has been reported from early days of introduction of ART, but multidrug-resistant tuberculosis (MDR-TB) and more recently extensively drug-resistant tuberculosis (XDR-TB) posing a threat to TB control program globally. Unfortunately, 3.3% of new TB cases and 20% of previously treated cases have Multi Drug Resistant TB (MDR-TB), 54% of which occurring in India, China and Russian Federation. An estimated 9.7% of people suffering from MDR-TB have Extensively Drug Resistant TB (XDR-TB)[19].

Pathogenesis

The majority of droplet nuclei containing MTB from infectious patients are trapped in upper airway and expelled by ciliated mucosal cells: only a fraction reaches alveoli. The mycobacteria then bind to cell surface of alveolar macrophages through complement receptors, mannose receptor or type A scavenger receptor. Following phagocytosis, mycobacteria reduce acidity in phagosome and a cell wall component, lipoarabinomannan impairs Ca^{+}/calmodulin pathway thus inhibiting phagosome-lysosome fusion. On successful arrest of phagosome maturation, the multiplication of bacilli begins and the macrophage eventually ruptures to release its bacilli, which are taken up by macrophages and continues infection cycle further expanding the infection[21].

During primary infection, MTB bacilli undergo hematogenous and lymphatic dissemination involving hilar and mediastinal lymph nodes forming primary Ghon's complex. Then bacilli gain access to blood stream and reach various organs. This

lympho-hematogenous dissemination results in extrapulmonary tuberculosis during primary infection or later in life during reactivation of disease[22].

EPTB can involve any site in the body. The most common site being the lymph node. However pleural, neurological, synovial, pericardial, abdominal, genitourinary involvement has been described.

Tubercular Lymphadenitis

From ancient times, lymph node TB has been called Scrofula or King's evil. It constitutes nearly 35% of EPTB cases. Cervical lymphadenitis is the most common and reported in 60-90% of tuberculous lymphadenitis cases. Involvement of cervical lymph node is due to spread of bacilli from primary focus of infection in Ghon's complex or from tonsils, adenoids, sinonasal/osteomyelitis of the ethmoid bone.

Initially, MTB bacilli multiply in lymph node causing marked hyperemia, swelling, necrosis and caseation of involved lymph node. The inflammation, progressive swelling and matting of other nodes around, resulting in adhesion to adjacent skin and rupture into surrounding tissue or through skin forming sinuses.

Mediastinal lymphadenitis can compress major blood vessels, phrenic nerve or recurrent laryngeal nerve or cause erosion of bronchus which is commonly seen in children.

Peripheral tuberculosis of lymph nodes is classified by Jones and Campbell into-

STAGE I:-Enlarged, firm, motile discrete nodes.

STAGE II:-Large rubbery nodes fixed to surrounding tissue.

STAGE III:-Central softening due to abscess formation.

STAGE IV:-Collar stud abscess formation.

STAGE V:-Sinus tract formation[23].

Pleural TB

The incidence of pleural TB is as high as 30% of all EPTB cases in high burden countries. The patients usually presents with acute febrile illness with non-productive cough and pleuritic chest pain; associated with night sweats, chills, weakness, dyspnea, weight loss.

The pathogenesis in pleural TB is presumed to be due to delayed hypersensitivity rather than direct infection of pleural space. This space is infected from initial lung parenchymal lesions and results in immunological response predominated by neutrophils (first 24 hours). This is followed by lymphocyte driven immune response forming pleural granuloma formation and release of Adenosine Deaminase (ADA).

Neutrophils remain the first line of defense for first 24 hours, followed by macrophages which peak at 96 hours and then by lymphocytes. A strong T-helper type-1 (Th 1) response is necessary to contain MTB. Activated CD_{3+} and CD_{4+} Th1

cells release interferon γ (IFN- γ) thus activating macrophages to kill MTB. The Th1 immunity in pleural TB is confirmed by the high levels of IFN- γ, interleukin-12 (IL-12) and elevated helper T cells in pleural fluid as compared to serum/peripheral blood.

The delayed hypersensitivity reaction to mycobacterial antigens affects pleura and increases the permeability of pleural capillaries, and thereby increasing fluid in pleural cavity. The fluid is drained through openings in the parietal pleura called stomata. Since diffuse involvement of parietal pleura with TB and damage to or obstruction of stomata leads to accumulation of pleural fluid.

Chronic TB empyema resolve leaving thickened, scarred and clarified pleura causing chronic chest pain, dyspnea and impaired lung function. Pleural fibrosis, a well-documented complication has been reported in 5-55% of pleural TB cases[24, 25].

Abdominal TB

The abdominal tuberculosis is diagnosed in 11% of patients with EPTB which was around 55-90% in era before effective ATT. The most common site of gastrointestinal tract involvement is the ileocecal region due to following reasons-

More lymphoid tissue (Peyer's patches)

Increased physiological stasis

Rate of fluid and electrolyte absorption is more

Low digestive activity

Other sites of involvement in decreasing order are ascending colon, jejunum, appendix, duodenum, stomach, esophagus, sigmoid colon and rectum. Hepatobiliary, splenic and pancreatic TB are rare and associated with military tuberculosis; often diagnosed in immunocompromised patients.

MTB bacilli gain entry to abdominal organs by two routes and cause disease due to reactivation of dormant focus. As a result of hematogenous spread from primary lung infection in children and as a part of military TB. Through ingestion of contaminated food and milk which infect Peyer's patches and are transported to mesenteric lymph node, where they remain dormant[26,27].

Central Nervous System (CNS) TB

It is serious and often fatal form of EPTB, predominantly affecting young children. CNS TB is difficult to diagnose. It presents in 2 major forms

TB meningitis- 0.5-1% of all TB cases

Intra-cranial tuberculoma-accounting up to 40% of brain tumors

MTB bacilli reach CNS during dissemination that occurs in active pulmonary disease. These bacilli cross physiological Blood Brain Barrier (BBB) via infected monocytes/neutrophils and cause a caseating focus in brain parenchyma or meninges. These foci are termed as 'Rich foci'. In later stage, the rupture of these foci in subarachnoid space triggers inflammatory T cell response with elevated levels of INF

γ and TNF-α in CSF. The subsequent inflammation leads to production of inflammatory infiltrates which obstructs CSF outflow causing hydrocephalus, and vasculitis leads to infarction, causing potentially irreparable neurological damage[28].

Bone and Joint TB

It accounts for 10-15% of all EPTB cases. It arises from reactivation of dormant MTB bacilli lodged in any bone (spine or large joints) during bacteremia of primary lung infection. These bacilli have affinity for spine and large joints because of their rich vascular supply. An extension of initial infection focus from the bone to the joint results in tuberculous arthritis. Rarely the bacilli can reach spine from the lung along the Batson paravertebral venous pleura or by lymphatic drainage to the para-aortic lymph nodes.

Non tuberculous mycobacteria (NTM) have been reported to cause osteo-articular TB following a traumatic injury or during surgical procedure like joint arthroplasty. NTM bone infection through hematogenous dissemination occur in patients with AIDS or transplant recipients. In recent years M. bovis skeletal infections have been reported in individuals who receive intravesical BCG vaccine therapy[29].

Genito-urinary TB (GUTB)

It accounts for 15% of all EPTB cases and 3-4% of all PTB cases. Its occurrence is 20 times more in kidney transplant recipients than in general population[12].

After hematogenous spread of bacilli from active site of infection (usually lungs), bacilli lodged in kidney (most common site of GUTB) and form metastatic lesions (tubercles). These foci of infection may

Heal spontaneously or due to treatment

Enlarge and rupture into nephrons

Remain dormant for many years

Usually the spread of infection is descending from kidney to other genito-urinary organs. It develops between 2nd and 4th decades of life; usually 5-25 years of inactivity after primary lung infection[30].

Miliary TB

Military TB account for less than 2% of all tuberculosis cases and up to 20 % of all EPTB cases among immunocompetent adults, however the autopsy studies have shown military TB ranges between 0.3%-13.3%.

TH_2 response plays a central role in immunopathogenesis of military TB by inhibiting protective response such as granuloma formation and containment of the disease activity at the site of infection. The production of interlukin 4 (IL-4) during TH_2 response, downregulates nitric oxide synthase (NOS), tall like receptor 2 and macrophage activation; thus sabotaging protective response of TH_1 cells. This process favors dissemination of MTB[31].

Histology

Any site of infection involved in PTB or EPTB has pathognomonic lesions known as tubercles. This is characteristic granulomatous inflammatory reaction against MTB bacilli from host's cell mediated immunity. These tubercles are microscopic to begin with and coalesce to become macroscopically visible granulomas. The granulomas contain MTB bacilli within macrophages, fibrin rich alveolar exudate, lymphocytes and multinucleated giant cells enclosed within fibroblastic rim. These granulomas formed are both caseating and non-caseating grsnulomas[32].

Immunology

Robert Koch (1880) demonstrated delayed hypersensitivity reaction in Guinea pigs and later in human patients using mycobacterial extracts. Seifert (1934) purified MTB extract which later become reference used as Purified Protein Derivative (PPD) in tuberculin test. In 1945 M. Chase demonstrated that immunity against MTB cannot be transferred to animals by immune serum but by transfer of CD_4 T lymphocytes.

It is now clear that protection against MTB is through mainly T lymphocyte activating the macrophages. Dendritic cells (DCs) present in proximal draining lymph nodes play a major role in priming naïve T cells. DCs takes part in surveillance around airways, vessels and in the loose connective tissue. The mycobacterial specific lipoglycan lipoarabinomannan (LAM) binds to receptor present on DC to gain entry. The lipoid adjuvant of LAM activate antigen presenting cells (APC) through toll like receptor-2 (TLR-2). Both DC and APC ultimately prime T lymphocytes, after which memory CD_4 and CD_8 cells play central role in immune response against MTB.

The activated CD_4 and CD_8 cells have ability to kill intercellular MTB through secretion of cytolytic molecules (e.g granulysin, perforin) and chemokines (e.g CCL5 which attracts infected macrophages). Natural killer cells (NK cells) acts as bactericidal against MTB which are part of innate immunity. NK cells do not require APCs for their activation and also improve function of and γ δ T cells. The γ δ T cells are mycobactericidal and potent secretors of INF-γ like macrophages.

The activated T lymphocyte release IFN γ, TNF- α, and Interleukin-2 which activates other resting monocytes/macrophages. γ IFN up regulates production of TNF, toxic oxygen species and nitric oxide in macrophages. These lead to granuloma formation and effective containment of MTB inside granuloma[33].

Clinical Features

EPTB is less common when compared to PTB, thus less commonly encountered by clinicians and difficult to diagnose clinically.

Miliary TB

The clinical features are usually non-specific and may present with fever, weight loss, night sweats, anorexia and weakness. The physical findings in descending order are fever, wasting, hepatomegaly. Pulmonary finding lymphadenopathy, splenomegaly. A granuloma in retinal choroid is strong suggestive feature of disseminated TB[31, .34].

TB Lymphadenitis

It resents as painless swelling in cervical region on in the supraclavicular fossa. Usually the process is bilateral and with progression of disease, the lymph node fuse and become matted. The overlying skin gets inflamed, ultimately enlarged lymph node rupture through inflamed skin forming sinus tract. Intra thoracic adenopathy may cause atelectasis by compressing bronchi or bronchiectasis (common in children)[23, 34].

Pleural TB

The presentation in tubercular pleurisy depends on number of bacteria infecting pleural space. If few MTB bacilli gain entry into pleural space, then it leads to hypersensitivity response causing pleural effusion. The process may resolve spontaneously or may lead to large effusion causing fever, pleuritic pain, dyspnea and weight loss. If large number of MTB bacilli gain entry from rupture of a cavity or the

adjacent parenchymal fistula, then it leads to tubercular empyema. The presentation of pleural TB in HIV seropositive patients is chronic with additional symptoms like tachypnea, night sweats, fatigue, diarrhea and have more hepatomegaly, splenomegaly, lymphadenopathy as compared to seronegative patients[25,34].

Abdominal TB

The clinical presentation depends on site of involvement as TB can affect any location from mouth to anus. The most common site of involvement is terminal ileum or caecum and manifest as pain abdomen, a palpable mass sometimes with weight loss, fever and loss of appetite. Tubercular peritonitis presents with classic doughy abdomen, ascites, pain abdomen and fever.

In esophageal TB, additional symptoms seen are dysphagia, odynophagia and retrosternal pain/discomfort. Patient also suffers from life threating complications like broncho-esophageal fistula/hematemesis.

Gastric TB is rare because of acidic pH, few lymphoid tissue in mucosa and rapid gastric emptying. Duodenal TB presents with dyspepsia, duodenal obstruction and duodenal ulceration. Other reported complications are perforation, fistulae and obstruction jaundice.

The common presenting features in rectal TB is hematochezia followed by constitutional symptoms and complication. It may also present as anal fissure, fistulae or perirectal abscess[27,34].

CNS TB

The most common manifestation of CNS TB are meningitis (95%). Tuberculomas (2%) and abscess (1%). Clinical features include those related cranial nerve involvement as well as headache, vomiting, decreased level of consciousness, neck stiffness and in the absence of medical care coma and death[34,35].

Skeletal TB

Pain is the most common presenting feature. The involved joint have limited motion of range with or without the presence of swelling. The patient may present with sinus tract.

Involvement of spine leads to chronic backache, fever and more than 50% of patient suffer from neurological symptom due to compression of spinal cord. Delayed diagnosis may further complicate the situation due to spinal deformity and sever, irreversible neurological sequelae like paraplegia[29,34].

Genito-urinary TB

Patient usually presents with local symptoms like dysuria, hematuria, frequency of micturition and flank pain. In women, genital involvement presents with pelvic pain, menstrual irregularities and infertility where as in men the most common

presentation is scrotal swelling/mass with or without pain. Symptoms of prostatitis, orchitis or epididymitis may also occur depending on site of involvement[30,34].

Lab Diagnosis

Definitive diagnosis of tuberculosis involves demonstration of M. tuberculosis by microbiological, cytopathological or histopathological methods[2].

The classic laboratory approach to the diagnosis of mycobacterial infections involves the phenotypic characterization of colonies growing on Lowenstein-Jensen medium. The current recommendation is that a combination of phenotypic and molecular assay are used for the rapid identification of mycobacteria, particularly for the identification of M. tuberculosis.[36] Accurate diagnosis of EPTB depends on the detection of mycobacteria by using direct and indirect approaches[7].

Microscopy

Mycobacteria are recovered from variety of pulmonary and extrapulmonary samples. At least 10,000 AFB should be present per ml of sample for them to be readily demonstrable in direct smears[37].

Microscopy is reliable, reproducible, inexpensive an indicator of infectiousness, a comprehensive tool for diagnosis/monitoring progress/defining cure and even feasible in the remote or tribal places[38].

WHO evaluation showed that the diagnostic accuracy of light – emitting diode (LED) microscopy is comparable to that of conventional fluorescence microscopy with much less expense.[7] RNTCP has provided light emitting diode based fluorescent microscope services in 200 medical colleges across India as a pilot study[38].

Acid Fast Staining

The cell wall of mycobacteria, because of their high lipid content, have the unique capability of binding the Fuchsin dye so that it is not removed (destained) by acid alcohol. The presence of acid fast bacilli (AFB) in the smear, combined with history of weight loss, fever, night sweats and radiological evidence of old pulmonary lesion helps in early diagnosis. Acid fast smears are also useful in monitoring response to treatment.

Two types of acid-fast stains are commonly used:

Carbolfuchsin stains: a mixture of fuchsin with phenol (carbolic acid)

Ziehl-Neelsen (hot stain)

Kinyoun (cold stain)

Fluorochrome stain: Auramine O, with or without a second fluorochrome, rhodamine[36].

Ziehl-Neelsen (hot stain):-

The reagents and dyes used are-

Carbol fuchsin- dissolve 3g of fuchsin in 10ml of 90-95% ethanol. Add 90ml of 5% aqueous solution of phenol.

Acid alcohol- add 3ml of concentrated HCL slowly to 97ml of 90-95% ethanol.

Methylene blue counterstain- dissolve 0.3g of methylene blue chloride in 100ml of distilled water.

Examine with 100 × oil-immersion objective. Mycobacteria are stained red and the background light blue (figure 5).

The heating process during staining helps to penetrate carbol fuchsin into the MTB bacilli. The smear is decolorized using 20% H_2SO_4 and counter stained with methylene blue. MTB bacilli resist decolourisation by 20% H_2SO_4 hence are known as AFB[36].

Ziehl-Neelsen staining is reliable, reproducible and cost effective and also useful in monitoring response to anti TB treatment. Smear microscopy has low and variable sensitivity values (0–40%) and could not differentiate between *Mycobacterium tuberculosis* and nontuberculous mycobacteria[39,40,41].

Kinyoun (cold stain):-

The reagents and dyes used are-

Carbol fuchsin- dissolve 4g of basic fuchsin in 20ml of 90-95% ethanol and then add 100ml of 9% aqueous solution of phenol (9g of phenol dissolved in 100ml of distilled water)

Acid alcohol- add 3ml of concentrated HCL slowly to 97ml of 90-95% ethanol.

Methylene blue counterstain- dissolve 0.3g methylene blue chloride in 100ml of distilled water.

Examine with 10 0 × oil immersion objective. Mycobacteria are stained red and the background light blue.

This technique is cold stain technique because increased phenol concentration replaces heating step and helps in penetration of carbol fuchsin[36].

Auramine Fluorochrome:-

The reagents and dyes used are-

Phenolic Auramine- dissolve 0.1g of Auramine O in 10ml of 90-95% ethanol and then add to a solution of 3g of phenol in 87ml of distilled water. Store the stain in a brown bottle.

Acid-alcohol- add 0.5ml of concentrated HCL to 100ml of 79% alcohol

Potassium permanganate- dissolve 0.5g potassium permanganate in 100ml of distilled water.

Examine with 25 X objective for scanning. The 40 X objective is used to confirm any suspicious forms. Mycobacteria are stained yellow-orange against a dark background in fluorescent microscope (figure 6). The fluorochrome stain offers the advantage of greater sensitivity compared with the ZN stain, since a significantly larger area of the smear can be scanned thus reducing the time needed. Fluorescent microscopy increases sensitivity by 10% over ZN staining[36].

RNTCP has supplied light emitting diode based fluorescent microscope (LED-FM) in 200 medical colleges across India to reduce the burden on laboratory technicians in high work load settings (> 25 slides per day)[38].

Culture

Isolation of M. tuberculosis from clinical samples by culture is the 'gold standard' for a definitive diagnosis of EPTB. Culture methods are much more sensitive because fewer bacilli (10-100 bacilli/ml of concentrated material) can be detected and provides the necessary isolates for conventional drug susceptibility test and species identification. (manju) The sensitivity of culture for identification of M TB ranges between 0-80% in different extrapulmonary specimens[9,42,43,44].

The most commonly used solid media for culture of M TB is LJ media which usually takes 4-8 weeks for visible growth[45].

Solid Media

Egg-based Media

It contain whole eggs or egg yolk, potato flour, salts and glycerol and are solidified by inspissation. They have good buffer capacity and a long shelf life and support good growth of most mycobacteria. Of the egg-based media, L-J medium is most commonly used in clinical laboratories.

Agar-based Media

In contrast to egg-containing media, agar based media are chemically better defined. Colonies may be observed in 10 to 12 days, in contrast to 18 to 24 days with egg-based media. Thinly poured 7H11 agar plates can grow micro colonies in 11 days and can be examined by focusing agar surface through the bottom of the plate at 10 X to 100 X magnification. This method is used as an alternative to broth cultures. It can also be used for susceptibility testing.

Selective Media

The addition of antimicrobial agents may be helpful in eliminating growth of contaminating organisms. If a selective medium is used for a particular specimen, it should not be used alone but in conjunction with a non-selective agar or egg-based medium. A LJ media is made selective by adding penicillin and nalidixic acid, this is called Gruft modification or it can be made selective with cycloheximide, lincomycin, and nalidixic acid. Mitchison selective 7H11 medium contains carbenicillin, polymyxin B, trimethoprim and amphotericin B[17].

Liquid Media

Broth media can be used for early isolation of mycobacteria and also for subsequent subculturing. Middlebrook 7H9, BACTEC 12B and Dubos Tween albumin broth are commonly used liquid media. 7H9 broth is used as a basal media and Tween 80 acts as a surfactant which disperses clumps of mycobacteria resulting in homogenous growth. The liquid media are currently used in semi-automated and automated like BACTEC 460TB system, BACTEC MGIT 960 system, BACTEC 9000MB, ESP culture system 2 and MB/BacT ALERT 3D system, BACTEC MYCO/F lytic blood culture bottle.

Mycobacteria Growth Indicated Tube (MGIT):-

The MGIT contains a modified Middlebrook 7H9 broth in conjunction with a fluorescence-quenching-based oxygen sensor (silicon rubber impregnated with a ruthenium pentahydrate) to detect growth of mycobacteria. The presence of oxygen in the medium quenches the fluorescence of the sensor. As mycobacteria or other organisms grow in the broth, leads to depletion in the oxygen level and the indicator fluoresces brightly when illuminated with UV light at 365nm. The broth is enriched with 0.5ml of OADC (Oleic Acid, Bovine Albumine, Dextrose, Catalyse) and 0.1ml of PANTA antibiotic mixture (Polymixine B, Amphotericin B, Nalidixic Acid, Trimethoprim, Azlocillin). In BACTEC MGIT 960 system the tubes are continuously monitored by the instrument. Sensitivity and time to growth detection of the MGIT system are similar to those of the BACTEC 460TB system and have been superior to those obtained with solid media in clinical evaluation. But contamination rates are slightly higher for MGIT system than for BACTEC 460TB system[17,46].

BACTEC 460TB system:-

It is a semi-automated system which uses 14C-labeled palmitic acid as carbon source in the medium and when metabolized by microorganisms to $14CO_2$, it is monitored by the instrument. The amount of $14CO_2$ and the rate at which the gas is produced are directly proportional to growth rate of the organism in the medium. The average detection time for smear positive specimen is 9 to 14 days in case of M. tuberculosis and less than 7 days for NTM. The positive vials can also be used for drug susceptibility testing. The disadvantages of this system include inability to observe colony morphology, difficulty in identifying mixed cultures, overgrowth by contaminants, cost and radioisotope disposal[17].

Automated Continuous Monitoring Systems:-

The BACTEC 9000 MB system uses fluorescence-quenching-based oxygen sensor same as the MGIT system to detect growth. In ESP Culture System 2, growth is detected by monitoring pressure changes in the headspace above the broth medium in a sealed bottle resulting from gas production by microorganisms.

The MB/BacT ALERT 3D (figure8) system employs a colorimetric CO_2 sensor in each bottle and reflected light to monitor the presence and production of CO_2 dissolved in the culture media. As the microorganism grow, the CO_2 is generated which diffuses through membrane to sensor and dissolves in water present in the sensor causing accumulation of hydrogen ions.

$$CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow H^+ + HCO3^-$$

The amount CO_2 produced is proportional to the growth of microorganism in the media, as the CO_2 levels increase, the concentration of hydrogen ions increases, thus reducing the pH of sensor causing color change from dark green to light green or yellow.

A light emitting diode system projects light on the sensor and the reflected light is measured by photodetector. The color change of sensor increases reflectance units which is monitored and recorded by the instrument to determine the positive or negative result[17].

BacT ALERT 3D system has been evaluated in many studies for rapid detection of growth and also for DST. A study by Carricajo A et al reported mean detection time of M TB complex from pulmonary and different extrapulmonary samples were 22.8 days with LJ medium and 16.2 days with the BacT ALERT 3D system.47 In study by Piersimoni et al mean detection time of M TB complex in smear positive samples by BacT ALERT 3D, B460 and LJ media was 11.5, 8.3 and 20.6 days respectively whereas in smear negative samples it was 19.9, 16.8 and 32.1 days respectively[48].

These studies have reported that mean detection time taken by BacT ALERT 3D system was 16-18 days compared to 22-32 days by using LJ media for M TB complex. Thus BacT ALERT 3D system reduces detection time by 25% [47,48,49].

In another story by Moore WAJ et al the median time to culture positive was 7 days, 13 days and 26 days for microscopic observation drug susceptibility culture, automated culture and LJ culture respectively[50]. (As shown in Figure – Time taken for culture positivity by different methods)

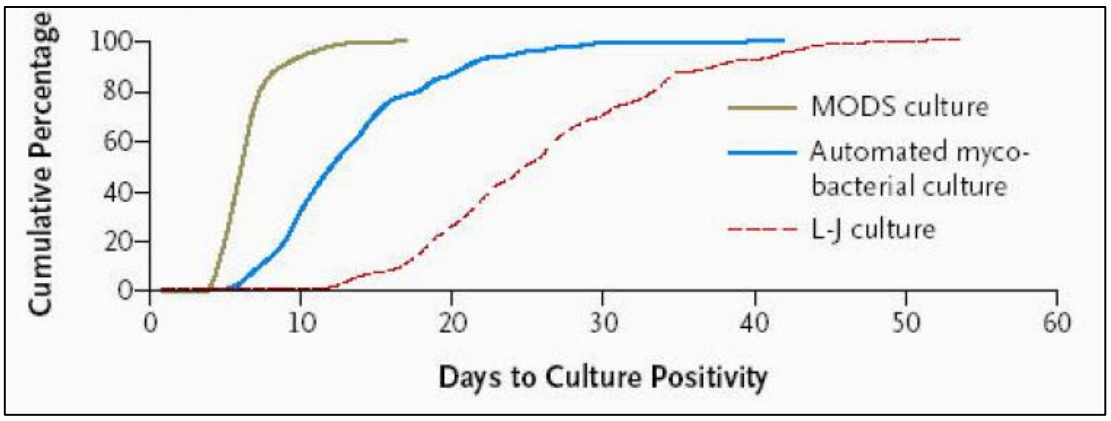


Figure 1. Mean detection time by different culture methods reported by Moore WAJ et al

The BACTEC MYCO/F LYTIC culture bottle has lytic agent to release mycobacteria phagocytosed by white blood cells. The incubation and monitoring is similar to other BACTEC blood culture bottles. It can also be used to culture other bacteria and fungi present in blood stream[36].

Gas-Liquid and High-Performance Liquid Chromatography (HPLC)

Analysis of fatty acids by gas-liquid chromatography is a rapid and reliable method for the culture confirmation mycobacteria and also identification of the species. The method is based on development of profiles of mycolic acids, which vary from one species to another. This technology to speciate mycobacteria is available only in few reference laboratories[36].

Denaturing HPLC is an alternative for other methods which utilizes a molecular probes like DNA sequence analysis, which is regarded as the gold standard for mutation detection, reverse line hybridization, single-strand conformation polymorphism, DNA macroarrays and real-time PCR. This technique is relatively inexpensive, same-day results can be obtained, potentially any mutation in the amplified fragment can be detected and it can be applied on a universal basis. Other studies have shown this method can be useful for detecting mutations on rpoB (RMP), katG (INH), pncA (pyrazinamide), rspL (streptomycin) and embB (ethambutol) gene [51].

Identification using Conventional Methods [36]

Rates of Growth

A rate of growth, the time of recovery varies from media to media- the average time of recovery of mycobacteria on egg based media is about 21 days, but ranges from as short as 3-5 days to as long as 60 days depending on the species. The growth of micro colonies on 7H10 agar is detectable from 3 to 12 days. In a study the average time to detection of micro colonies of MTB was 11 days on middlebrook 7H11 agar, 16 days with MB/BacT bottles and 19.5 days with LJ media. Some mycobacterial species belonging to rapid grower group grows within 7 days on LJ media.

Pigment production

Mycobacterium species have capability of producing colony pigmentation in the dark (scotochromogen) or only after exposure to light (photochromogen) doesn't finalize species identification but narrows possibilities. Even after exposure to light MTB fails the pigment production, beyond a light buff color (figure 7).

Non Tuberculous Mycobacteria

Non tuberculous mycobacteria (NTM) has been classified on the basis of pigment production and rate of growth by Runyon-

GROUP I- Photochromogens

GROUP II- Scotochromogens

GROUP III- Nonchromogens

GROUP IV- Rapid growers

Niacin accumulation test

All mycobacteria produce niacin, but only MTB and M. simiae lack the enzyme required to further convert the niacin to niacin ribo-nucleotide. Reagent impregnated filter paper strips incubated in test medium produces yellow color which is indicative of niacin accumulation.

Nitrate reduction test

MTB produces nitroreductase which catalyzes the reduction of nitrate to nitrite. Production of red color after adding sulfanilic acid and N-naphthyl ethylenediamine to an extract of the unknown culture is indicative of the presence of nitrite and a positive test.

Tween 80 Hydrolysis

This test is useful in identifying mycobacteria which possess lipase that splits tween 80 into oleic acid and sorbitol. M. kansasii and M. gordonae hydrolyze tween ^{80}.

Catalase test

The enzyme catalase splits H_2O_2 to release O2 which is indicator by the presence of effervescence. The catalase activity after heating the culture at 68^0C for 20 min (heat stable catalase) is not seen in most strains of MTB. Semi quantitative assessment of catalase activity by measuring the height achieved by the column of bubbles produced when H_2O_2 is added to tube culture. A column higher than 45mm is considered a positive test.

Arylsulfatase activity

The enzyme arylsulfatase in mycobacteria breaks tripotassium phenolphthalein sulphate to release phenolphthalein. This free phenolphthalein turns the media pink in the presence of sodium bicarbonate. This test differentiate rapidly growing mycobacteria (positive) from group III nonphotochromogenic mycobacteria (negative).

Urease activity

The presence of urease activity is used to differentiate M. scrofulaceum (positive) from M. gordonae (negative).

Pyrazinamidase

The enzyme pyrazinamidase deaminates pyrazinamide to form pyrazinoic acid, which produce a red band in the culture media. This test is useful in distinguishing MTB, M. bovis, M. kansasii and M. marinum from other species of mycobacteria.

Iron uptake

M. fortuitum and M. smegmatis take up soluble iron salts from culture media to produce rusty brown appearance on addition of 20% ferric ammonium citrate. Other mycobacteria species lack this property.

Growth on MacConckey

MacConckey supports the growth of rapidly growing mycobacteria. However, most other mycobacterium species cannot grow on this media.

Growth in 5% sodium chloride

M. triviale and some strains of M. falvescens, M. fortuitum and M. abscessus can grow on egg based culture media containing 5% NaCl when incubated at 28^0C.

Table 1. Biochemical Reactions of Mycobacterium species

	Niacin accumulation	Nitrate reduction	Catalase		Arylsulfatase	Urease	Pyrazinamidase	Iron uptake	Growth on MacConckey
			Semiquantitaitve	68^0C					
M. tuberculosis	+	+	<45	-	-	+	+	-	-
M. bovis	-	-	<45	-	-	+	-	-	-
M. ulcerans	-	-	>45	+	-	V	-	-	-
M. africanum	-	-	>45	-	-	V	-	-	-
M. kansasii	-	+	>45	+	-	+	+	-	-
M. marinum	-	-	>45	-	V	+	+	-	-
M. simiae	+	-	>45	+	-	+	-	-	-
M. genavense	-	-	>45	+	-	+	+	-	-
M. asiaticum	-	-	>45	+	-	-	-	-	-
M. scrofulaceum	-	-	>45	+	-	+	V	-	-
M. szulgai	-	+	>45	+	-	+	-	-	-
M. zenopi	-	-	<45	+	+	-	+	-	-
M. gordonae	-	-	>45	+	-	-	V	-	-
M. avium complex	-	-	<45	-	-	-	+	-	V
M. malmoense	-	-	<45	V	-	V	V	-	-
M. haemophylum	-	-	<45	-	-	-	+	-	-
M. fortuitum	-	-	>45	+	+	+	+	+	+
M. chelonei	-	-	>45	+	+	+	+	-	-
M. abscessus	-	-	>45	+	+	+	+	-	+

Molecular methods

There is a movement in clinical laboratories away from the conventional time consuming and tedious test for species identification of mycobacteria recovered in culture e.g. nucleic acid probes have been produced to identify MTB, mycobacterium avium intracellulare, M. kansasii and M. gordonae. There are 4 major applications used in clinical laboratories:

1. Use of DNA probes for culture confirmation of isolates recovered from clinical specimens.

2. Use of DNA sequencing for identification of mycobacteria.

3. Use of nucleic acid amplification tests (NAAT) for direct detection of MTB from clinical specimens.

4. DNA finger printing and strain typing of mycobacterium species.

Nucleic acid probes

This is a first nucleic acid based technology used to identify mycobacteria in positive cultures with very high accuracy, sensitivity and specificity. In this technique ribosomal RNA (rRNA) present in the cells and in culture in high quantities acts as genetic target. The radiolabeled (acridine ester) single stranded DNA probes hybridizes with rRNA forming stable DNA-RNA complex. After the inactivation of unhybridized probe, light generated is recorded by an instrument which is proportional to the amount of probe present. A predetermined threshold is used to determine positivity. This technique requires two hours.

In situ hybridization

This technology uses an oligonucleotide probe labeled with fluorescein and the interpretation is made by direct observation using fluorescence microscopy. It is popularly known as fluorescence *in situ* hybridization (FISH). If the detection of hybridized group is done by secondary reaction and color, the reaction is known as chromogenic *in situ* hybridization (CISH). FISH has been used to detect MTB in cultures and in direct respiratory samples that contain AFB.

Nucleic acid amplification methods

In late 1990's FDA approved amplicor M. tuberculosis PCR assay (Roche diagnostics) and amplified M. tuberculosis direct test (AMTD) for respiratory specimens. These assays perform well on smear positive specimens but sub optimally on smear negative respiratory specimens, when compared with culture. Lately many in-house PCR and more recently real-time PCR assays have been developed and tested.

A study by Laraque F et al tested performance of NAA on respiratory samples (N= 4642) and found that NAA had a sensitivity of 96% and specificity of 95.3% in specimen tested positive for AFB on smear[52].

In another study by Guerra RL et al the effect of NAA results in clinical care of Pulmonary TB was evaluated. A total of 638 cases were included of which 270 were

positive for MTB on culture. NAA had a sensitivity of 92.3% and specificity of 99.8%. NAA had decreased length of unnecessary therapy from 31 days to 6 days[53].

Polymerase Chain Reaction:

PCR technique is now widely used in the research and diagnostic fields. This technique is based on amplification of specific DNA sequence to a large number of copies that can be detected by separation on gel electrophoresis. The amplification is achieved by using synthetic oligonucleotide primers which are complementary to specific DNA sequence. This process leads to a million fold amplification of target DNA through multiple cycles of:

Denaturation

Annealing

Extension

Resulting in an exponential increase in the number of copies of the target. A number of target genes of mycobacterial DNA have been evaluated for diagnosis by PCR and various other genotypic methods.

The different DNA amplification targets used are – IS6110, devR, rpoB, IS986 and genes encoding MPB-64 (mpb64), 38kDa (pstS1), 65kDa (hsp65), 30kDa (fbpB), ESAT-6 (esat6), and CFP-10 (cfp 10) proteins. Any stretch of nucleic acid can be amplified by using DNA polymerase, provided that the specific sequence data are known to allow the designing of appropriate primers.

The target most frequently amplified is the IS 6110 repetitive element which is present in multiple copies (up to 20) in most strains of *M. tuberculosis*. Species specific and genus specific PCR methods are being used with various targets and modifications of PCR[54].

Advantages of PCR:

1. Most sensitive and rapid method of detection, can even detect when the bacilli number is as less than 10 {1-10 AFB/ml}
2. Determine rapidly whether AFB identified by microscopic examination in clinical specimens are *M. tuberculosis* or atypical mycobacteria.
3. Identify the presence of genetic modifications known to be associated with drug resistance.

Disadvantages of PCR:

1. False positive reactions- due to carry over contamination
2. False negative reactions- due to presence of inhibitors that interfere with the PCR
3. High cost
4. Amplification of DNA from both live and dead bacilli. So it cannot be used for monitoring therapy response.
5. Inhibition of amplification and reproducibility of the assay

The effectiveness of PCR for tuberculosis depends on experience and accuracy of the personnel conducting the assay.

In many studies sensitivity of PCR has been compared with microscopy and culture results. Some of the study results are as follows-

Table 2. PCR sensitivities in different studies

Study	**PCR Sensitivity (%) in different studies**		
	Overall	**Smear + Culture +**	**Smear – Culture +**
Abe et al [55]	84	96	50
Clarridge et al [56]	86	94	62
Beige et al [57]	98	100	94
Nolte et al [58]	91	95	57
Cheng et al [59]	72	100	-

The disadvantages of PCR were addressed in a multi-laboratory study conducted by Noordhoek and coauthors. They had sent 200 sputum, saliva and water samples containing known numbers of M bovis BCG along with negative controls to 7 laboratories. Each laboratory used IS6110 insertion sequence as the target and their own protocol for PCR. High levels of false positive results ranging from 3% to 20% were reported. This was due to lack of monitoring of each step of the procedure. To overcome these problems there is a necessity of careful quality control during every step of assay.

Real time PCR is a technique that reduces the detection time and also quantifies the amount of M TB present in the clinical sample. The whole process of

amplification and detection takes place in single reaction vessel in a closed system. Thus it reduces risk of amplicon contamination of laboratory. Since this technique is completely automated there is no need of post amplification processing and electrophoresis for detection of amplicons[36].

Line probe assay

It is a reverse-hybridization technology made available commercially by Innogenetics and Roche diagnostics. It uses nitrocellulose strip on which multiple probes are immobilized. The amplicons are applied to the strip. Line or dots are formed at the site of amplicon-probe hybridization. The pattern formed at the end of reaction is then compared with standard key to interpret the results of that particular reaction. This technology is reverse of Southern blot technique with advantage of numerous probes can tested simultaneously. Radioisotopes are not used which is another advantage of this technology over Southern blot. These assays are relatively simple when compare to DNA sequencing with simpler post-analytic analysis. It is used for species identification as well as detection of mutations that lead to drug resistance[36].

Transcription mediated amplification:

TMA amplifies rRNA via DNA intermediated producing billions of copies of RNA within an hour followed by detection using acridinium ester labeled DNA probes. Results are read in a luminometer in terms of Relative Light Units (RLU). Samples with values of 30,000 RLU are considered positive.

TMA is sensitive enough to detect as little as 2.5 (femptogram) of RNA in clinical samples. Since there are 3-5fgof rRNA per M. tuberculosis cell, this assay is capable of detecting the rRNA contained in a single cell and is useful in conditions where culture is not feasible considerable time or in Paucibacillary conditions[60].

DNA sequencing:

This technology is very useful in the identification of slow growing organisms like mycobacteria. It is a complicated technique than simple probe hybridization and requires experience in sequence alignment, editing software and genetic data basis. This technique hypervariable A region of the 16S gene complex is most commonly targeted for rapid and accurate identification of mycobacteria. MicroSeq (Applied Biosystems, Inc, foster city, CA) has made this technology commercially available. The rpoB gene was popularly used as sequencing target because it provides identification information as well as information about the susceptibility to Rifampin[36].

Spoligotyping:

This technique is used studying genetic diversity and epidemiologic study of M TB strain circulating in a particular region. In this technology, DNA polymorphism is detected at direct repeat locus (DR locus) of M TB genome by hybridization assay which is then used for phylogenetic analysis. The predominant spoligopatterns reported from Indian studies are CAS, EAI, Beijing, manu. The technique further characterizes MTB strain and its importance in determining clinical manifestation[61].

DNA microarray analysis:

High density oligonucleotide arrays (DNA microarrays) offer the possibility of rapid examination of large amounts of DNA sequences with a single hybridization step. This approach has recently been applied to simultaneous species identification and detection of mutations that confer drug resistance in mycobacterium. The DNA microarray method holds great promise for the future because it is easy to perform it can be readily automated and it allows for identification of a large number of mycobacterial species in one reaction. This technique is expensive and currently used only for research work[36].

GENEXpert MTB/RIF assay:

WHO has recommended GENExpert MTB/RIF assay for the early diagnosis of TB and for the detection of resistant to Rifampicin in 2011. This simple cartridge based nucleic acid amplification test has revolutionized TB control program. It requires only 130 TB bacilli per ml of sputum for a positive result. It is also used for urine, stool, blood and other body fluids thus improving the diagnosis of extra pulmonary tuberculosis and detection of drug resistance[62]. This assay works on heminested PCR principle in a closed, completely automated cartridge based system which targets 81 bp fragment of rpoB gene for identification of M TB strain and subsequent probing of this region for mutations that detect Rifampicin resistance[63].

An overall sensitivity of GENEXpert MTB/RIF assay in the diagnosis of pulmonary TB was 88%, pooled sensitivity of 98% for smear and culture positive cases; 68% for smear negative cases[64].

Susceptibility testing:

Drug resistant TB was first described by Pyle in 1947 against streptomycin. Resistance to other anti-TB drugs was reported through 1960s, 1970s, 1980s but wasn't given much importance to this problem until emergence of multi-drug resistance TB in the United States in early 1990s. Multi-drug resistance TB(MDR TB) is defined as resistance to at least isoniazid(H) and rifampicin(R).

Extensively drug resistant TB(XDR TB) is defined as the resistance to rifampicin and isoniazid, plus any fluoroquinolone and at least one of three injectable second-line drugs(i.e. amikacin, kanamycin, or capreomycin)[66].

Drug susceptibility tests are done by two methods

Phenotypic methods

Direct method-

Digested, concentrated specimen is used as inoculum and inoculated on drug free and drug containing media. The advantage of this method is availability of results sooner than indirect method and better represents the patient's original bacterial population.

Indirect methods-

1. **Absolute concentration method-** In this method, a standardized inoculum is tested on drug free media and drug containing media. Graded concentrations of each drug are tested, and resistance is expressed in terms of the lowest concentration of the drug that inhibits growth i.e. minimum inhibitory concentration (MIC).
2. **Resistance ratio method- in this method,** clinical isolate is tested against standard laboratory strain (H37Rv). Parallel sets of media containing 2 fold dilutions of the drug are inoculated with test strain and standard strain. Resistance is expressed as MIC of test strain and MIC of standard strain in the same set. A ratio of 4 or more for a test strain indicates resistance to that antibiotic.
3. **Proportion method-** This method estimates proportion of mutants resistant to a given drug. Several 10 fold dilutions of inoculum are inoculated on drug free media (control media) and drug containing media. The dilution that yields isolated countable (50-100) colonies is taken and colony count is adjusted by multiplying by the dilution of the inoculum. The total number of viable colonies on control media and total number of mutant colonies resistant to the drug concentrations tested is estimated. The proportion of resistant bacilli is determined by expressing resistant bacilli to total population of bacilli used (in percentage). This method is used in

    BACTEC 460

    MGIT 960

    MB/BacT system

    ESP 2 system

Genotypic methods

The advantage of using molecular tests for the detection drug resistance is that results are made available within 24 to 48 hours Mutations associated with resistance to many of the anti-TB drugs have been described.

Table 3 Various gene loci conferring drug resistance in MTB [67]

Drug	**Gene**	**Functional role of gene product**	**Cellular Targets**
Rifampicin	*rpoB*	B subunit of RNA polymerase Transcription	Nucleic acids
Isoniazid	*kat G* *OxyR-ahpc* *Kas A*	Catalase-peroxidase/ activation of pro-drug Alkyl-hydro-reductase B- ketoacyl acyl carrier protein	Cell wall
Ethionamide	*eta A* *eth A*	Enoyl-ACP reductase/synthase mycolic acid biosynthesis	Cell wall
Streptomycin	*Rpsl* *Rrs*	Ribosomal protein S12/ translation 16s r RNA /translation	Protein synthesis
Fluroquinolone	*gyr A* *gyr B*	DNA gyrase	Nucleic acids
Pyrazinamide	*pnc A* Overexpression of RpsA	Amidase/Activation of pro-drug Ribosomal protein S1-translation- render more resistance to PZA	Unknown
Àminoglycosides	*rpsL* *rrs*	Inhibits protein synthesis by binding to the 30S subunit	Protein synthesis
PAS	*dfr A*	dihydrofolate synthase	Nucleic acids
Ethambutol	*emb CAB*	Arabinosyltransferase/ Arabinan:polymerization	Cell wall

In molecular tests, variations in nucleic acid amplification tests are employed. For the amplification of desired target gene PCR is employed followed by second assay to detect mutation in amplified product. For second assay, hybridization or DNA sequencing is used.

DNA sequencing-

DNA sequencing of PCR amplified products are most widely used and are becoming the GOLD-STANDARD for the detection of drug resistance in *M.tuberculosis*. It has been performed by both manual & automated procedures, DNA sequencing is used for characterization of the mutation responsible for drug resistance. This technique is mainly used for drugs like rifampicin, isoniazid, streptomycin and ciprofloxacin. Drugs included in the whole genome drug resistance library are amikacin, capreomycin, ethambutol, ethionamide, isoniazid, kanamycin, moxifloxacin, ofloxacin, pyrazinamide, rifampicin, streptomycin, para-aminosalicylic acid, linezolid, clofazimine and bedaquiline.

Hybridization assays

For hybridization assays such as the INNO-LiPA® Rif.TB (Innogenetics) and GenoType® MTBDR(plus) (Hain LifeScience GmbH) line-probe assays are commercially available. A gene associated with resistance is amplified by PCR,and labaled PCR amplicons are then hybridized to oligonucleotide probes immobilized on a nitrocellulose strip. Mutations are detected by lack of binding to wildtype probes or by binding to probes specific for commonly occurring mutations.

Molecular beacons-

The technique uses fluorescent dye labeled probes. Hybridization of these probe with their target emit fluorescence and can discriminate between targets differing only by a single nucleotide.(cdc report molecular methods)

Treatment of patients with tuberculosis:

The Revised National Tuberculosis Control Programme (RNTCP) has launched directly observed treatment short course (DOTS) strategy in 1997 and expanded across the country in a phased manner. The whole country was covered under the strategy by March 2006. Under this programme, designated microscopy centers have been established for every one lakh population in the general areas and for every 50,000 population in the tribal areas. Until now more than 13,000 microscopy centers are established in the country. The free treatment services for TB is made available at government hospitals, community health centers, primary health centers.

Objectives of the programme:

To reduce the incidence of and mortality due to TB

To prevent further emergence of drug resistance and effectively manage drug-resistant TB cases

To improve outcomes among HIV-infected TB patients

To involve private sector on a scale commensurate with their dominant presence in health care services

To further decentralize and align basic RNTCP management units with NRHM block level units within general health system for effective supervision and monitoring[68].

Drug regimen [69.70]

Drug Sensitive TB-

The RNTCP has introduced daily regimen for treatment of drug sensitive TB among people living with HIV and pediatric TB patients in 104 district. Rest of the country follows intermittent regimen as per existing guidelines until the daily regimen is scaled up to the entire country.

For new TB cases, 8 weeks of intensive phase (IP) consist of 4 drugs i.e. Isoniazid (H), Rifampicin (R), Pyrazinamide (Z) and Ethambutol (E). For continuation phase (CP) (16 weeks) only Pyrazinamide is stopped.

For previously treated cases of TB the IP is of 12 weeks, where injection streptomycin (S) is stopped after 8 weeks and the remaining 4 drugs (HRZE) are continued for another 4 weeks. There is no need for extension of IP. Pyrazinamide (Z) is stopped at the start of CP, while the rest of the drugs i.e. HRE are continued for another 20 weeks in the CP.

The CP in both new and previously treated cases is extended by 12 to 24 weeks in cases like CNS TB, Skeletal TB, Disseminated TB based on clinical decision of the treating physician.

Table 4 Drug sensitive TB treatment

Type of cases	Intensive phase	Continuation phase
New	HRZE (2 months)	HRE (4 months)
Previously treated	HRZES (2 months) + HRZE (1 months)	HRE (5 months)

MDR TB (without additional resistance)

These patients receive 6-9 months of IP with kanamycin (km), levofloxacin (Lfx), ethambutol (Eto), pyrazinamide (Z), ethionamide (E) and cycloserine (Cs) and 18 months of CP with levofloxacin (Lfx), ethambutol (Eto), ethionamide (E) and cycloserine (Cs).

Table 5 MDR TB treatment

Type of cases	Intensive phase	Continuation phase
Rifampicin resistant + Isoniazid sensitive or unknown	Km, Lfx, Eto, Cs, Z, E, H (6-9 months)	Lfx, Eto, Cs, Z, H (18 months)
MDR TB	Km, Lfx, Eto, Cs, Z, E (6-9 months)	Lfx, Eto, Cs, Z (18 months)

XDR TB

The XDR TB cases are treated with injection Capreomycin (Cm), Moxifloxacin (Mfx), Linezolid (Lz), Para Aminosalicylic acid (PAS), Clofazimine (Cfz), High dose Isoniazid (H) and Co-Amoxyclav (Amx/clv) for 6-12 months. In CP only injectable Capreomycin is stopped and remaining drugs are continued for another 18 months.

Table 6 XDR TB treatment

Type of cases	Intensive phase	Continuation phase
XDR TB	Cm, Mfx, Lz, PAS, Cfz, High dose H, Amx/ clv (6-12 months)	Cm, Mfx, Lz, PAS, Cfz, High dose H, Amx/ clv (18 months)

Bedaquiline (BDQ) conditional access programme: Introduction of new anti-TB drug under RNTCP.

This drug belongs to diarylquinoline class and targets ATP synthase. It has strong mycobactericidal activity. The drug has high volume and extensive tissue distribution with large affinity for plasma proteins. The drug is finally metabolized in liver. The drug has not demonstrated any cross resistance with existing first line and second line anti-TB drugs. It has significantly improved the time to culture conversion in MDR TB patients. WHO has recommended its use in conjunction with other WHO recommended MDR TB treatment.

RNTCP has introduced this drug at 6 sites in the country through conditional access programme.

MATERIALS AND METHODS

Study Design: Cross Sectional study

Source of Data

Patients with strong clinical suspicion of extra-pulmonary tuberculosis attending OPD and admitted in R L JALAPPA HOSPITAL AND RESEARCH CENTRE, TAMAKA, KOLAR from January 2015-August 2016 were included in this study.

Inclusion criteria

Patients with strong clinical suspicion of extra-pulmonary tuberculosis willing to give consent were included in the study.

Exclusion criteria

All cases already on Anti-tubercular Therapy or had been confirmed as having tuberculosis was excluded from study.

Sample size

A standard protocol was prepared and data related to socio-demographic features, occupation, history of presenting illness, other systemic illness, therapy received were

recorded as per the proforma. A total of 71 samples were collected during the period of January 2015-August 2016 (1 year 8 months).

Statistical analysis

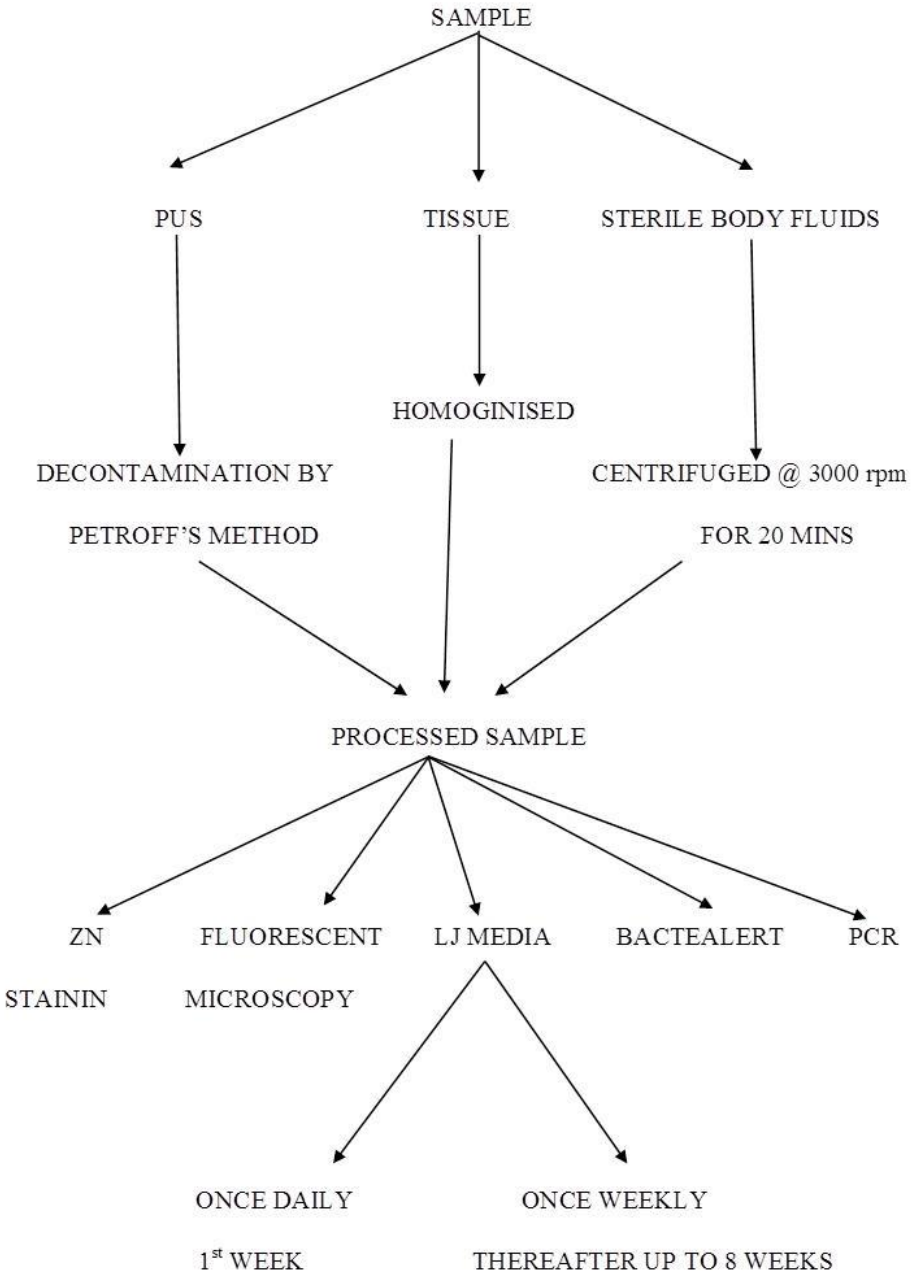
Statistical analysis was done using 'SPSS 22' software and the sensitivities, specificities, positive predictive values, negative predictive values were calculated considering LJ culture as gold standard. Cohen's kappa value was also obtained to assess the reproducibility and level of agreement between the PCR assay and other diagnostic tests employed

The clinical specimens included in this study were pus (15), endometrial biopsy (14), lymph node aspirate (10), peritoneal fluid (9), pleural fluid (7), tissue (5), CSF (5), synovial fluid (3), urine (3).

Depending on the nature of samples, concentration methods were employed. Sediments thus obtained were divided in 3 aliquots. One aliquot was used for microscopy that is ZN staining and fluorescent staining, second aliquot was used for inoculation on LJ media and in BacT ALERT culture bottle and third aliquot was used for PCR.

The tests were conducted in following order.

Processing of sample



Procedures

Processing of sample:

Procedure of processing tissue/biopsy sample:

Sterile homogenizer & 9% saline was used to homogenize tissue. If tissue has been collected & processed aseptically, homogenate was inoculated directly on LJ media & liquid media using aseptic technique.

If tissue has not been collected or handled aseptically or if in doubt, the homogenate was decontaminated using modified Petroff's method & sediment thus obtained was used for microscopy, inoculated on LJ media & liquid culture; & used for PCR assay.

Procedure of processing pus sample:

If pus was thick or collected & handled aseptically, then it was decontaminated by modified Petroff's method. The sediment thus obtained was used for microscopy, inoculated on LJ media & liquid culture; & used for PCR assay.

Procedure of processing body fluids:

The decontamination was not required for body fluids like cerebro-spinal fluid, peritoneal fluid, pleural fluid, synovial fluid or in case of bone marrow aspirate. The sample was centrifuged @ 3000 rpm for 15mins. Supernatant was discarded and deposit was used for microscopy, inoculated on LJ media & liquid culture; & used for PCR assay.

Decontamination: Modified Petroff's method-

Sample was transferred to McCartney bottle

↓

Equal volume of 4% NaOH added

Incubated @ 37^0 ↓ for 20 mins

Sterile distilled water added & centrifuge @ 3000 rpm for 20 mins

Supernatant discarded ↓

Sediment used for microscopy, inoculation on LJ media & liquid culture; & for PCR

Microscopic examination:

Smear preparation

Loopful /drop of specimen deposit was transferred to clean glass slide

↓

Spread over with the help of sterile loop

↓

Air dried smear then heat fixed and used for

↙ ↘

Zeihl-Neelsen staining | Fluorescent staining

Zeihl-Neelsen staining:

Heat fixed smear flooded with 1% carbol fuchsin

For 5-7 mins ↓ With intermittent heating till the vapours rise, 3-5 times

Slide washed in tap water & decolorized with 20% sulphuric acid

For 2-4 mins ↓

Slide washed in tap water

(Decolourisation repeated until smear became colourless)

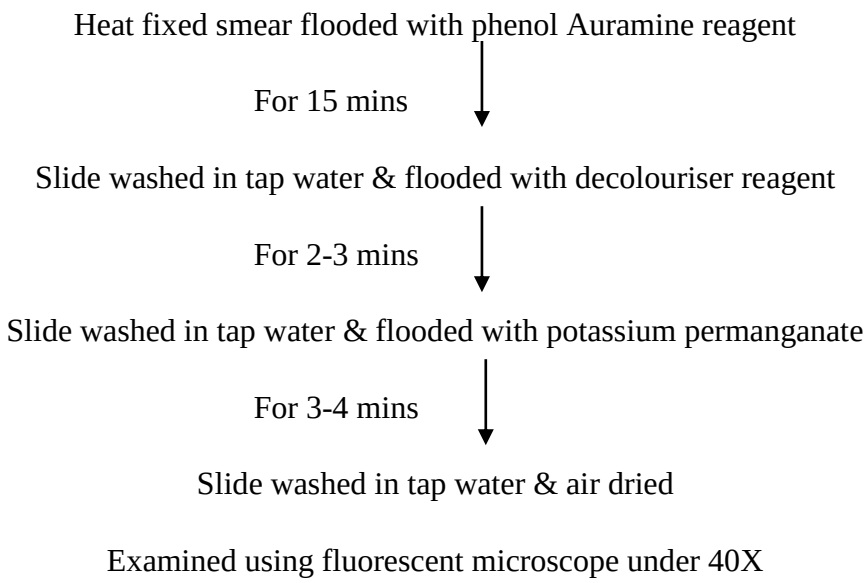
Smear counterstained with 0.1% methylene blue reagent

For 30 secs ↓

Slide washed & air dried

Examined using light microscope under 40X & then under 100X

Fluorescent staining:



Procedure for culture

One aliquot of sediment obtained after initial processing of sample was used to inoculate on LJ media and in BacT ALERT MP bottle. The LJ bottle was incubated at 37^0C and monitored once daily in 1st week and once weekly thereafter up to 8 weeks.

The BacT ALERT MP bottle top was cleaned with alcohol soaked cotton ball. Aseptically 0.5ml of reconstitution fluid added to bottle in case of sterile specimen and in case of non-sterile specimen like tissue/ biopsy, 0.5ml of mycobacteria antibiotic solution (containing Polymixin B, Amphotericin B, Nalidixic acid, Trimithoprim, Azlocillin, Vancomycin and a bulking agent) was added to the bottle. The inoculated culture bottle was loaded in BacT ALERT 3D system for the incubation and monitoring the growth of mycobacteria. All the samples positive by BacT ALERT 3D system were confirmed by PCR assay.

Procedure of Polymerase Chain Reaction

The procedure has 3 steps-

1. DNA extraction
2. DNA amplification
3. DNA detection

DNA extraction

Method: The DNA was extracted by Qiagen Spin Column method

Sample was centrifuged at 300 x g / 190 rpm for 5 min

Added 100 µL of sample sediment into a 1.5 ml micro centrifuge tube

Added 20 µL of Proteinase K

Adjusted the volume to 220 µL with PBS

Added 200 µL Buffer AL.

Mixed thoroughly by vortexing

Incubated samples at 56^0C for 10 min

Added 200 µL ethanol (96-100%)

Mixed thoroughly by vortexing

Pipetted the mixture into a DNeasy Mini spin column placed in a 2 ml collection tube.

Centrifuged at 6000 x g (8000 rpm) for 1 min

Discarded the fluid along with collection tube

Placed the spin column in a new 2 ml collection tube

Added 500 µL Buffer AW1

Centrifuged for 1 min at ≥ 6000 x g

Discarded the fluid along with collection tube

Placed the spin column in a new 2 ml collection tube

Added 500 µL Buffer AW2, and centrifuged for 3 min at 20,000 x g (14,000 rpm)

Discarded the fluid along with collection tube

Transferred the spin column to a new 1.5 ml micro centrifuge tube

Eluted the DNA by adding 200 µL Buffer AE to the center of the spin column membrane

Incubated for 1 min at room temperature (15-25 ^{0}C)

Centrifuged for 1 min at ≥ 6000 x g

Stored the extracted DNA at - 80 ^{0} C

DNA Amplification: Master mix preparation

Target gene: IS 6110 (245 base pairs)

The species specific primer sequences used for M. tuberculosis complex IS6110 TB PCR were:

Forward primer: 5’ CGT GAG GGC ATC GAG GTG GC 3’

Reverse primer: 5’ GCG TAG GCG TCG GTG ACA AA 3’

Different master mix combinations were prepared and tested on DNA extracted (Qiagen method) from H37Rv strain of M TB. This was taken as positive control for the PCR assay. We got a distinct band at 245 bp using following master mix reagents-

Standardized Master mix: Reaction volume 25 µL

Table 7 Composition of standardized master mix

Reagents	**Volume in µL**
Forward primers FP	1
Reverse primers RP	1
PCR Buffer	2.5
$MgCl_2$	1
dNTP ‘s	2.5
Taq polymerase	0.5
Distilled water (RNAase/ DNAase free)	15
Extracted DNA	1.5
Total	**25**

Selected the appropriate reaction volume depending upon the number of clinical samples, positive control, negative control

- Transferred 23.5 µL of Master mix volume to the sterilized and labelled PCR tubes (0.2ml micro centrifuge tube)

- Added 1.5 µL of extracted DNA from clinical samples, from positive control and from negative control to the respective PCR tubes so that final reaction mixture was 25 µL.
- Mixed the contents with vortexing and subjected to thermocycler.

DNA amplification: Thermocycler **(figure 9)**

The cycling Parameters for M. tuberculosis complex IS6110 TB PCR were as follows

Table 8 Temperature and duration of steps of amplification of PCR

Initial denaturation	94^0C for 5 minutes	
Denaturation	94^0C for 30 seconds	35 cycles
Annealing	55^0C for 30 seconds	
Extension	72^0C for 45 seconds	
Final extension	72^0C for 10 minutes	

Detection of Amplification products

Detected the amplified products on 2% Agarose Gel Electrophoresis (figure 10) and visualized by ethidium bromide (0.04µL/ml) under ultra violet illumination.(figure 11)

Weighed 2 grams of Agarose

Added 100 ml of 1 X TAE Buffer & mixed

Heated the solution mixture in a micro oven for 5 mins

Cooled the solution to 60 ^{0} C and added 4 µL Ethidium bromide

Mixed well

Poured the solution into the Gel apparatus tray which was sealed at 2 sides by Insulation Tape

Placed the combs gently in the Gel matrix

Allowed the gel to set for 15 -30 mins at Room Temperature

Placed the gel along with comb in the Electrophoresis tank filled with TAE buffer

Covered the gel with TAE buffer to a depth of 1mm

Removed the combs gently from Gel matrix after 30 mins

Added molecular weight marker (DNA ladder) to the first well

Mixed 10µl of amplified DNA products with 3 µl gel loading buffer on a paraffin film paper

Added 10µl of mixture in the appropriate well

Ran rlectrophoresis for 60 mins at 100 volts

Removed the gel matrix from the tank gently & place it on UV Illuminator and Gel Doc System

Analyzed the product size (245bp) using DNA ladder as a Reference Standard

Photographed and documented the results

RESULTS

In the present study, 71 clinical specimens from patients with strong clinical suspicion of extra-pulmonary tuberculosis attending OPD and admitted in R L Jalappa hospital and research centre, Tamaka, Kolar were processed as per the methodology and their results were compiled and analyzed.

Table 9 Sex wise distribution of the 71 patients (n=71)

Gender	Number	Percentage
Males	40	56%
Females	31	44%

Table 9- A total of 71 cases were studied of which 40 (56%) were male and 31 (44%) were females.

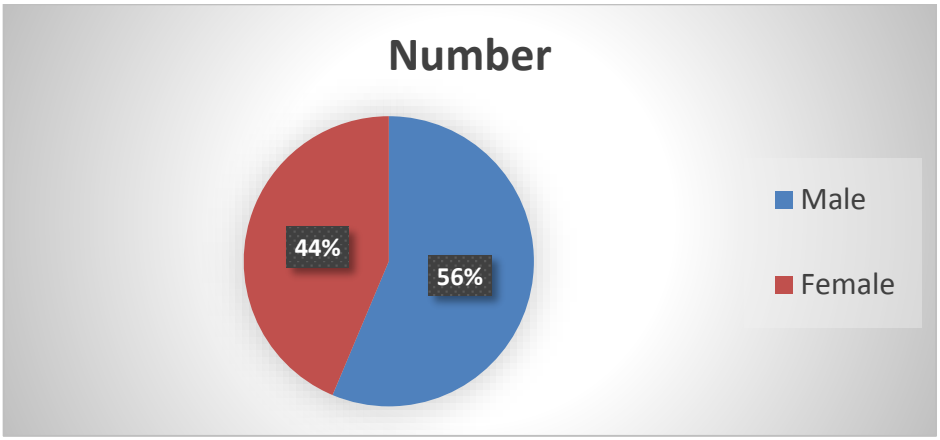


Figure 2 Sex wise distribution

Table 10 Age wise distribution of patients

Age group	Number	Percentage
0-10	1	1.4
11-20	8	11.2
21-30	15	21.1
31-40	17	23.9
41-50	8	11.2
51-60	14	19.7
61-70	6	8.4
71-80	2	2.8

Table 10- The majority of the patients belonged to the age group of 31-40 years accounting for 23.9% followed by 21-30 years age group accounting for 21.1%. The least number of patients belonged to the age group of 0-10 years (1.4%)

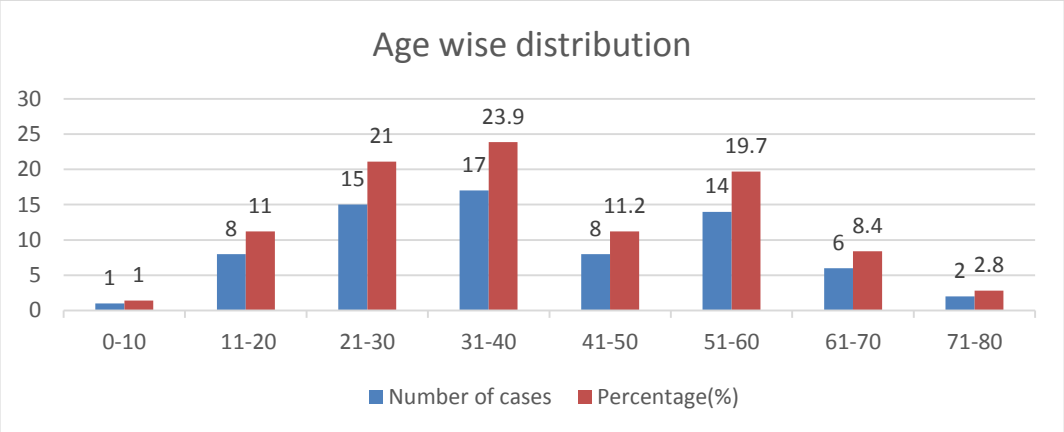


Figure 3 Age wise distribution of patients

Table 11- Distribution of clinical specimens from the 71 patients processed

Nature of specimen	No of specimens	Percentage
Pus	15	21.1
Endometrial Biopsy	14	19.7
Lymph node aspirate	10	14
Peritoneal fluid	9	12.6
Pleural fluid	7	9.8
CSF	5	7
Tissue	5	7
Synovial fluid	3	4.2
Urine	3	4.2

The majority of specimens in study were pus (21.1%), followed by endometrial biopsy (19.7%).

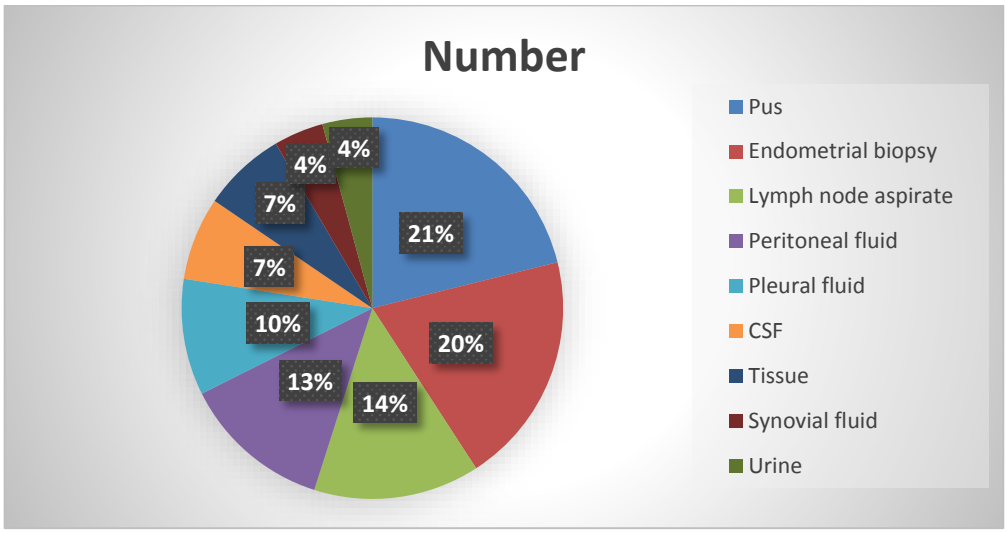


Figure 4 Distribution of clinical specimens

Analysis of individual specimen type

Table 12 Distribution of pus samples

SI No	Age/Sex	Specimen	Staining methods		Culture methods		
			ZN	AR	LJ	BacT	PCR
1	36/M	Pus(tuberculoma)	-ve	-ve	-ve	-ve	-ve
2	44/F	Pus(scalp wound)	+ve	+ve	-ve	-ve	-ve
3	60/M	Pus(Epididymo-orchitis)	-ve	-ve	-ve	-ve	-ve
4	56/M	Pus(Pott'spine)	-ve	-ve	-ve	-ve	-ve
5	22/M	Pus(discharge from wound-in situ nail)	-ve	-ve	-ve	-ve	-ve
6	35/M	Pus(Pott'spine)	-ve	-ve	-ve	-ve	-ve
7	1.5/F	Pus(injection abscess)	+ve	+ve	-ve	-ve	-ve
8	25/F	Pus(Pott'spine)	+ve	+ve	-ve	-ve	-ve
9	50/F	Pus(Pott'spine)	-ve	-ve	-ve	-ve	-ve
10	48/M	Pus(injection abscess)	+ve	+ve	+ve	+ve	-ve
11	43/M	Pus	-ve	-ve	-ve	-ve	-ve
12	35/M	Pus (Pott'spine)	-ve	-ve	+ve	-ve	-ve
13	41/M	Pus(Pott'spine)	-ve	-ve	-ve	+ve	+ve
14	22/M	Abscess from neck	-ve	-ve	-ve	-ve	-ve
15	58/M	Pus(Pott'spine)	+ve	+ve	+ve	+ve	+ve

Table 12- shows the total number of pus samples processed. Out of 15 samples, one sample from Pott's spine was positive by all methods, 3 samples were positive only by ZN and Fluorescent microscopy. One sample was positive by ZN and Fluorescent microscopy, LJ culture and BacT ALERT culture. One sample was positive by BacT ALERT culture and PCR. One sample was positive only by LJ culture.

Table 13- Distribution of Endometrial biopsy samples

SI No	Age/Sex	Specimen	Staining methods		Culture methods		
			ZN	AR	LJ	BacT	PCR
1	33/F	Endometrial biopsy	-ve	-ve	-ve	-ve	-ve
2	30/F	Endometrial biopsy	-ve	-ve	-ve	-ve	-ve
3	28/F	Endometrial biopsy	-ve	-ve	-ve	-ve	-ve
4	20/F	Endometrial biopsy	-ve	-ve	-ve	-ve	-ve
5	25/F	Endometrial biopsy	-ve	-ve	-ve	-ve	-ve
6	29/F	Endometrial biopsy	-ve	-ve	-ve	-ve	-ve
7	28/F	Endometrial biopsy	-ve	-ve	-ve	-ve	-ve
8	23/F	Endometrial biopsy	-ve	-ve	-ve	-ve	-ve
9	27/F	Endometrial biopsy	-ve	-ve	-ve	-ve	-ve
10	36/F	Endometrial biopsy	-ve	-ve	-ve	-ve	-ve
11	35/F	Endometrial biopsy	-ve	-ve	-ve	-ve	+ve
12	35/F	Endometrial biopsy	-ve	-ve	-ve	-ve	-ve
13	33/F	Endometrial biopsy	-ve	-ve	-ve	-ve	-ve
14	30/F	Endometrial biopsy	-ve	-ve	-ve	-ve	-ve

Table 13 - shows the total number of endometrial biopsy samples processed. Out of 14 samples none was positive by microscopy or culture. Only one sample was positive by PCR.

Table 14 Distribution of Lymph Node aspirate

SI No	Age/Sex	Specimen	Staining methods		Culture methods		
			ZN	AR	LJ	BacT	PCR
1	60/M	Lymph Node aspirate	+ve	+ve	-ve	+ve	+ve
2	40/F	Lymph Node aspirate	-ve	-ve	-ve	-ve	-ve
3	56/F	Lymph Node aspirate	-ve	-ve	+ve	+ve	+ve
4	20/M	Lymph Node aspirate	-ve	-ve	-ve	-ve	-ve
5	18/M	Lymph Node aspirate	-ve	-ve	-ve	-ve	-ve
6	54/F	Lymph Node aspirate	-ve	-ve	-ve	-ve	-ve
7	37/M	Lymph Node aspirate	-ve	-ve	-ve	-ve	-ve
8	47/F	Lymph Node aspirate	-ve	-ve	-ve	-ve	+ve
9	20/M	Lymph Node aspirate	-ve	-ve	-ve	-ve	-ve
10	20/M	Lymph Node aspirate	+ve	+ve	+ve	+ve	+ve

Table 14- Shows number of lymph node aspirate samples processed. Out of 10 samples processed one sample was positive by all methods, one sample was positive by Microscopy, BacT ALERT culture and PCR. Another sample was positive by both culture methods and PCR; one sample was positive only in PCR assay.

Table 15 Distribution of Pleural fluid

SI No	Age/Sex	Specimen	Staining methods		Culture methods		
			ZN	AR	LJ	BacT	PCR
1	35/M	Pleural fluid	-ve	-ve	-ve	+ve	+ve
2	27/M	Pleural fluid	-ve	-ve	-ve	+ve	+ve
3	37/M	Pleural fluid	-ve	-ve	-ve	-ve	-ve
4	18/M	Pleural fluid	-ve	-ve	-ve	-ve	-ve
5	38/M	Pleural fluid	-ve	-ve	-ve	-ve	-ve
6	61/M	Pleural fluid	-ve	-ve	-ve	-ve	-ve
7	55/M	Pleural fluid	-ve	-ve	-ve	-ve	-ve

Table 15- shows number of pleural fluid samples processed. Out of 7 samples 2 were positive by Bac T ALERT culture and PCR.

Table 16 Distribution of Tissue samples

SI No	Age/Sex	Specimen	Staining methods		Culture methods		PCR
			ZN	AR	LJ	BacT	
1	60/F	Tissue (Tuberculoma)	-ve	-ve	-ve	-ve	-ve
2	20/M	Tissue(Pott's spine)	-ve	-ve	-ve	-ve	-ve
3	60/M	Tissue(wound-chronic osteomyelitis)	+ve	+ve	-ve	+ve	-ve
4	70/M	Tissue(middle ear Tb)	-ve	-ve	-ve	-ve	-ve
5	11/F	Tissue(mastoid Tb)	-ve	-ve	-ve	-ve	-ve

Table 16- A total of 5 tissue samples were processed of which only one sample was positive by ZN and Fluorescent microscopy; and BacT ALERT culture.

Table 17 Distribution of Synovial fluid

SI No	Age/Sex	Specimen	Staining methods		Culture methods		
			ZN	AR	LJ	BacT	PCR
1	40/F	Synovial fluid	-ve	-ve	+ve	+ve	+ve
2	65/M	Synovial fluid	-ve	-ve	-ve	-ve	-ve
3	47/M	Synovial fluid	-ve	-ve	-ve	-ve	-ve

Table 17- shows number of Synovial fluid samples processed. Out of 3 samples processed one sample was positive by LJ culture, BacT ALERT culture and PCR.

Table 18 Distribution of Peritoneal fluid

SI No	**Age/Sex**	**Specimen**	**Staining methods**		**Culture methods**		
			ZN	AR	LJ	BacT	PCR
1	23/F	Peritoneal fluid	-ve	-ve	-ve	-ve	-ve
2	73/M	Peritoneal fluid	-ve	-ve	-ve	-ve	-ve
3	60/M	Peritoneal fluid	-ve	-ve	-ve	-ve	-ve
4	26/F	Peritoneal fluid	-ve	-ve	-ve	-ve	-ve
5	23/F	Peritoneal fluid	-ve	-ve	-ve	-ve	-ve
6	70/M	Peritoneal fluid	-ve	-ve	-ve	-ve	-ve
7	39/M	Peritoneal fluid	-ve	-ve	-ve	-ve	-ve

8	65/M	Peritoneal fluid	-ve	-ve	-ve	-ve	-ve
9	65/F	Peritoneal fluid	-ve	-ve	-ve	-ve	-ve

Table 18- shows number of peritoneal fluid samples processed. Out of 9 samples processed none of the samples were positive by any of the five methods.

Table 19 Distribution of CSF

SI No	Age/Sex	Specimen	Staining methods		Culture methods		
			ZN	AR	LJ	BacT	PCR
1	60/M	CSF	-ve	-ve	-ve	-ve	-ve
2	54/M	CSF	-ve	-ve	-ve	-ve	-ve
3	40/M	CSF	-ve	-ve	-ve	-ve	-ve
4	80/M	CSF	-ve	-ve	-ve	-ve	-ve
5	45/M	CSF	-ve	-ve	-ve	-ve	-ve

Table 19- shows number of CSF samples processed. Out of 5 samples processed none of the samples were positive by any of the five methods.

Table 20 Distribution of Urine

SI No	Age/Sex	Specimen	Staining methods		Culture methods		
			ZN	AR	LJ	BacT	PCR
1	35/F	Urine	-ve	-ve	-ve	-ve	-ve
2	58/F	Urine	-ve	-ve	-ve	-ve	-ve
3	60/M	Urine	-ve	-ve	-ve	-ve	-ve

Table 20-A total of 3 urine samples were processed of which none of the samples were positive by any of the five methods.

Table 21 Distribution of samples which were positive by one or more methods

SI no	Age/ Sex	Specimen	Staining method		Culture methods		
			ZN	AR	LJ	BacT	PCR
1	44/F	Pus(scalp wound)	+ve	+ve	-ve	-ve	-ve
2	41/M	Pus(Pott's spine)	-ve	-ve	-ve	+ve	+ve
3	35/M	Pleural fluid	-ve	-ve	-ve	+ve	+ve
4	60/M	Lymph node aspirate	+ve	+ve	-ve	+ve	+ve
5	27/M	Pleural fluid	-ve	-ve	-ve	+ve	+ve
6	35/F	Endometrial biopsy	-ve	-ve	-ve	-ve	+ve
7	47/F	Lymph node aspirate	-ve	-ve	-ve	-ve	+ve
8	60/M	Tissue(wound-chronic osteomyelitis)	+ve	+ve	-ve	+ve	-ve
9	40/F	Synovial fluid	-ve	-ve	+ve	+ve	+ve
10	1.5/F	Pus(injection abscess)	+ve	+ve	-ve	-ve	-ve
11	58/M	Pus(Pott's spine)	+ve	+ve	+ve	+ve	+ve
12	56/F	Lymph node aspirate	-ve	-ve	+ve	+ve	+ve
13	20/M	Lymph node aspirate	+ve	+ve	+ve	+ve	+ve
14	25/F	Pus(Pott's spine)	+ve	+ve	-ve	-ve	-ve
15	48/M	Pus(injection abscess)	+ve	+ve	+ve	+ve	-ve
16	35/M	Pus (abscess)	-ve	-ve	+ve	-ve	-ve
	Total number of positive cases		8	8	6	10	10

Table 21- Total 71 samples were processed out of which, 16 (22.5%) samples were positive by one or more methods employed for the detection of acid fast bacilli. A total of 8 samples were positive by ZN staining and fluorescent staining, 6 were positive by LJ culture, 10 were positive by BacT ALERT culture and 10 were positive by PCR.

Table 22 positivity rate of individual method employed in detection of M TB

Sl No	**Diagnostic test**	**No. positive**	**Positivity rate (%)**
	ZN staining	8	11.26
2	Fluorescent microscopy	8	11.26
3	LJ culture	6	8.45
4	BacT ALERT culture	10	14.08
5	PCR assay	10	14.08

Table 22- The positivity rates by microscopy, LJ culture, BacT ALERT culture and PCR were 11.26%, 8.45%, 14.08% and 14.08% respectively.

Table 23 sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) of ZN staining

		LJ culture				
		+ve	-ve	Total	Sensitivity	50%

ZN staining	+ve	3	5	8	Specificity	92.3%
	-ve	3	60	63	PPV	37.5%
		6	65	71	NPV	95.2%

Table 23- shows a sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) of ZN staining was 50%, 95.08%, 37.5% and 95.2% respectively when compared with LJ culture as gold standard.

Table 24 sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) of Fluorescent staining

		LJ culture				
		+ve	-ve	Total	Sensitivity	50%
Fluorescent staining	+ve	3	5	8	Specificity	92.3%
	-ve	3	60	63	PPV	37.5%
		6	65	71	NPV	95.2%

Table 24- shows a sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) of fluorescent staining was 50%, 95.08%, 37.5% and 95.2% respectively when compared with LJ culture as gold standard.

Table 25 sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) of BacT ALERT culture

		LJ culture			

		+ve	-ve	Total	Sensitivity	83.3%
BacT ALERT culture	+ve	5	5	10	Specificity	92.3%
	-ve	1	60	61	PPV	50%
		6	65	71	NPV	98.4%

Table 25- shows a sensitivity of 83.3%, specificity of 92.3%, positive predictive value (PPV) of 50%, negative predictive value (NPV) 98.36% of ZN staining when compared with LJ culture taken as gold standard.

Table 26 sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) of PCR assay

		LJ culture		Total		
		+ve	-ve		Sensitivity	66.7%
PCR	+ve	5	3	8	Specificity	90.8%
	-ve	5	58	63	PPV	40%
		10	61	71	NPV	96.7%

Table 26- shows a sensitivity of 50%, specificity of 95.08%, positive predictive value (PPV) of 62.5%, negative predictive value (NPV) 92.06% of ZN staining when compared with LJ culture as gold standard.

Table 27 Number of positives by LJ and BacT ALERT culture from different clinical samples

SI No	Sample	No of sample processed	LJ culture (%)	BacT ALERT (%)
1	Synovial fluid	3	1 (33.3%)	1 (33.3%)
2	Lymph node aspirate	10	2 (20%)	3 (30%)
3	Pleural fluid	7	0	2 (28.57%)
4	Pus	15	3 (20%)	2 (13.33%)
5	Tissue	5	0	0
6	Endometrial Biopsy	14	0	0
7	CSF	5	0	0
8	Peritoneal fluid	9	0	0
9	Urine	3	0	0

Table 27- Shows percentage of culture positives on LJ media and from different clinical samples. The percentage of isolation of M TB by BacT ALERT and LJ culture from synovial fluid was 33.3%, whereas in lymph node it was 30% by BacT ALERT compared to 20% by LJ culture. In pleural fluid, percentage of isolation by BacT ALERT was 28.57% whereas isolation by LJ was none; however, in pus samples, isolation percentage was higher by LJ culture (20%) compared to BacT ALERT (13.33%).

Table 28 time taken for LJ culture positivity

Sl. No.	Age/ Sex	Specimen	Time taken for positivity (days)
1	40/F	Synovial fluid	40
2	58/M	Pus(Pott's spine)	34
3	56/F	Lymph node aspirate	48
4	20/M	Lymph node aspirate	49
5	48/M	Pus(injection abscess)	05
6	35/M	Pus (abscess)	42

Table 28- Shows number of LJ culture positive and time taken for culture positivity. The mean detection time for culture positivity was 36.3 days.

Table 29 time taken for BacT ALERT culture positivity

Sl. No.	Age/ Sex	Specimen	Time taken for positivity (days)
	41/M	Pus(Pott's spine)	21
	35/M	Pleural fluid	17
	60/M	Lymph node aspirate	19
	27/M	Pleural fluid	18
	60/M	Tissue(wound-chronic osteomyelitis)	04
	40/F	Synovial fluid	14
	58/M	Pus(Pott's spine)	16
	56/F	Lymph node aspirate	20
	20/M	Lymph node aspirate	13
	48/M	Pus(injection abscess)	04

Table 29- Shows number of BacT ALERT culture positive and time taken for culture positivity. The mean detection time for culture positivity was 14.6 days.

Table 30 Comparison of recovery rate of Mycobacteria by LJ culture and BacT ALERT

Oraganism (n= 11)	**Recovery of Mycobacteria by**	
	LJ Culture (%)	**BacT ALERT**
M TB complex (9)	6 (66.66%)	8 (88.88%)
NMT (2)	1 (50%)	2 (100%)
Total (11)	7 (63.63%)	10 (90.9%)

Table 30 shows recovery rate of Mycobacteria by LJ culture and BacT ALERT. The recovery rate was higher by BacT ALERT culture (90.9%) compared to LJ culture (63.63%).

Calculation of kappa value-

The Cohen's kappa statistics was applied to assess the reproducibility and the level of agreement between the PCR assay and other diagnostic tests which were evaluated in this study. The data was analyzed using 'SPSS statistics 22' software.

Table 31 Comparison of PCR and Microcopy for calculating kappa value

		PCR		Total
		+ve	-ve	
Microscopy	+ve	3	5	8

	-ve	7	56	63
Total		10	61	71

Symmetric Measures					
		Value	Asymptotic Standardized Error[a]	Approximate T[b]	Approximate Significance
Measure of Agreement	Kappa	.238	.156	2.021	.043
N of Valid Cases		71			
a. Not assuming the null hypothesis.					
b. Using the asymptotic standard error assuming the null hypothesis.					

Table 31-In our study, there was ‘fair’ level of agreement between microscopy and PCR, with a kappa value of 0.238.

Table 32 Comparison of PCR and LJ culture for calculating kappa value

		PCR		Total
		+ve	-ve	
LJ culture	+ve	4	2	6
	-ve	6	59	65
Total		10	61	71

Symmetric Measures					
		Value	Asymptotic Standardized Error[a]	Approximate T[b]	Approximate Significance
Measure of Agreement	Kappa	.441	.163	3.870	.000
N of Valid Cases		71			
a. Not assuming the null hypothesis.					
b. Using the asymptotic standard error assuming the null hypothesis.					

Table 32-In our study, there was 'moderate' level of agreement between LJ culture and PCR, with a kappa value of 0.441.

Table 33 Comparison of PCR and BacT ALERT culture for calculating kappa value

		PCR		Total
		+ve	-ve	
BacT ALERT	+ve	4	2	6
	-ve	6	59	65
Total		10	61	71

Symmetric Measures					
		Value	Asymptotic Standardized Error[a]	Approximate T[b]	Approximate Significance
Measure of Agreement	Kappa	.767	.111	6.465	.000
N of Valid Cases		71			
a. Not assuming the null hypothesis.					
b. Using the asymptotic standard error assuming the null hypothesis.					

Table 33- In our study, there was 'substantial' level of agreement between microscopy and PCR, with a kappa value of 0.767.

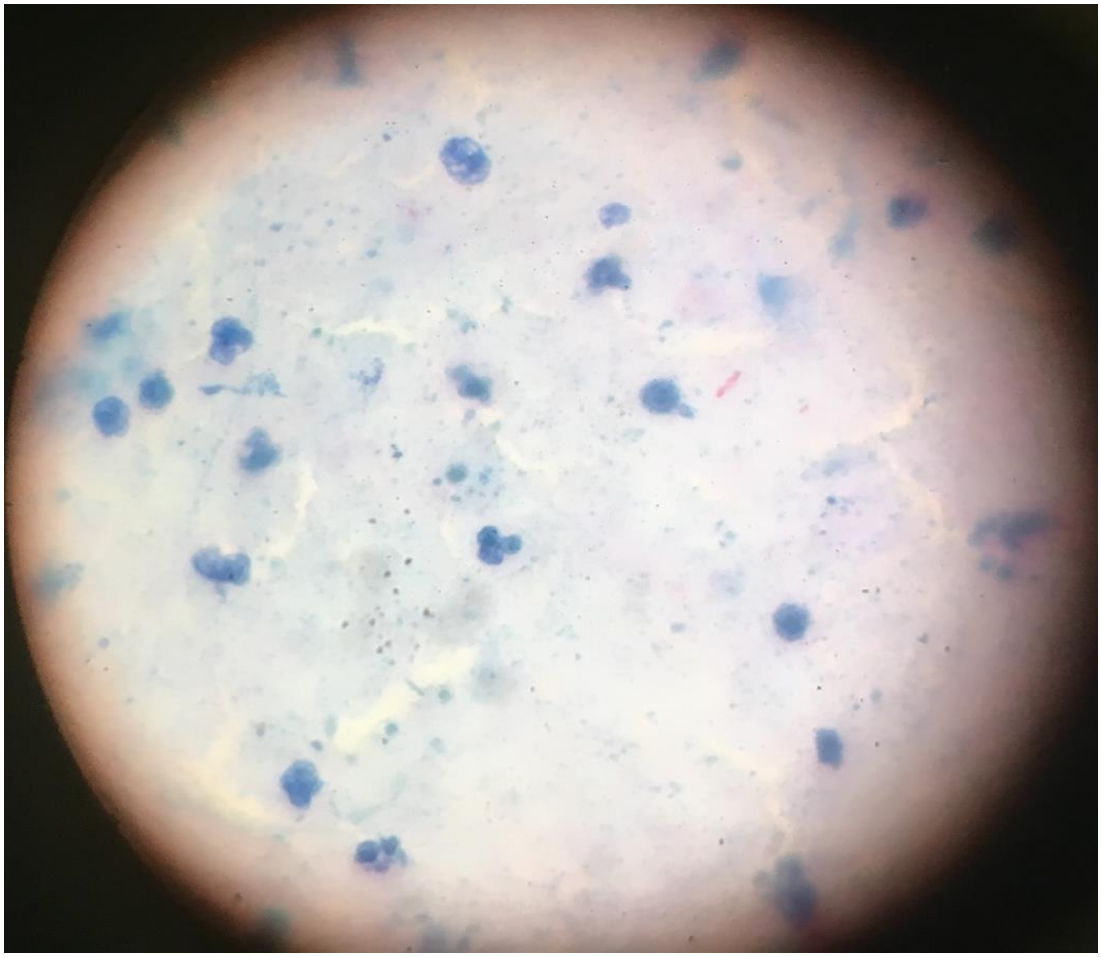


Figure 5 Ziehl Neelsen staing showing pink colored acid fast bacilli from pus sample.

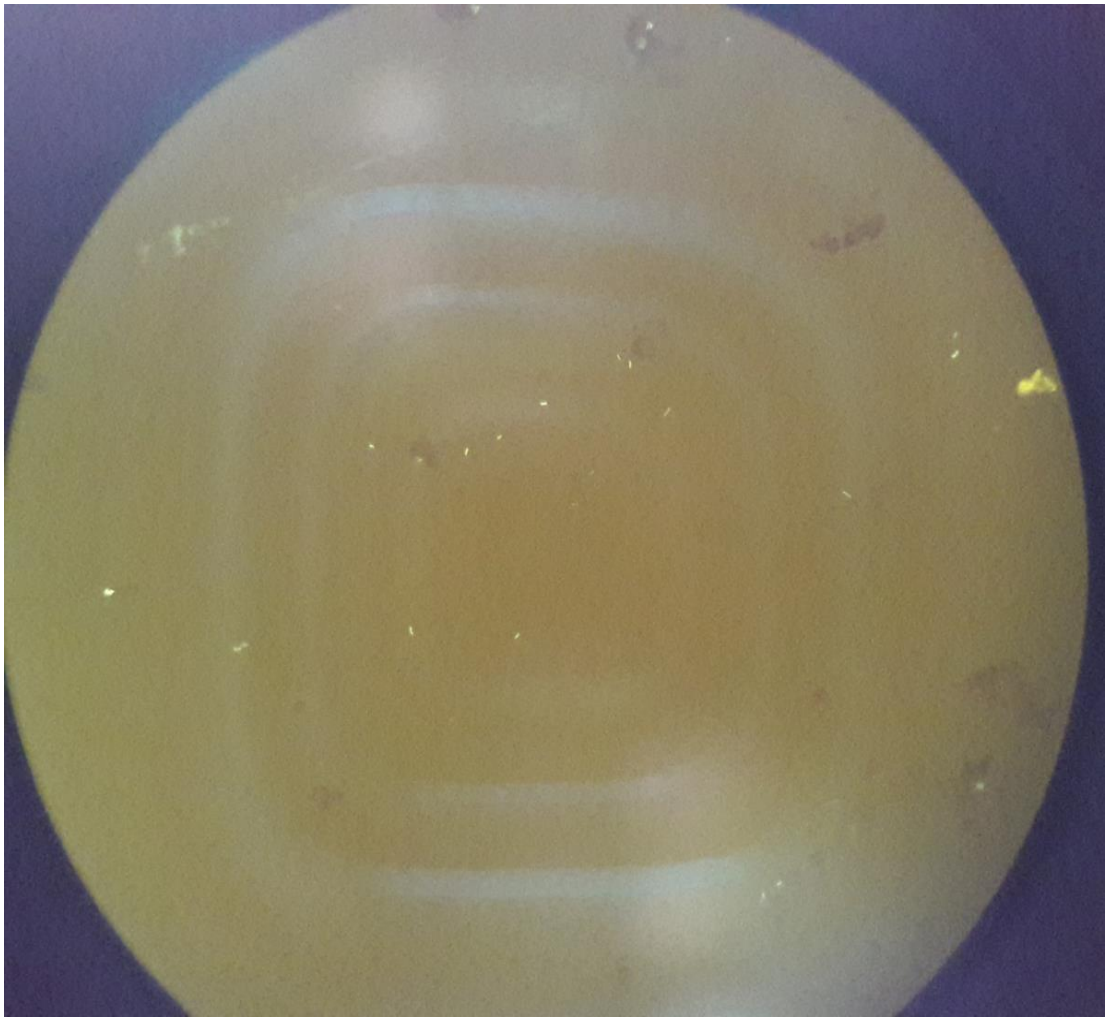


Figure 6 Fluorescent staining showing curved bacilli focused under 40X objective lens

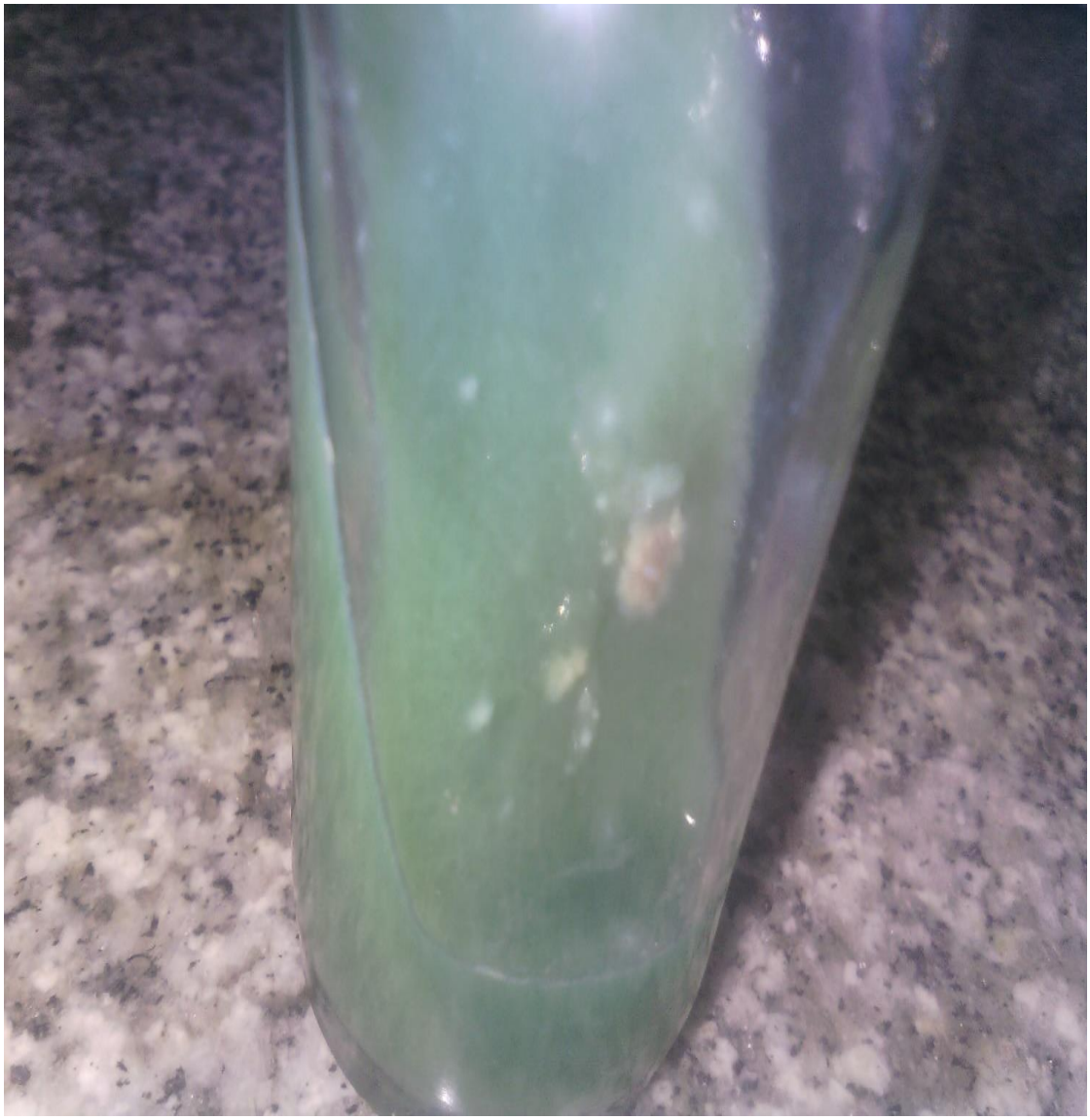


Figure 7 Characteristic rough, tough and buff colored colonies of M TB on LJ media.

Figure 8 BacT ALERT 3D liquid culture system

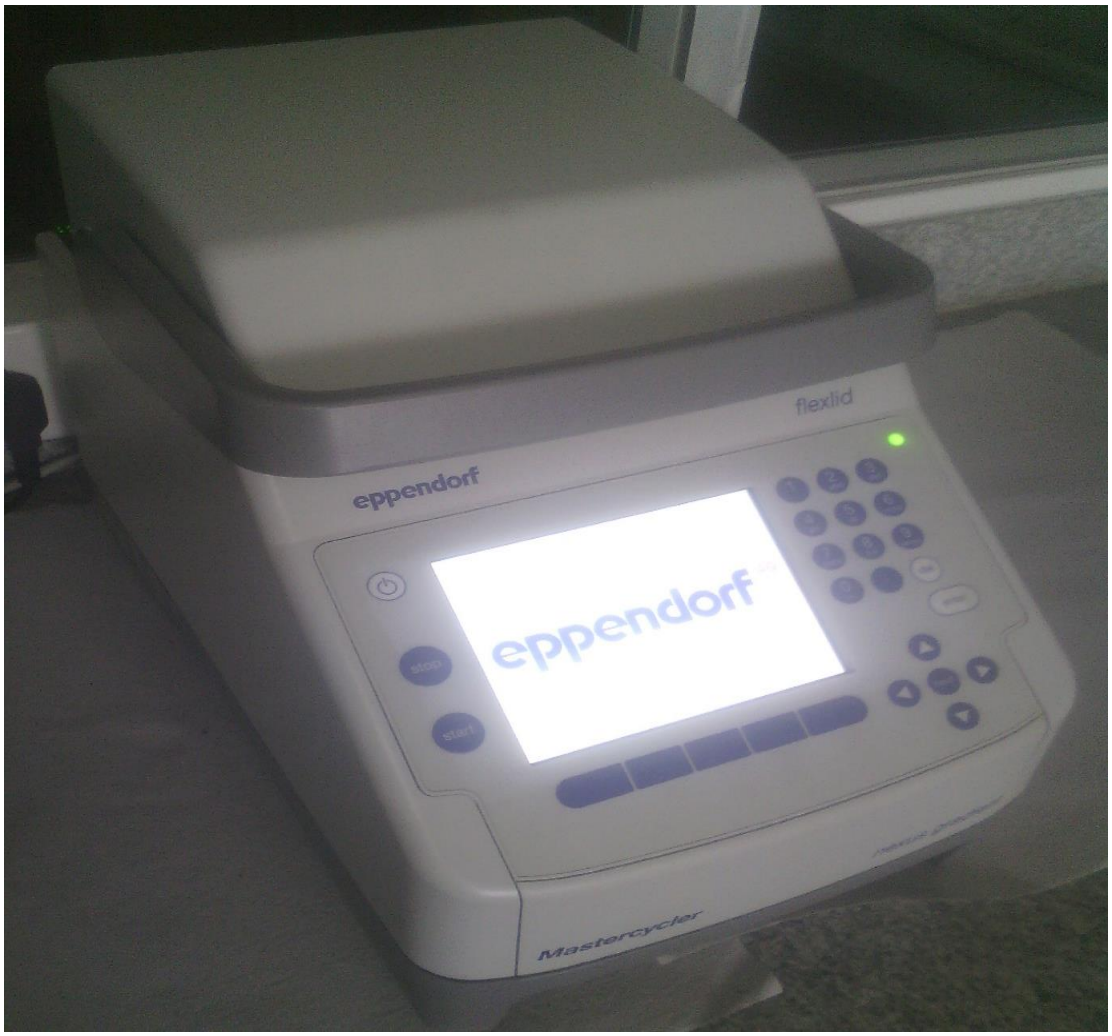


Figure 9 Thermocycler

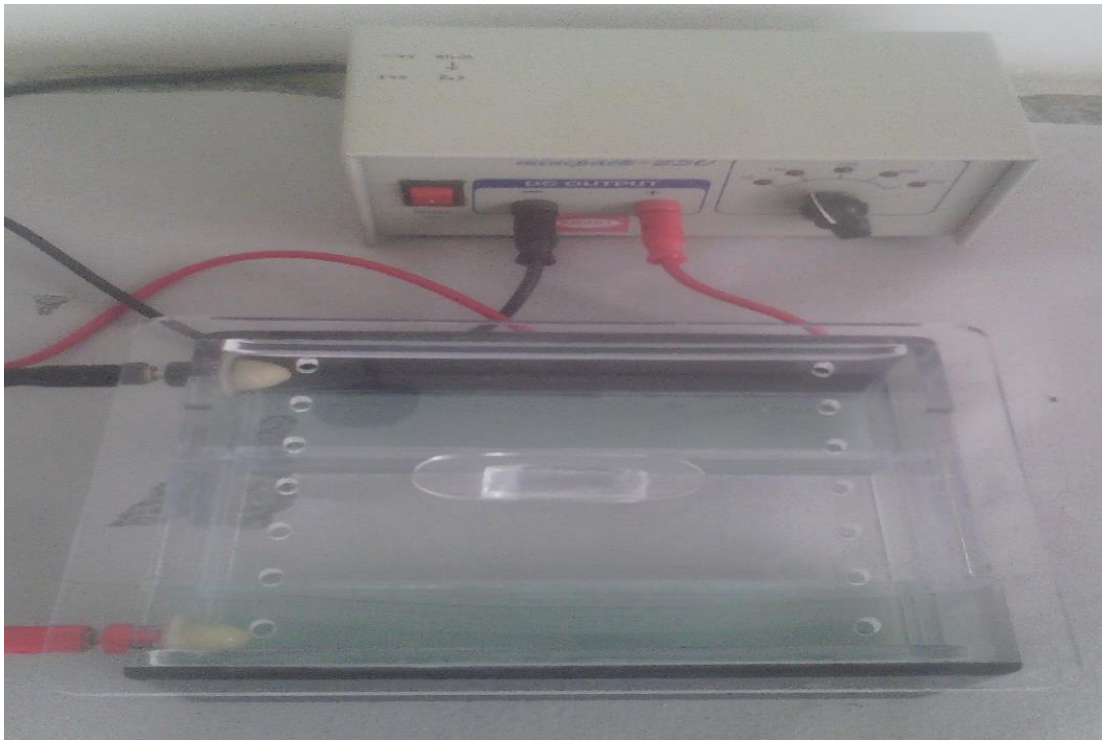


Figure 10 Gel electrophoresis apparatus

Figure 11 - UV illuminator

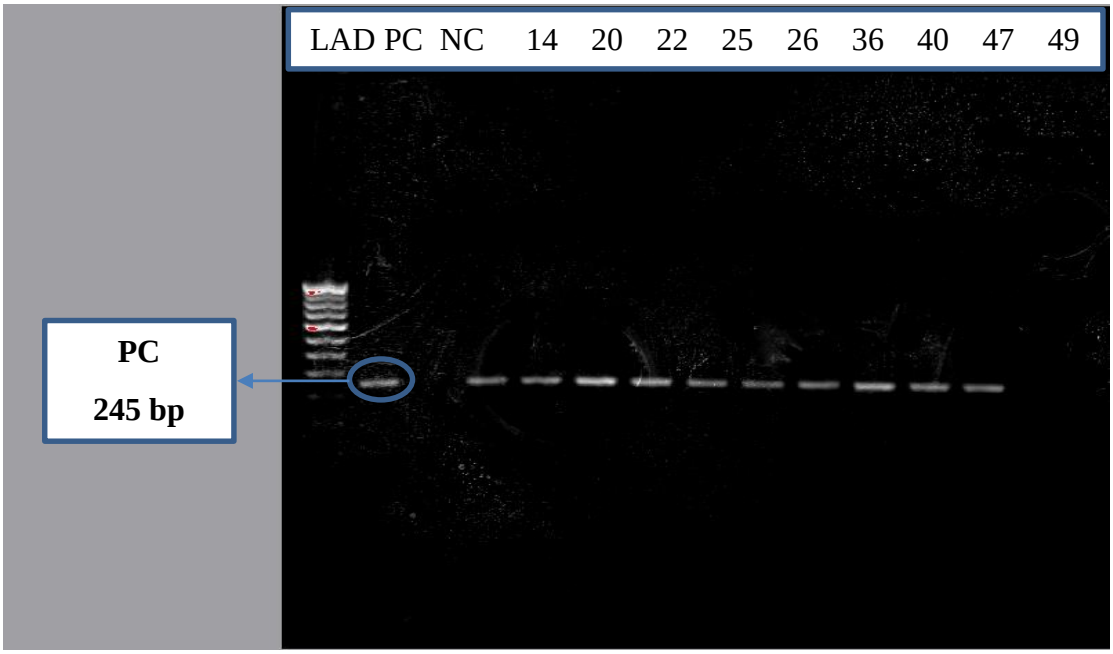


Figure 12 Showing Gel documentation picture of samples positive by PCR assay

(LAD- Ladder 100 bp, PC- positive control, NC- negative control)

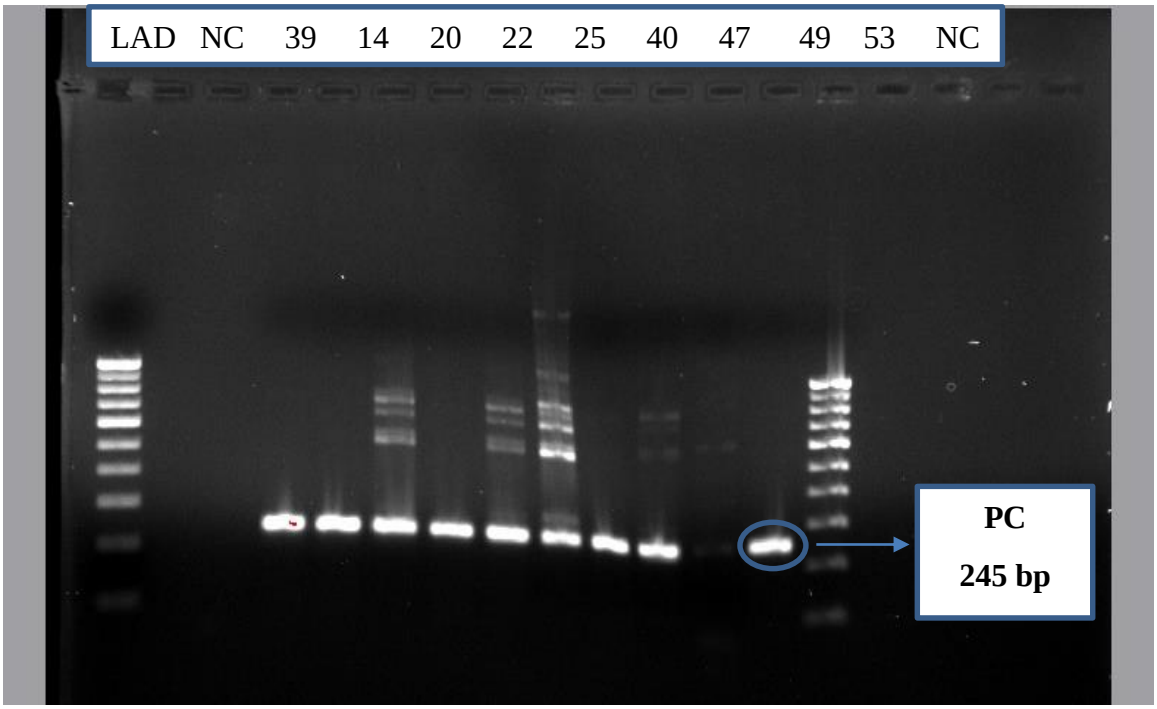


Figure 13 Gel documentation picture of PCR assay done on positive BacT ALERT

(LAD- Ladder 100 bp, PC- positive control, NC- negative control)

DISCUSSION

Tuberculosis is one of the oldest diseases affecting mankind. It is among the leading causes of morbidity and mortality worldwide. It is primarily a disease that affect lungs and cause pulmonary tuberculosis but can affect any organ in the body manifesting as extrapulmonary tuberculosis[71]. In clinical practice the diagnosis of EPTB is difficult because of its non-specific, misleading and variety of clinical manifestations[8]. Microbiological investigations used in most of the clinical laboratories for detection of AFB are microscopy and culture. However, the sensitivity of microscopy and culture on LJ media are low in EPTB owing to its paucibacillary nature. Culture is gold standard for confirming the diagnosis of M TB infection but it is time consuming, requiring 6-8 weeks[72]. Recent introduction of liquid culture has reduced the time taken for culture positivity and also increased the rate of isolation of M TB[36]. The role of PCR in early diagnosis of EPTB has been evaluated with the hope of shortening the time required for diagnosis of EPTB.

In the present study, 71 clinical specimens from patients with strong clinical suspicion of extra-pulmonary tuberculosis attending OPD and also admitted in R L Jalappa hospital and research centre, Tamaka, Kolar from January 2015-August 2016 were included. The clinical samples were processed and evaluated by ZN staining, fluorescent microscopy, LJ culture, BacT ALERT culture and PCR.

In our study there were 40 (56%) males and 31 (44%) females. It shows male preponderance with female:male ratio of 1:1.27 (table 9, figure-2). Hajia M et al has reported 68.5% of their subjects were males while 51.5% females which is comparable

to our study[72]. In studies done by Sharma S K et al [9], Arora V K and Gupta R et al[73] showed female preponderance in cases of extrapulmonary tuberculosis.

The majority of the patients belonged to the age group of 31-40 years accounting for 23.9% followed by 21-30 years age group accounting for 21.1% (Table-10, figure-3). This observation correlates with studies conducted by Sharma S K et al [9], Arora V K et al[73], Musellim et al [74] indicating that younger age groups are at a higher risk of EPTB.

The clinical specimens included in this study were pus (15), endometrial biopsy (14), lymph node aspirate (10), peritoneal fluid (9), pleural fluid (7), tissue (5), CSF (5), synovial fluid (3), urine (3) as shown in the table-11(figure-4).

Out of the 15 pus samples processed, 7 were positive by one or more methods. One samples was positive by all the 5 methods, 1 sample was positive by both the staining techniques (ZN and fluorescent method) and both the culture methods (LJ and BacT ALERT) but negative by PCR. Later this isolate was identified as NTM. One sample was positive by BacT ALERT culture and PCR; 1 sample was positive only by LJ culture. Three samples were positive only by the staining techniques (ZN and fluorescent method) (table 12).

Among the 14 endometrial biopsy studied none were positive by staining techniques and culture methods. Only 1 sample was positive by PCR. This could be due to presence of killed or dormant bacilli in clinical sample (table 13).

A total of 10 lymph node aspirates were processed of which 4 samples were positive for AFB. Among the 4 positive, 3 were positive by BacT ALERT culture and PCR but negative by microscopy and LJ culture. One sample was positive only by PCR as shown in table 14.

Out of 7 pleural fluid samples processed 2 were positive by BacT ALERT culture and PCR but negative by other methods (table 15).

Out of 5 tissue samples from different clinical conditions processed only one sample was positive by both the staining techniques and both the culture methods but negative by PCR. Later this isolate was identified as NTM (table 16).

Out of the 3 synovial fluid samples (table 17) processed, one sample was positive by both the culture methods and PCR but could not be detected by ZN staining or fluorescent staining. None of the peritoneal fluid (9), CSF (5) and urine (3) samples processed were positive by any of the methods (tables-18,19,20).

Out of 71 samples processed, 16 samples (22.5%) were positive by one or more methods employed for the detection of acid fast bacilli (Table-21). Microscopy showed the positivity of 11.26% by both the methods (ZN and fluorescent staining method). A study done by Sudhindra KS et al reported equal positivity rates of ZN and fluorescent staining method[13].

Our study showed least positivity rate by LJ culture i.e. 8.45%. The low positivity rate may be attributed to paucibacillary nature of the disease and the sampled site may not represent the site of active infection[75]. Low positivity rates were also reported by Chhina D et al (2.1%)[76], Ajantha GS et al (5%)[77], Sharma K et al (11.3%)[78] and Siddiqui MAM et al (15%)[79].

In our study BacT ALERT had positivity rate of 14.08% (Table-22) which is much higher than reported by Angeby KAK et al (3.44%)[49], Carricago A et al (4.07%)[47] and Piersimoni C et al (7.07%)[48]. PCR was positive in 10 samples accounting for 14.08% of positivity rate. In studies by Hajia M et al[72], Pednekar SN et al [80] and Chawla K et al [81] reported higher positivity rates of 41%, 53% and 74 % respectively.

Isolation of M TB by culture remains the cornerstone for definitive diagnosis but the major drawback is long turnaround time of 6-8 weeks due to slow growth of M TB on LJ media. Introduction of liquid culture has increased the percentage of M TB recovery and reduced the detection time when compared with conventional methods[36].

We have considered LJ culture as gold standard and calculated sensitivity and specificity of ZN staining, fluorescent microscopy, LJ culture and PCR assay.

The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) of ZN staining was 50%, 95.08%, 37.5% and 95.2% respectively (table 23) which is much higher when compared to studies done by others. The sensitivity and specificity of ZN staining reported by other studies are-

Table 34 Sensitivity and specificity of ZN staining reported by other studies

Sl No.	Study	No. of samples	Sensitivity (%)	Specificity (%)
1	Siddqui MAM et al [79]	100	5	100
2	Sharma K et al [78]	150	7.33	-
3	Pednekar SN et al [80]	100	15.6	100
4	Negi SS et al [82]	156	33.79	100
5	Oberoi A et al [83]	255	34.78	100

The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) of fluorescent staining was 50%, 95.08%, 37.5% and 95.2% respectively (table 24) showing there was no difference observed between the results obtained by ZN staining and fluorescent staining. However fluorescent microscopy has the advantage of screening slides under low power thus reducing the time taken. RNTCP has provided light emitting diode based fluorescent microscopy in 200 medical colleges across country under the project LIGHT. It has increased the detection of AFB in sputum samples reported in 2012 compared to that in 2011[38]. There is an

advantage of reduced fatigue for observers and also improves the diagnostic value especially in paucibacillary samples that are likely to be missed on ZN-stained smears[84].

Among 8 cases which were positive by ZN staining and fluorescent microscopy, 2 were also positive by both culture methods and PCR. One lymph node aspirate was positive by BacT ALERT and PCR but failed to grow on LJ media. This could be due to nonuniform distribution of bacilli in the aliquots apportioned for the diagnostic tests[85]. Two samples were PCR negative but culture positive and later diagnosed as NMT. Three samples were negative by both culture methods and PCR. The reason for smear positive but culture and PCR negative could be the availability of small quantity of sample for processing.

In our study sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) of BacT ALERT culture was 83.3%, 92.3%, 50% and 98.4% respectively (table 25) which correlates with study by Martinez MS et al [86] who reported sensitivity of 87.7% and specificity of 99.2%..

The percentage of isolation of M TB by BacT ALERT and LJ culture from synovial fluid was 33.3%, whereas in lymph node it was 30% by BacT ALERT compared to 20% by LJ culture. In pleural fluid, percentage of isolation by BacT ALERT was 28.57% whereas isolation by LJ was none; however, in pus samples, isolation percentage was higher by LJ culture (20%) compared to BacT ALERT (13.33%). Endometrial biopsy, peritoneal fluid, tissue samples from different

conditions, CSF and urine samples did not yield growth of M TB. Since the number of synovial fluid samples processed were less, the isolation percentage by both culture methods in present study is high (Table-27) In study by Ghadage et al has reported maximum isolation of M TB by LJ culture in pus (33%), followed by pleural fluid (26.3%), fine needle aspiration biopsy (25%) and CSF (12.5%)[87].

BacT ALERT culture was positive in additional 5 samples when compared to LJ culture. The recovery rate was higher by BacT ALERT culture (90.9%) compared to LJ culture (63.63%) as shown in table-30. Though BacT ALERT had higher recovery rate, in one case of suspected Pott's spine only LJ culture was positive. This underlines the need to use the combination of liquid and solid media especially in the diagnosis of EPTB.

PCR was positive in 10 samples. The sensitivity, specificity, positive predictive value and negative predictive value of PCR assay was 66.66%, 90.76%, 40% and 96.72% respectively as shown in table 26. These results are comparable to other studies as shown in the table-35

Table 35 Sensitivity and specificity of PCR reported by other studies

Sl No.	**Study**	**No. of samples**	**Sensitivity (%)**	**Specificity (%)**
1	Oberoi A et al [83]	255	73.9	97.29
2	Siddqui MAM et al [79]	100	70	100

PCR was positive in 2 sample (endometrial biopsy, lymph node aspirate) where both the staining techniques and both the culture methods were negative. This could be due to paucibacillary nature of EPTB.

However in one case of suspected Pott's spine, only LJ culture was positive but other methods failed to detect M TB. This false negative PCR result could be due to nonuniform distribution of bacilli in the aliquots apportioned for the diagnostic tests[85]. Ineffective extraction of DNA or the presence of PCR inhibitor[88].

The mean turnaround time for ZN staining was 40 mins as compared to fluorescent staining (30 mins). The mean turnaround time for culture positivity was 36.3 days with LJ culture (range 5 to 49 days) and 14.6 days with BacT ALERT culture (range 4 to 21 days) (Table 28 & 29). The use of BacT ALERT culture has reduced the mean detection time by 2.5 times when compared to LJ culture. PCR mean detection time was 5 hours. Thus, PCR reduces detection time when compared with culture. PCR provides additional information when positive microscopy results are combined with PCR results and it also differentiates M TB from NMT.

The Cohen's kappa statistics was applied to assess the reproducibility and the level of agreement between the PCR assay and other diagnostic tests. (Table-31,32,33) In our study, the level agreement between BacT ALERT and PCR was 'substantial' with a kappa value of 0.767 whereas 'moderate' level of agreement between LJ culture and PCR with kappa value of 0.441.

The ability of PCR to detect even one organism from clinical sample makes it very attractive diagnostic tool in the diagnosis of EPTB. In the present study, PCR had sensitivity of 66.7%, specificity of 90.76% and short detection time (5 hours). The sensitivity of PCR from clinical samples reported from different studies varies between 55% and 90%, which is more compared to any other test used in the diagnosis of EPTB. This makes PCR a valuable screening test, especially when limitations of conventional diagnostic modalities have negative impact on patient care. Though PCR has been reported to have high sensitivity and specificity, it has few drawbacks. It is very expensive, needs expertise and proper standardization and risk of false negative and false positive results.

PCR has high sensitivity, specificity, substantial level of agreement with BacT ALERT culture and shorter turnaround time; hence in the era of evidence based clinical practice, it adds meaningful evidence to the results of conventional method employed in the diagnosis of EPTB, to rule-in or rule-out the disease. Therefore, use of PCR in combination with other diagnostic modalities helps to provide maximum information to clinicians in the diagnosis of EPTB.

SUMMARY

In the present study, 71 clinical specimens from patients with strong clinical suspicion of extra-pulmonary tuberculosis attending OPD and also admitted in R L Jalappa hospital and research centre, Tamaka, Kolar from January 2015-August 2016 were included. The clinical samples processed and evaluated by ZN staining, fluorescent microscopy, LJ culture, BacT ALERT culture and PCR. The clinical specimens included in this study were pus (15), endometrial biopsy (14), lymph node aspirate (10), peritoneal fluid (9), pleural fluid (7), tissue (5), CSF (5), synovial fluid (3), urine (3)

Of the 71 cases studied 40 were male (56%) and 31 were females (44%). The majority of the patients belonged to the age group of 31-40 years accounting for 23.9% followed by 21-30 years age group. The positivity rates by microscopy, LJ culture, BacT ALERT culture and PCR were 11.26%, 8.45%, 14.08% and 14.08% respectively.

We have considered LJ culture as gold standard and calculated sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of ZN staining, fluorescent microscopy, LJ culture and PCR assay.

The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) of both staining methods was 50%, 95.08%, 37.5% and 95.2% respectively showing there was no difference observed between the results obtained by

ZN staining and fluorescent staining. However fluorescent microscopy had an advantage over ZN staining, of screening slides under low power thus reducing the time taken (ZN staining turnaround time 40 mins Vs fluorescent staining 30 mins.)

The mean turnaround time for culture positivity was 36.3 days with LJ culture (range 5 to 49 days) and 14.6 days with BacT ALERT culture (range 4 to 21 days). The use of BacT ALERT culture has reduced the mean detection time by 2.5 times when compared to LJ culture. The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) of BacT ALERT culture was 83.3%, 92.3%, 50% and 98.4% respectively. BacT ALERT culture was positive in additional 5 samples when compared to LJ culture. The recovery rate was higher by BacT ALERT culture (90.9%) compared to LJ culture (63.63%). Though BacT ALERT had higher recovery rate, in one case of suspected Pott's spine only LJ culture was positive. This underlines the need to use the combination of liquid and solid media especially in the diagnosis of EPTB.

PCR was positive in 10 samples. The sensitivity, specificity, positive predictive value and negative predictive value of PCR assay was 66.66%, 90.76%, 40% and 96.72% respectively. PCR was positive in 2 sample (endometrial biopsy, lymph node aspirate) where both the staining techniques and both the culture methods were negative. Thus increasing the detection rate of M TB compared to culture. PCR mean detection time was 5 hours. Thus, PCR reduces detection time when compared with culture. PCR provides additional information when positive microscopy results are combined with PCR results and it also differentiates M TB from NMT

In our study, the level agreement between BacT ALERT and PCR was ‘substantial’ with a kappa value of 0.767 whereas ‘moderate’ level of agreement between LJ culture and PCR with kappa value of 0.441.

PCR has high sensitivity, specificity, substantial level of agreement with BacT ALERT culture and shorter turnaround time. Therefore, use of PCR in combination with other diagnostic modalities is a useful tool to detect additional EPTB cases to be missed otherwise.

CONCLUSION

The diagnosis of EPTB is, many a times, a clinico-microbiological dilemma. The sensitivity of microscopy and culture on LJ media are low. BacT ALERT culture sensitivity and recovery rate was better compared to LJ culture with lesser turnaround time. Thus BacT ALERT is a better option for isolation of M TB. PCR has a great potential to improve the clinicians ability to diagnose extrapulmonary tuberculosis. This will ensure early treatment to patients and prevent further transmission of disease. The present study revealed that PCR using IS6110 could detect more number of positives in extrapulmonary tuberculosis compared with conventional methods. As of today all the available diagnostic tests need to be utilized and the results need to be carefully correlated with the clinical findings to effectively diagnose EPTB. Since PCR detects positive cases in extrapulmonary cases which may be missed by smear and/or culture, it's a valuable tool and needs to be utilized routinely in the diagnosis of EPTB.

BIBILOGRAPHY

1. Ryan KJ, Ray CG. Sherris Medical Microbiology. 5th ed.USA:McGraw Hill company, Inc; 2010. 489-505.
2. Shrma SK, Mohan A. Tuberculosis: from an incurable scourge to a curable disease- journey over a Millennium. Ind J Med Res. 2013; 137:455-493.
3. Pommerville JC. Alcamo's Fundamentals of Microbiology. 7th ed. Canada: Jones and Bartlett; 2004. 284-289.
4. Lee JY. Diagnosis and Treatment of Extrapulmonary Tuberculosis. Tuberc Respir Dis 2015; 78: 47-55.
5. World Health Organization. Definitions and Reporting Frame Work for Tuberculosis: 2013 revision (updated December 2014). Geneva: World Health Organization : 2013
6. World Health Organization Global Tuberculosis Report 2015. Available from: http://:www.health-e.org.2a/wp-contact/uploads/2015/10/global-TB-report-2015
7. Purohit M, Mustafa T. Laboratory Diagnosis of Extrapulmonary Tuberculosis (EPTB) in Resource- Constrained Setting: State of the Art, Challenges and the Need. J Clin Diagn Res 2015;9:EE01-EE06.
8. Lange C, Mori T. Advances in the Diagnosis of Tuberculosis. Journal of Asian Pacific Society of Respiriology 2010;15:220-240.
9. Sharma S K, Mohan A. Extrapulmonary Tuberculosis. Indian J Med Res 2004;120:316-53.
10. World Health Organization. Commercial Serodiagnostic Tests for Diagnosis of Tuberculosis, 2011. Geneva: WHO Press: 2011.
11. Sekar B, Selvaraj L, Alexis A, Ravi S, Arunagiri K, Rathinavel L. The Utility of IS Sequence Based Polymerase Chain Reaction in Comparison to Conventional

Methods in the Diagnosis of Extra-Pulmonary Tuberculosis. Indian J Med Microbiol 2008;26:352-5.

12. Pingle P, Apte P, Trivedi R. Evaluation of Microscopy, Culture and PCR Methods in the Laboratory Diagnosis of Genito-urinary Tuberculosis. American Journal of Infectious Diseases and Microbiology 2014;2:17-21.
13. Sudhindra KS, Prakash RC, Fathima F, Venkatesh VN, Rao A, Vijaynath V. Evaluation of PCR in Early Diagnosis of Tuberculosis. J Med Res Pract 2012;1:24-28.
14. Arvind N, Chand P, Baliga S, Bharati B, Shenoi S, Saralaya V. A Comprehensive Comparison of PCR Based Assay Versus Microscopy and Culture in the Diagnosis of Tuberculosis in Different Clinical Specimens. Int J Biol Med Res 2014;5:3763-68.
15. Cambau E, Drancourt M. Steps towards the Discovery of Mycobacterium tuberculosis by Robert Koch, 1882. Clin Microbiol Infect 2014;20:196-201.
16. Tille PM. Bailey & Scott's Diagnostic Microbiology. 13th ed:Mosby Inc;2014.484-512
17. Pfyffer GE, Palicova F. Mycobacterium: General characteristics, laboratory detection, and procedures. In Versalovic J, Carroll KC, Funke G, Jorgensec JH, Landry ML, Warnock DW. Mannual of Clinical Microbiology. 10th ed:ASN press;2011.472-502.
18. Bhardwaj AK, Kumar D, Raina SK, Sharma S, Chander V. Assessment of extra pulmonary tuberculosis (EPTB) cases from selected tuberculosis units (TUs) of Himachal Pradesh, India. Int J Health 2015;3:29-33.
19. Global tuberculosis Report 2015- World Health Organization. Avialable from: http://:www.who.int/tb/publications/global_report/gtbr15_main_text.pdf

20. Shirvastva AK, Brahmachari S, Pathak P, Kumar R, Patel U, Mandil A. Clinico-Epidemiological profile of extrapulmonary tuberculosis in central India. Int J Med Res Rev 2015;3:223-230
21. Raviglione MC, O'Brien RJ. Tuberculosis In Lango DL, Kasper DL, Jameson JL, Fauci AS, Hauser SL, Loscalzo J, editors. Harisson's principles of internal medicine. 18th ed.USA:McGraw Hill company, Inc;2012.1342-1344
22. Fitzgerald D, Haas DW. Mycobacterium tuberculosis. In Mandell GL, Bennet JE, Dolin R, editors. Principles and Practice of Infectious Diseases.6th ed.USA:Elsevier Churchill Livingstone;2005.2852-86
23. Mohapatra PR, Janmeja AK. Tuberculous Lymphadenitis. JAPI 2009;57:585-590.
24. Vorster MJ, Allwood BW, Diacon AH, Kongelenberg CFN. Tuberculous puleral efuusion: advances and contraverses. Available at http://dx.doi.org/10.3978/j.issn.2072-1439.2015.02.18
25. Ferrer J. Pleural tuberculosis. Eur Respir J 1997;10:942-947
26. Abraham P, Mistry FP. Tuberculosis of the Gastrointestinal tract. Ind J tub 1992;7992;251.
27. Rathi P, Gambhire P. Abdominal tuberculosis. J Assoc Physicians India 2016;64:38-47.
28. Be NA, Kim KS, Bishai WR, Jain SK. Pathogenesis of Central Nervous System tuberculosis. Curr Mol Med 2009;9:94-99.
29. Pigrau-Sarrallah C,Rodiguez-Pardo D. Bone and Joint tuberculosis. Eur Spine J 2013;22:556-566.
30. Hemal AK. Genitourinary tuberculosis. In Sharma SK, Mohan A. Editors. Tuberculosis. New Delhi: Jaypee brothers medical publishers: 2001;325-37.

31. Sharma SK, Mohan A, Sharma A. Miliary Tuberculosis: A new look at an old foe. J clin tuberc other mycobact dis 2016;3:13-27.
32. McAdam AJ, Milner DA, Sharpe AH. Infectious diseases In Robbins and Cotran pathologic basis of disease. South Asia edition, Reed Elsevier India pvt ltd 2014;1:374-376.
33. Nicode LP. Immunology of tuberculosis. Swiss Med Wkly 2007;137:357-362.
34. Wani RLS. Clinical manifestations of Pulmonary and extra-pulmonary tuberculosis. SSMJ 2013;6:52-56.
35. Labh K, Sun X. Various manifestation of central nervous system tuberculosis: A review. Biomed Lett 2016;2:1-7.
36. Winn W, Allen S, Janda W, Koneman E, Procop G, Schreckenberger P, Woods G. Koneman's Color Atlas and Textbook of Diagnostic Microbiology. 6th ed:2006.1064-1124.
37. Kapil A. Ananthanarayan & Paniker's textbook of Microbiology. 9th ed. Chennai;Universities Press (India) Pvt ltd:2013:352.
38. Shirvastava SR, Shirvastava PS, Ramasamy J. Revised National tuberculosis Control Program: Progress in the Diagnostic Front. J Mahatma Gandhi Inst Med Sci 2014;19:164-165.
39. Liu KT, Su WJ, Perng RP. Clinical utility of polymerase chain reaction for diagnosis of smear-negative pleural tuberculosis. J Chin Med Assoc 2007;70:148–151.
40. Haldar S, Bose M, Chakrabarti P, et al. Improved laboratory diagnosis of tuberculosis — the Indian experience. Tuberculosis 2011;91:414–426.

41. Derese Y, Hailu E, Assefa T, Bekele Y, Mihret A, Aseffa A, et al. Comparison of PCR with standard culture of fine needle aspiration samples in the diagnosis of tuberculosis lymphadenitis. J Infect Dev Ctries 2012;6:53–57.
42. Padmavathy L, Rao L, Veliath A. Utility of polymerase chain reaction as a diagnostic tool in cutaneous tuberculosis. Indian J Dermatol Venereol Leprol 2003;69:214–216.
43. Takahashi T, Tamura M, Asami Y, Novel wide-range quantitative nested real-time PCR assay for Mycobacterium tuberculosis DNA: clinical application for diagnosis of tuberculous meningitis. J Clin Microbiol 2008;46:1698–1707.
44. Abbara A, Davidson RN. Etiology and management of genitourinary tuberculosis. Nat Rev Urol 2011;8:678–688.
45. Mehta PK, Kalra M, Khuller GK, Behera D, Verma I. Development of an ultrasensitive polymerase chain reaction-amplified immunoassay (Immuno-PCR) based on mycobacterial RD antigens: implications for the serodiagnosis of tuberculosis. Diagn Microbiol Infect Dis 2012;72:166–174.
46. Samant H, Desai D,Abraham P, Joshi A,Gupta T,Rodrigues C et al. Acid-fast bacilli culture positivity and drug resistance in abdominal tuberculosis in Mumbai, India. Indian J Gastroenterol 2014;33:414–419
47. Carricajo A, Fonsale N, Vautrin A C, Aubert G. Evaluation of BacT/Alert 3D Liquid Culture System for Recovery of Mycobacteria from Clinical Specimens Using Sodium Dodecyl (Lauryl) Sulfate-NaOH Decontamination J Clin Microbiol 2001;39:3799-3800
48. Piersimoni C, Scarparo C, Callegaro A Tosi CP, Nista D, Bornigia S et al.Comparison of MB/BacT ALERT 3D System with Radiometric BACTEC System and Lo¨wenstein-Jensen Medium for Recovery and Identification of

Mycobacteria from Clinical Specimens: a Multicenter Study. J Clin Microbiol 2001;39:651-657

49. Angeby KA, Werngren J, Toro JC et al. Evaluation of the BacT/ALERT 3D system for recovery and drug susceptibility testing of Mycobacterium tuberculosis. Clin Microbiol Infect 2003; 9: 1148 –52.
50. Moore DAJ, Evans CAW, Gilman RH, Caviedes L, Coronel J, Vivar A, et al. Microscopic Observation drug susceptibly assay for the diagnosis of TB. N Engl J Med 2006;355:1539-50
51. Yip CW, Leung KL, Wong D, Cheung DTL, Chu MY, Tang HS et al. Denaturing HPLC for high-throughput screening of rifampicin-resistant Mycobacterium tuberculosis isolates. Int J Tuberc Lung Dis 2006;10:625–630
52. Laraque F, Griggs A, Slopen M, Munsiff SS. Performance of Nucleic acid performance test for Diagnosis of tuberculosis in a large Urban setting. Clin Infect Dis 2009; 49: 46-54
53. Guerra RL, Hooper NM, Baker JF, Alborz R, Armstrong DT, Maltas G et al. Chest 2007; 132:946-951
54. Raj A, Singh N, Gupta KB, Chaudhary D, Yadav A, Chaudhary A. Comparative Evaluation of Several Gene Targets for Designing a Multiplex-PCR for an Early Diagnosis of Extrapulmonary Tuberculosis. Yonsei Med J 2016;57:88-96
55. Abe C, Hirano K, Wada M, Kazumi Y, Takahashi M, Fukasawa Y et al. Detection of Mycobacterium tuberculosis in clinical Specimens by Polymerase Chain Reaction and Gene-Probe Amplified Mycobacterium Tuberculosis Direct Test. J Clin Microbiol 1993;31:3270-74

56. Clarridge JE, Shawar RM, Shinnick TM, Plikaytis BB. Large –Scale Use of Polymerase Chain Reaction for Detection of Mycobacterium tuberculosis in a Routine Mycobacteriology Laboratory. J Clin Microbiol 1993;31:2049-56
57. Beige J, Lokies J, Schaberg T, Finckh U, Fischer M, Mauch H et al. Clinical Evaluation of a Mycobacterium tuberculosis PCR Assay. J Clin Microbiol 1995;33:90-95
58. Nolte FS, Metchock B, McGowan JE, Edwards A, Okwumabua O, Thurmond C et al. Direct Detection of Mycobacterium tuberculosis in Sputum by Polymerase Chain Reaction and DNA Hybridization. J Clin Microbiol 1993;31:1777-82
59. Cheng VCC, Yam WC, Hung IFN, Woo PCY, Lau SKP, Tang BSF et al. Clinical evaluation of the polymerase chain reaction for the rapid diagnosis of tuberculosis. J Clin Pathol 2004;57:281–285
60. Shenai S, Rodrigues C, Mehta AP. Newer Rapid Diagnostic Methods for Tuberculosis: A Preliminary Experience. Indian J Tuberc 2004;51:219-230
61. Kandhakumari G, Stephen S, Sivakumar S, Narayanan S. Spoligotype patterns of Mycobacterium tuberculosis isolated from extra pulmonary tuberculosis patients in Puducherry, India. Indian J Med Microbiol 2015;33:267-270
62. Mbaave TP, Igbudu TJ, Egwuda L, Audu O, Jombo GT. GeneXpert MTB/RIF Sputum MTB Detection and Human Immunodeficiency Virus Seroprevalence among Patients with Presumptive Pulmonary Tuberculosis at a New Teaching Hospital in North-Central Nigeria. Int.J.Curr.Microbiol.App.Sci 2016;5:564-570
63. Zeka AN, Tasbakan S, Cavusoglu C. Evaluation of the GeneXpert MTB/RIF Assay for Rapid Diagnosis of Tuberculosis and Detection of Rifampin Resistance in Pulmonary and Extrapulmonary Specimens. J Clin Microbiol 2011;11:4138-41

64. Pai M. Extrapulmonary Tuberculosis: New Diagnostics and New Policies. Indian J Chest Dis Allied Sci 2014;56:71-73

65. Nachega JB, Chaisson RE. Tuberculosis Drug Resistance: A Global Threat. Clin Infect Dis 2003;36:24–30

66. Drug-resistant TB available from: @ www.cdc.gov/tb/topic/drtb/

67. Campbell PJ, Morlock GP, Sikes RD, Dalton TL, Metchock B, Starks AM et al. Molecular Detection of Mutations Associated with First- and Second-Line Drug Resistance Compared with Conventional Drug Susceptibility Testing of Mycobacterium tuberculosis. Antimicrob Agents Chemother 2011;55:2032-42

68. Revised National Tuberculosis Control Programme, Available from http://dghs.gov.in/content/1358_3_RevisedNationalTuberculosisControlProgramme.aspx

69. TB India 2016 Available from http://tbcindia.nic.in/index1.php?lang=1&level=2& sublinkid=4569&lid=3174

70. National Strategic Plan for Tuberculosis Control 2012–2017. Available from: http://www.tbfacts.org/wp-content/uploads/2016/01/NSP-2012-2017.pdf

71. Mercy EG, Aditya T, Amarendra KE, Dan MM. Review on Mycobacterium Tuberculosis. RRJMB 2016:9-17

72. Hajia M, Rahbar M, Amini R. Is PCR assay reliable for diagnosis of extrapulmonary tuberculosis? Afr J Microbiol Res 2009;3:877-881

73. Arora VK, Gupta R. Trends of extrapulmonary tuberculosis under revised national tuberculosis control program; a study from south Dehli. Indian J Tuberc 2006;53:73-83

74. Musellim B, Erturan S, Duman ES, Ongen G. Comparison of extrapulmonary and pulmonary tuberculosis case; Factors influencing the site of reactivation. Int J Tuberc Lung Dis 2005;9:1220-1223
75. Srivastava I, Bhatambare G.S, Deshmukh A.B, Bajpai P, Bajpai T, Singh T. Genital Tuberculosis: Evaluating Microscopy, Culture, Histopathology and PCR for Diagnosis All Play Their Role. Int J Curr Microbiol App Sci 2014; 3: 439-445
76. Chhina D, Mohi G, Gupta R, Deeplyall K, Kaur J. Role of gen-probe amplified MYCOBACTERIUM TUBERCULOSIS Direct Test (AMTD) in the diagnosis of extrapulmonary tuberculosis. IJPRBS 2015; 4:135-143
77. Ajantha GS, Shetty PC, Kulkarni RD, Biradar U. PCR as a Diagnostic Tool for Extra-pulmonary Tuberculosis. JCDR 2013;7:1012-1015
78. Sharma K, Appannanavar SB, Modi M, Singh M, Sharma A, Varma S. Role of multiplex polymerase chain reaction using IS6110 and Protein b for the diagnosis of extrapulmonary tuberculosis: North India. Indian J Pathol Microbiol 2015;58:27-30
79. Siddqui MAM, Anuradha PR, Nagamani K, Vishnu PH. Comparison of conventional diagnostic modalities, BACTEC culture with polymerase chain reaction for diagnosis of extrapulmonary tuberculosis. J Med Allied Sci 2013;3:53-58
80. Pednekar SN, Desai SL, Pol S, Kagal AS, Dharmashale SN, Bharadwaj RS. A Comprehensive Comparision Of Mpb64 Based Pcr Assay Versus Microscopy And Culture In The Diagnosis Of Clinically Suspected Cases Of Extrapulmonary Tuberculosis. Int J Recent Advances in Multidisciplinary Research 2015;2:0436-0440

81. Chawla K, Johar R, Vishwanath S, Mukhopadhyay C. Role of PCR in the Diagnosis of Pulmonary and Extra-Pulmonary Tuberculosis. NJLM 2015;4:43-46
82. Negi SS, Khan SFB, Gupta S, Pasha ST, Khare S, Lal S. Comparison of the Conventional Diagnostic Modalities, Bactec Culture And Polymerase Chain Reaction. Indian J Med Microbiol 2005;23:29-33
83. Oberoi A, Aggarwal A. Comparison of the conventional diagnostic techniques, BACTEC and PCR. JK Sci 2007;9:179-181
84. Anmam V, Kulkarni MH, Puranik RB. Comparison of modified fluorescent method and conventional Ziehl-Neelsen method in detection of acid fast bacilli in Lymph node aspirate. CytoJournal 2009;6:13
85. Chakravorty S, Sen MK, Tyagi JS. Diagnosis of Extrapulmonary Tuberculosis by Smear, Culture, and PCR Using Universal Sample Processing Technology. J Clin Microbiol 2005;49:4357-4362
86. Martinez MS, Sardinas M, Garcia G, Mederos LM, Diaz R. Evaluvation of BacT ALERT 3D System for Mycobacteria isolates. Journal of Tuberculosis Research 2014;9:59-64.
87. Ghadage DP, Muley VA, Pednekar S, Bhore AV. Evaluation of polymerase chain reaction using primer MPB 64 for diagnosis of clinically suspected cases of extra-pulmonary tuberculosis. J Sci Soc 2014;41:89-93
88. Zakham F, Bazoui H, Akrim M, Lamrabet S, Lahlou O, Elmzibri M et al. Evaluation of conventional molecular diagnosis of mycobacterium tuberculosis in clinical specimens from Morocco. J Infect Dev Ctries 2012;6:40-45

ANNEXURE-1

PROFORMA

Study Title: Evaluation of microbiology, culture and PCR assay in the diagnosis of Extrapulmonry Tuberculosis

Name: **Date:**
Age/Sex:

Address:

Occupation: **Contact no.:**

Brief history of presenting illness:

Past history of tuberculosis: YES/NO

Family history:

Drug history:

<u>Investigations</u>

Nature of specimen

CSF	Pleural fluid	Peritoneal fluid	Synovial Fluid	Pericardial fluid	Urine	Tissue	Miscellaneous

Biochemical- for CSF, Proteins-

Sugar-

Pathology- for Body fluid, lymphocyte count-

For Tissue (histopathology report)-

Radiology- Chest X-ray-

CT scan-

Laboratory results

1. **Microscopy-**
    a. **Ziehl Neelsen staining-**
    b. **Auramine staining-**

2. **Culture-**
    a. **Lowenstein Jensen medium**

Date of inoculation	**Results**

    b. **BacT/ALERT**

Date of inoculation	**Results**

3. **PCR**

Diagnosis

ANNEXURE-2 MASTER CHART

Sl No	Age	Sex	Specimen	Staining methods		Culture methods		PCR
				ZN	AR	LJ	BacT	
1	33	f	endo biopsy	n	n	n	n	n
2	30	f	endo biopsy	n	n	n	n	n
3	28	f	endo biopsy	n	n	n	n	n
4	20	f	endo biopsy	n	n	n	n	n
5	60	f	tissue	n	n	n	n	n
6	36	m	pus	n	n	n	n	n
7	23	f	peritoneal fluid	n	n	n	n	n
8	25	f	endo biopsy	n	n	n	n	n
9	29	f	endo biopsy	n	n	n	n	n
10	20	m	tissue	n	n	n	n	n
11	28	f	endo biopsy	n	n	n	n	n
12	44	f	pus	p	p	n	n	n
13	60	m	pus	n	n	n	n	n
14	41	m	epidural pus	n	n	n	p	p
15	35	f	urine	n	n	n	n	n
16	73	m	ascitic fluid	n	n	n	n	n
17	23	f	endo biopsy	n	n	n	n	n
18	27	f	endo biopsy	n	n	n	n	n
19	36	f	endo biopsy	n	n	n	n	n
20	35	m	pleural fluid	n	n	n	p	p
21	56	m	pus	n	n	n	n	n
22	60	m	pus	p	p	n	p	p
23	30	f	endo biopsy	n	n	n	n	n
24	22	m	pus	n	n	n	n	n
25	27	m	pleural fluid	n	n	n	p	p
26	35	f	endo biopsy	n	n	n	n	p
27	35	f	endo biopsy	n	n	n	n	n

28	35	m	pus	n	n	n	n	n
29	40	f	pus	n	n	n	n	n
30	33	f	endo biopsy	n	n	n	n	n
31	60	m	CSF	n	n	n	n	n
32	37	m	pleural fluid	n	n	n	n	n
33	54	m	CSF	n	n	n	n	n
34	60	m	peritoneal fluid	n	n	n	n	n
35	40	m	CSF	n	n	n	n	n
36	47	f	tissue	n	n	n	n	p
37	26	f	peritoneal fluid	n	n	n	n	n
38	23	f	peritoneal fluid	n	n	n	n	n
39	60	m	tissue	p	p	n	p	n
40	40	f	synovial fluid	n	n	p	p	p
41	70	m	tissue	n	n	n	n	n
42	22	m	abscess from neck	n	n	n	n	n
43	70	m	peritoneal fluid	n	n	n	n	n
44	1.5	f	pus	p	p	n	n	n
45	39	m	peritoneal fluid	n	n	n	n	n
46	80	m	CSF	n	n	n	n	n
47	58	m	pus	p	p	p	p	p
48	37	m	cervical C.N	n	n	n	n	n
49	56	f	pus	n	n	p	p	p
50	65	m	synovial fluid	n	n	n	n	n
51	20	m	tissue	n	n	n	n	n
52	18	m	pleural fluid	n	n	n	n	n
53	20	m	tissue	p	p	p	p	p
54	38	m	pleural fluid	n	n	n	n	n
55	25	f	pus	p	p	n	n	n
56	50	f	pus	n	n	n	n	n
57	48	m	pus	p	p	p	p	n
58	47	m	synovial fluid	n	n	n	n	n

59	20	m	L.N. aspirat	n	n	n	n	n
60	18	m	L.N. aspirat	n	n	n	n	n
61	65	m	peritoneal fluid	n	n	n	n	n
62	61	m	pleural fluid	n	n	n	n	n
63	54	f	submandibular gland aspirate	n	n	n	n	n
64		m	pleural fluid	n	n	n	n	n
65	58	f	urine	n	n	n	n	n
66	11	f	tissue	n	n	n	n	n
67	45	m	CSF	n	n	n	n	n
68	43	m	pus	n	n	n	n	n
69	65	f	peritoneal fluid	n	n	n	n	n
70	60	m	urine	n	n	n	n	n
71	35	m	pus	n	n	p	n	n

m- male

f- female

ZN- Ziehl Neelsen staining

AR- Auramine Rhodamine staining

LJ- Lowenstein Jensen media

PCR- Polymerase Chain Reaction

p- positive

n-negative