EVALUATION OF MULTIPLE LABORATORY METHODS FOR THE DIAGNOSIS OF EXTRAPULMONARY TUBERCULOSIS

BY

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UNDER THE GUIDANCE OF

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DECLARATION BY THE CANDIDATE

I hereby declare that this dissertation entitled **"EVALUATION OF MULTIPLE LABORATORY METHODS FOR THE DIAGNOSIS OF EXTRAPULMONARY TUBERCULOSIS"** is a bonafide and genuine research work carried out by me under the guidance of **Dr. T.V.RAO,** Professor, Department of Microbiology, Sri Devaraj Urs Medical College, Kolar.

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ETHICAL COMMITTEE CERTIFICATE

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List of abbreviations

HIV	Human immuno deficiency virus
RNTCP	Revised National Tuberculosis Control Programme
EPTB	Extra pulmonary tuberculosis
PTB	Pulmonary tuberculosis
M tb	Mycobacterium tuberculosis
ZN	Ziehl Neelsens
CSF	Cerebro spinal fluid
L J	Lowenstein Jensens
RSC	Rapid slide culture
LN TB	Lymph node tuberculosis
TBM	Tuberculous Meningitis
VDR	Vitamin D receptors
TNF	Tumor necrosis factor
IL	Inter Leukin
MBL	Mannose binding lectin
AFB	Acid fast bacilli
HBM	Human blood medium
HPLC	High pressure liquid chromatography
RFLP	Restriction fragment length polymorphism
NTM	Non tuberculous Mycobacteria
NAOH	Sodium Hydroxide

Abstract

Introduction

Tuberculosis remains a worldwide public health problem even after 100 years of the discovery of the causative agent, Mycobacterium tuberculosis. The emergence of HIV infections has further complicated the disease burden as it has rapidly increased the risk of pulmonary and extra pulmonary tuberculosis.

Extra pulmonary tuberculosis is an important clinical entity. The term has been used to describe isolated occurrences of tuberculosis at body sites other than lungs. The precise diagnosis is very important because early detection of cases and effective treatment if instituted at the right time, completely cures the patients of the disease.

Objectives

1. To find out the sensitivity and specificity of Ziehl Neelsen's staining, Auramine staining and Rapid Slide Culture technique, comparing them with growth on Lowenstein Jensen's medium as gold standard.
2. To do a qualitative estimation of antibodies against 38kda, 16kda, 6kda antigens of Mycobacterium tuberculosis in the serum of patients suspected of extra pulmonary tuberculosis.
3. To evaluate the role of the above multiple tests, for the etiological diagnosis of extra pulmonary tuberculosis.

Material and methods:

The present study included 66 clinical specimens from patients suspected of extra pulmonary tuberculosis inclusive of HIV infected patients at R.L.Jalappa Hospital and Research Centre, attached to Sri Devaraj Urs Medical College, Tamaka, Kolar. The Patients whose sputum was positive for acid fast bacilli and diagnosed with pulmonary tuberculosis were excluded from the study.

The following extra pulmonary specimens Pleural fluid (29), Pus samples (11), Lymph node aspirates (6), Biopsy specimens (6),Cerebrospinal fluid (CSF)(4), Synovial fluid (3) , Ascitic fluid (3), Urine(2), Bone marrow aspirate(2) were collected. These specimens were subjected to ZN and Fluorescent method of staining, culturing by LJ and RSC method, and comparing growth on LJ medium as the gold standard. Blood specimens were collected for serological study for detection of antibodies against 38 kda, 16kda, and 6kda antigens of Mycobacterium tuberculosis. The results were compiled and analysed. Statistical analysis was done and the sensitivities, specificities, were compared with the gold standard and the positive predictive values and the negative predictive values were obtained.

RESULTS AND DISCUSSION:

In the present study 57.5% were males and 42.5% were females and most of the patients belonged to the age group of 21-30 years. Mycobacterium tuberculosis was isolated in 24.1% and Non Tuberculous Mycobacterium (NTM) was isolated in 6% of the 66 samples processed. Highest number of isolation was from lymph node aspirates (83.3%) . In pleural fluid the percentage of isolation was 13.8%, pus 27.8%, synovial fluid 33.3%, urine 50%, ascitic fluid 33.3% and biopsy specimen 16.7%, 2%

of the samples were HIV seropositive. 4(6%) of the samples were smear negative and culture positive. 3% of the samples were smear positive and culture negative .

The ZN method of staining had a sensitivity of 80%, specificity of 95.65%, positive predictive value of 88.89% and negative predictive value of 91.67%. The fluorescent method of staining had a sensitivity of 80% and a specificity of 95.65% positive predictive value of 88.89% and negative predictive value of 91.67%. RSC had a sensitivity of 80%, specificity of 100%, positive predictive value of 100% and negative predictive value of 92%.

RSC had sensitivity comparable with the sensitivity of smear microscopy by ZN and Fluorescent methods. RSC had a positive predictive value of 100% which indicates that the diagnostic potential of the test is good. However from the above findings it can be concluded that with a positive predictive value of 100% and sensitivity of 80% RSC is as good as LJ culture. The growth is obtained in 7 days and is useful in the early confirmation of viable Mycobacterium tuberculosis which makes it an ideal diagnostic test in a country like India where tuberculosis is rampant. From the present study it can be inferred that antibody estimation does not play an important role in diagnosis of extra pulmonary tuberculosis.

KEY WORDS: Extrapulmonary tuberculosis , M tuberculosis ,ZN staining, LJ culture, RSC.

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1. INTRODUCTION

Tuberculosis remains a worldwide public health problem even after hundred years of discovery of the causative agent; Mycobacterium tuberculosis and in spite of effective treatment[1].Tuberculosis is an ancient disease, the presence of which has been inferred from as early as 4000 BC from Egyptian tomb paintings and by examination of mummies for spinal tuberculosis[2]. Approximately nine million people develop acute tuberculosis every year world over and this disease kills 1.3 million per year[3]. The emergence of Human immunodeficiency virus (HIV) infection has further complicated the disease burden as it has rapidly increased the risk of tuberculosis[3].

Tuberculosis can involve any organ system in the body while the pulmonary tuberculosis is the most common presentation. However extra pulmonary tuberculosis (EPTB) is also an important clinical entity. The term Extrapulmonary tuberculosis has been used to describe isolated occurrences of tuberculosis at body sites other than the lungs[4].Extrapulmonary tuberculosis constitutes about 15% to 20% of all cases of tuberculosis[4].

Patients with extrapulmonary tuberculosis may manifest with constitutional symptoms such as fever, anorexia, weight loss, malaise and fatigue. In India patients with extrapulmonary tuberculosis especially when the disease is located at an obscure site may present with pyrexia of unknown origin and this may be the only diagnostic clue in them[4].

The Diagnosis of extrapulmonary tuberculosis is very important because early detection of cases and effective treatment instituted at the right time completely cures the patient of this disease. The clinical presentation of extrapulmonary tuberculosis is atypical; tissue samples for the confirmation of diagnosis can sometimes be difficult to procure[4].

A persistent difficulty in tuberculosis management in developing countries is; there is no specific, sensitive, inexpensive and rapid method of diagnosis. The common tests done to detect extrapulmonary tuberculosis are smear microscopy by Ziehl Neelsen's staining (ZN) technique and culture by Lowenstein Jensen's medium. However, the smear positivity rate is low; it is 5-37% with respect to cerebrospinal fluid (CSF), <10% in pleural fluid, < 1% in pericardial fluid[4].

Culturing for the isolation of Mycobacterium tuberculosis proved to be a very sensitive technique. Among several egg based culture media for the recovery of Mycobacteria, conventional Lowenstein Jensen's (LJ) medium is most commonly used in microbiological laboratories. Culture on Lowenstein–Jensen's medium increases the detection rate: 12% to 70% from pleural fluid, 25% to 60% from pericardial fluid and 40% to 50% from cerebrospinal fluid[4]. It is also observed that the detection rate of urine examination by smear and culture for detecting the tubercle bacilli is low; and hence it becomes difficult to diagnose the condition[4]. Fluorescent microscopy using Auramine staining technique has been used to increase the sensitivity of detection of

Mycobacterium tuberculosis in sputum smears. Fluorescent microscopy showed that on an average, in comparison with Ziehl Neelsen's staining, there was a 10% increase in sensitivity in a systematic review of 43 studies[8].

The increase in incidence of HIV infections has further increased the incidence of extrapulmonary tuberculosis[5]; It is found that among the HIV-TB co–infected patients, EPTB is about 30% to 70% more common than pulmonary tuberculosis (PTB)[5]. The most common form of extrapulmonary tuberculosis is lymph node tuberculosis in India[6]. This is followed by infection of the pleura, osteoarticular, abdominal and genital tuberculosis. This pattern is common to other developing countries.

EPTB has a broader spectrum of clinical manifestations and even in countries where PTB is still a prevalent health problem, it often presents a diagnostic challenge, most often leading to delay in diagnosis[7]. The study is undertaken to see if different methods of diagnosis, can improve the sensitivity of diagnosis of Extrapulmonary tuberculosis.

Recently there is renewed interest in Rapid Slide Culture (RSC) [9] which was first used by Sir Robert Koch. An advantage of rapid slide culture is that it only involves picking up microcolonies with an ordinary bright light microscope without the need of sophisticated equipment or complicated procedure[10]. Though culture on Lowenstein

Jensen's medium remains the gold standard, Rapid Slide Culture is useful for early detection of viable Mycobacterium tuberculosis[10].

Recently serological tests have been used as an adjunct in the diagnosis of extrapulmonary tuberculosis to improve sensitivity. The 38 kda antigen is a surface exposed lipoprotein, a phosphate lipoprotein. Serodiagnostic tests based on the 38 kda antigen have been used to detect antibodies in different types of tuberculosis,bone and joint tuberculosis, tuberculous pleuritis and tuberculous meningitis. In a study conducted to evaluate the 38 kda antigen based immunochromatographic assay for the diagnosis of tuberculous pleuritis a sensitivity of 50.7% and a specificity of 57.7% was reported[11].The 38kda antigen has also been used to detect specific antibodies in the CSF of patients with tuberculous meningitis[11].

It is thought that a non conventional test may add to the diagnosis of extrapulmonary tuberculosis, however a negative non conventional test may not exclude tuberculosis in patients. Thus in a large number of patients diagnosis of extrapulmonary tuberculosis is delayed or missed. In this connection it may be useful to evaluate the value of the results of a number of tests in the diagnosis of extrapulmonary tuberculosis.

2. OBJECTIVES OF THE STUDY

1. To find out the sensitivity and specificity of Ziehl Neelsen's staining, Auramine staining and Rapid Slide Culture technique, comparing them with growth on Lowenstein Jensen's medium as gold standard.

2. To do a qualitative estimation of antibodies against 38kda, 16kda, 6kda antigens of Mycobacterium tuberculosis in the serum of patients suspected of extra pulmonary tuberculosis.

3. To evaluate the role of the above multiple tests, for the etiological diagnosis of extra pulmonary tuberculosis.

3. REVIEW OF LITERATURE

3.1. History of tuberculosis

Tuberculosis is one of the oldest human diseases ever known to mankind, a major cause of suffering and death since times in memorial. This disease is not only associated with medical implications but also has social and economical impact. Bunyan described it as 'The captain of men of death[2].

This disease referred by Oliver Wendell as the "White plague" is also known as Rajayakshema in the Vedas[12]. The word tuberculosis is a derivative of the Latin word tubercula which means a "small lump". They found changes resembling tuberculosis in the skeletal remains of Neolithic men. Evidence of tuberculous lesions of bone has also been found in Egyptian mummies dating back to 3400BC. It dates back to the era of Hippocrates where the earliest classical description of tuberculosis is found in Greek literature[12].

Tuberculous cervical lymphadenitis has been called scrofula, Kings Evil. During the middle ages there are records of healing touch of Monarchs, being used to treat scrofula. The credit goes to Fracastorius who found the germ theory of disease and he was the one who believed that tuberculosis was contagious . It was in 1720 that an English physician Benjamin Martin published that tuberculosis could be caused by certain species of animalcula or minute living creatures which gained entry into the body and produced pathognomonic lesions.

On 24th march 1882 it was Robert Koch who announced the discovery of the tubercle bacilli during the monthly evening meeting at Berlin physiological society. Lehmann and Neumann in 1896 introduced the generic name Mycobacterium, this was because of the mould like growth of the organism in liquid medium (Mould- Fungus).

3.2 Genus definition.

The genus Mycobacterium is the only genus in the family Mycobacteriacea and it belongs to the order Actinomycetales. Though many animals are susceptible to this infection only 100 Mycobacterial species are identified[13].

Standards for including a species in this Genus

1. Acid- alcohol fastness
2. Presence of mycolic acid containing 60-70 carbon atoms
3. G+C content of DNA (61-71mol %) [13]

3.3 MYCOBACTERIUM TUBERCULOSIS COMPLEX

This is a complex which refers to closely related groups of variants of a single species where in it causes tuberculosis affecting man and may other mammals.

M.tuberculosis -- Mostly human tuberculosis

M. caprae --- Goats / Veterinary surgeons

M. bovis ---- Principal cause of tuberculosis in cattle and many other mammals

M. microti ------ Rare pathogen of voles and other small animals and does not affect human being

M. africanum --- Appears to be intermediate between bovine and human type

3.4 MORPHOLOGY

The tubercle bacilli are straight or slightly curved, rod shaped measuring about 3x0.3micrometer in size. In clinical specimens they may occur singly or in clumps. They are nonmotile, nonsporing and noncapsulated.[14]

Mycobacterium are acid fast and alcohol fast. The cell resists decolourization with acid and alcohol, once they are stained with a specific dye. This is attributed to the presence of mycolic acid in the cell wall, integrity and viability of the cell wall[13].

The mycobacterial cell wall has higher lipid content which accounts for about 60% of the cell wall weight, the cell wall of mycobacteria has several distinct layers, the layers above the cell membrane is composed of peptidoglycan layer and above this is the arabinogalactan which is covalently linked to long chain fatty acids termed mycolic acid[12].

3.5 HABITAT AND GROWTH REQUIREMENTS.

Mycobacterium is isolated from water, soil, dust and sphagnum vegetations. Most of the Mycobacteria are non fastidious, are obligate aerobes they derive their energy from oxidation of many simple carbon compounds. The generation time in vitro is about 2 hours the

growth appears in about 2 weeks, may be delayed upto 6 to 8 weeks. The basic nutritional requirements are Carbon, Nitrogen, Oxygen, Phosphorous, Sulphur, Iron, Sodium, Potassium, Magnesium, and trace elements (primarily Zinc and Manganese).

The source of carbon is in the form of carbohydrates that is glucose, fructose, sucrose, mannose, trehalose, inositol, mannitol. It requires organic acid, long chain oleic and palmitic acids and hydrocarbons[3].

The optimum temperature for growth is $37c^{0.}$ The optimum ph is 6.4-7.0 .Increase in Co_2 (5% to10%) enhances growth, addition of Glycerol to the medium encourages the growth of human strains and not bovine strains.

3.6 Culture Medium

Culture methods provide definitive diagnosis by establishing the viability and identity of the organism. Culture is considered as the gold standard. The different culture mediums used for growth of Mycobacterium tuberculosis are solid and liquid mediums.

SOLID MEDIUMS

1. Lowenstein Jensens Medium.
2. Pawlowskys medium (potato medium)
3. Tarshis medium (Blood medium)

LIQUID MEDIUMS

1. Dubo's medium
2. Middle brooks medium
3. Sula's medium

Most commonly used solid medium is Lowenstein Jensens medium which contains egg, asparagines, glycerol, malachite green and some mineral acids

3.7. CULTURAL CHARACTERISTICS

The type of growth seen in culture mediums can be described as eugonic and dysgonic. Humans strains grow more luxuriantly in culture: eugonic than do bovine strains: dysgonic.

Colony characteristics:- On solid medium human type of tubercle bacilli gives rise to discrete, raised, irregular dry and wrinkled colonies which are creamy white to begin with and then develop buff coloured colonies. The bovine type grows as flat white smooth moist colonies which break up more readily when touched.

In liquid medium it grows as a pellicle which is wrinkled on top of the medium, if the medium is left undisturbed they will grow as floccules throughout the medium, by adding tween 80 which is a wetting agent, diffuse growth can be obtained. The differentiation between virulent and avirulent strains can be made out by the growth in the liquid medium; large serpentine cords are formed in case of virulent strains. Growth is more dispersed in avirulent strains[12].

3.8. EPTB Epidemiology:

Tuberculosis is classified as pulmonary and extra pulmonary. EPTB is described as an isolated occurrence of tuberculosis at body sites other than lungs. Tuberculosis may affect any organ in the body and although most cases of clinical TB involve the respiratory system, the

EPTB constituted about 15% to 20% 0f all cases of TB and with an increase in incidence of 50% in HIV positive individuals[12].

Mode of transmission

Tuberculosis infection is primarily acquired by the inhalation of dried residues of droplets containing tubercle bacilli that have been expelled in an aerosol created during coughing sneezing or talking. These droplet nuclei remain suspended in the air for prolonged period and those particles ranging between 1 to 10 micro meters in diameter are significantly small to reach the alveoli and initiate infection[15].

3.9. PATHOGENESIS

Following inhalation of the virulent tubercle bacilli in droplet nuclei the organism reaches the alveolar spaces where they are phagocytosed by alveolar macrophages. These organisms multiply within the macrophages, with minimal reaction and spreads to regional lymph nodes in the hilum of the lungs. In the primary infection the tubercle bacilli always spreads from the initial site via the lymphatics to the regional lymph nodes resulting in a Ghon's complex.

Then the bacilli spreads farther and reaches the blood stream Once the organism reaches the blood stream they are disseminated to various organs. Asymptomatic lymphohaematogenous dissemination of the primary infection occurs before the acquisition of hypersensitivity to tubercular protein and sets a stage for later reactivation to present clinically as extrapulmonary tuberculosis[16]. The common sites of

extrapulmonary infections are Lymphnodes, pleura, osteoarticular, meningeal, genitourinary tuberculosis.

Tuberculous Lymphadenitis.

Tuberculous lymphadenitis is one of the most commonest form of extrapulmonary tuberculosis[6]. Lymphnode tuberculosis(LNTB) is considered to be the local manifestation of a systemic disease. Cervical lymphadenopathy is the most common form of (LNTB)[17]. It has been increasingly observed in India with growing epidemics of HIV infections. It is rated that lymphadenopathy may be the only manifestion of the disease.

LNTB is relatively more common in younger patients (10-30year). The lymphnode affected may be discrete or multiple malted nodes[13].There is periadenitis of the lymphnodes resulting in the fixation of the lymphnodes. The lymphnodes coalesce and break down due to the formation of caseous pus, this may result in the formation of sinuses, healing may occur following calcification and scarring.

Lymphadenopathy may be the sole manifestation of the disease without the associated features of high grade fever, loss of weight, cough and other respiratory symptoms.

Pleural effusion

Tuberculous pleural effusion is categorised as extrapulmonary despite an intimate anatomic relationship between pleural and the lungs[12].Tuberculous pleural effusion is the most common exudative pleural effusion prevalent in India in contrast to the west where

malignant pleural effusion is more frequent. It remains an important treatable cause of exudative pleural effusion[18].Mycobacterium tuberculosis invades the pleural cavity chiefly through rupture of sub pleural caseous foci. Mycobacterial protein antigens induces a delayed hypersensitivity reaction.The fluid accumulated is an exudate with lymphocytic predominance[20]. Acid fast staining was positive in less than 10% of patients in most reports, where as culture for Mycobacterium tuberculosis were positive in 12 to 70% of cases[19] Tuberculous pleural effusion usually presents as an acute illness. Most patients complain of pleuritic chest pain, non–productive cough and dyspnoea. Majority of the patients manifests with fever, though a few may not have fever. Occasionally, the onset may be less acute, with only mild chest pain, low-grade pyrexia, cough, weight loss and loss of appetite.

Neurological tuberculosis:

Neurological Tuberculosis comprises 5 to 10% of cases of EPTB and occurs more frequently in children. This condition is associated with increased mortality and morbidity of all the forms of EPTB[4].

Tuberculous meningitis (TBM) accounts for 70 to 80% of cases of neurological tuberculosis and is still an important public health problem in developing countries.TBM is invariably secondary to tuberculosis elsewhere in the body It is generally believed that the critical event in the development of meningitis is the rupture of a sub

ependymally located tubercle (Rich focus) resulting in the delivery of infectious material into the subarachnoid space[12].

Skeletal tuberculosis

Evidence of osteoarticular tuberculosis has been found in prehistoric humans.In India it accounts for nearly 1-5% of cases[21]. In immunocompetent individuals the osteoarticular involvement occurs in 10% of patients with extrapulmonary tuberculosis[12]. TB commonly affects the spine and the hip joints, other sites of involvement includes knee joints, foot bones, elbow joints and hand bones, it rarely affects the shoulder joints.

Skeletal tuberculosis occurs due to haematogenous spread and affects almost all bones. The disease process can start either in the bone or in the synovial membrane, there after it spreads to other structures[12]

Genito urinary tuberculosis

Urogenital TB complicates 3 to 4% of all cases of pulmonary TB and it constitutes at least 30% of all cases of extrapulmonary TB.[12] Kidney being the most common genitourinary site of involvement Heamatogenous dissemination from an active site of infection after pulmonary infection leads to the formation of metastatic lesions in the kidney the intensity of infection depends on the infecting dose, virulence of the organism and the resistance of the host.

Tuberculosis of the urinary tract is easily overlooked. Many patients present with lower urinary symptoms typical of conventional bacterial

cystitis and suspicion of tuberculosis arouses only when there is no response to the usual antibacterial agents[49].

The diagnosis of tuberculosis of the urinary tract is based on the finding of pyuria in the abscence of infection as judged by culture on routine media[49].The diagnosis is finally established by culture on Lowenstein Jensens medium. Altenative strategy to diagnose tuberculosi by use of nucleic acid amplification methods to detect fragments of Mycobacterial DNA in urine have been developed. The detection of transrenal DNA makes it possible to assay the total body burden of mycobacterial infection in any age group and in extrapulmonary tuberculosis with urine samples which can be collected noninvasively[51].

The primary focus of genital TB in the female genital tract is the fallopian tube in 100% cases[22]. Spread from the fallopian tube to the uterus can occur in up to 50% of patients with tubal infection. In men the prostate, seminal vesicles and epididymis may be involved, where caseating granulomas, abscesses and calcification may ensue[12].

Genital tract TB has been reported in patients presenting with infertility, chronic pelvic pain and menstrual irregularities. The world wide incidence of genital TB in infertile women is 5% to 10%. The incidence of genital TB has been estimated to range from 1 to 7% in India[12].

Abdominal tuberculosis

It includes tuberculosis of the gastro intestinal tract, peritoneum, omentum, mesentry, lymph nodes and other solid intra abdominal organs. Tuberculous peritonitis constitutes 4% to 10%of all patients with EPTB. It is an endemic disease among socio economically poorer communities in developed and developing countries. The diagnosis of abdominal tuberculosis is made difficult because of the paucity of Mycobacterium tuberculosis in peritoneal fluid[43].

Tuberculosis of the breast:

Breast tuberculosis is a rare form of tuberculosis. The first case of mammary tuberculosis was reported by Sir Ashley cooper in 1829.The significance of breast tuberculosis is due to its rare occurrence and mistaken identity with breast cancer and pyogenic breast abscess[23].

Breast tissue is remarkably resistant to tuberculosis[23] .The breast may become infected in a variety of ways haematogenous, lymphatic,direct inoculation, spread from contiguous structures and ductal infection.Breast tuberculosis most commonly presents as a lump affecting the women of the reproductive age group.The second common mode of presentation is Tubercular breast abscess.

Disseminated tuberculosis:

Disseminated tuberculosis has been reported in underlying illness such as diabetes ,alcohol abuse,parentral drug abuse, or other conditions with immunosupresion.It commonly posses diagnostic difficulty as the primary symptoms and clinical findings are often not

characteristic. In such circumstances where it is difficult to establish the sites involved with disease, analysis of bone marrow may yield important insight. Clinical confirmation of the diagnosis of disseminated tuberculosis is established by culture confirmation[50].

3.10. **PATHOLOGY**

Tuberculosis: It is a chronic granulomatous inflammation. The pathognomonic lesion in tuberculosis is a "tubercle" in the infected tissue. There is a fibrin rich alveolar exudate containing a mixture of inflammatory cells. When there is an accelerated inflammatory process, an area of caseous necrosis surrounded by epithelioid cells, granulation tissue and eventually fibrosis[16]. Histological sites of active involvement are marked by characteristic granulomatous inflammatory reaction that forms both caseating and non caseating tubercles, individual tubercles are microscopic it is when these granulomas coalesce they become macroscopically visible. The granulomas are usually enclosed within a fibroelastic rim punctuated by lymphocytes and multinucleated giant cells.

3.11. **IMMUNOLOGY**

The important mechanism of protection against tuberculosis is the cell mediated immunity. Since the main route of entry of the causative agent is the respiratory tract, alveolar macrophages are the important cell types which combat the pathogen. There is an interaction between the macrophage and the Mycobacterium where in there is binding of M tuberculosis to the surface receptors of macrophages, fusion of

phagolysosome, mycobacterial growth inhibition and killing through free radical based mechanisms such as reactive oxygen and nitrogen intermediates followed by, recruitment of accessory immune cells for local inflammatory response and presentation of antigens to T cells for development of acquired immunity. The components of innate immune response are the natural resistance associated macrophage protein (Nramp), neutrophils and natural kill cells[24].The NRAMP1 gene codes for the natural resistance associated macrophage protein, belonging to a divalent metal transporter and play an important role in the homeostasis of iron and other metals. This protein is implicated in the control of growth of intracellular pathogens[44].

In a period of 2 to 6 weeks after the entry of the organism a cell mediated immunity develops and there is an influx of lymphocytes and activated macrophages into the lesion resulting in a granuloma formation.

The specific acquired immune response through the CD4 T cells is mainly responsible for protective function. The effect of cytotoxicity is brought about by CD_8 cells. Humoral immune response is seen though not implicated in protection. The activated CD_4 helper T cells can either be TH_1 or TH_2 cells. Th_1 dependent cytokines like gamma interferon's activate macrophages resulting in protective immunity and containment of the infection. TH_2 cytokines like IL-4, 5 and 10 induces delayed type of hypersensitivity reaction, tissue destruction and progressive disease[24]. The recent discovery of the importance of

the TLR protein family has provided a new insight into the link between innate and adaptive immunity. The interaction between Mycobacterium tuberculosis and TLR are complex and it appears that distinct Mycobacterial components may interact with different members of the TLR family. Mycobacterium tuberculosis can immunologically activate cells via TLR2 or TLR4 in a CD_{14} independent ligand specific manner.

3.12. **IMMUNOGENETICS OF TUBERCULOSIS**

An important area in understanding the immunology of tuberculosis is host genetics. It has been found that Mycobacterium tuberculosis is associated with HLA- DR2 and DQ1, antigens with the susceptibility to pulmonary tuberculosis. The polymorphic BSMI, ApaI, TaqI and TokI gene variants of VDR (Vitamin D receptors) showed differential susceptibility or resistance with male and female subjects.

The Non HLA gene polymorphisms such as Mannose binding lectin (MBL), Vitamin D receptors, TNF α and β, IL-1 receptor antagonist and Nramp genes revealed that functional mutant homozygotes of MBL are associated with the susceptibility to pulmonary tuberculosis[24].

3.13. **HIV AND TUBERCULOSIS.**

With the global rise of human immuno deficiency viral infections over the last decade there is an increased association of EPTB in HIV infected individuals. HIV increases the susceptibility of patients to

tuberculous infection and disease. Tuberculosis can occur in all stages of HIV infection, reduced Th_1 responses observed in these patients is thought to increase their susceptibility. It is found that individuals who are already infected with tuberculosis, co-infection with HIV increases the risk of endogenous tuberculous activation. Tuberculosis is a leading cause of HIV related morbidity and mortality. In 2001, approximately 11% 0f worldwide new tuberculosis cases in adults in the age group 15-49 years were attributable to HIV infections. Of 1.9 million deaths due to tuberculosis worldwide, 18% were due to HIV infection[25].

3.14. **CLINICAL FEATURES**

Patient with EPTB may manifest with nonspecific symptoms like fever, anorexia, weight lose, malaise, fatigue. In India patients with EPTB especially when the disease is located at an obscure site this may be the only diagnostic clue in them. In addition patients with EPTB manifests with symptoms and signs related to the organ system involved.

3.15. **DIAGNOSIS OF EXTRAPULMONARY TUBERCULOSIS.**

Diagnosis of tuberculosis is mainly based on clinical feature histopathology, demonstration of AFB and isolation of Mycobacterium tuberculosis from the clinical specimen. Serology, tuberculin testing, radiological examination and other imaging methods are supplemental.

Laboratory methods

Microscopy:-

Mycobacteria are recovered from variety of pulmonary and extrapulmonary samples. To be detected microscopically there must be 10^4 organisms per ml[13,26].

Acid fast staining:

In 1882 Ehrlich discovered that Mycobacteria stained with Fuchsin in the presence of aniline oil as a mordant resists decolourization by mineral acids. It was Ziehl who changed the mordant to carbolic acid and in 1883 Neelsen increased the concentration of carbolic acid and incorporated it with the dye to form carbolfuchsin thus the standard stain for demonstrating acid fastness:

The Ziehl Neelsen stain.

The primary stain in Ziehl- Neelsens technique is concentrated carbol fuchsin, heat along with phenol present in carbol fuchsin helps in penetration of the dye, 25% sulphuric acid acts as the decolourizing agent. This is counter stained with 1% methylene blue. The bacilli are stained pink and the background along with pus cells and other bacteria appear blue. Ziehl-Neelsen method of staining has a sensitivity and specificity of 33.79% and 100% respectively[47]

Factors influencing results of smear are:

a) Thickness of smear
b) Extent of decolourization
c) Type of counterstaining
d) The person examining the smear

Other methods of staining:

Kinyouns staining technique: Here the only modification is there is no application of heat. It is otherwise called cold staining method. Only disadvantage is that the surface active agents that increase permeability of the dye through the waxy capsule may render the cell more susceptible to acid decolourizers. Hence less stringent decolourizer such as 1% HCL may be used.

Fluorescent Microscopy (Auramine Phenol)

In this technique the smears are stained with special dyes called fluorochromes which have the property of absorbing light rays of shorter wave length and emitting light rays of longer wavelength as a result of which the stained particles appear like bright objects (Fluoresce). Here Auramine is used as a flurochrome where in the organism appears as bright yellowish orange rods. It is less laborious for the technician and is popular as screening method. 10^4 bacilli/ml of specimen is required for positivity. However this requires fluorescent microscope which may not be available in all laboratories[28].

3.16. **CULTURE:**

The most reliable way of establishing diagnosis of Mycobacterium tuberculosis is by culture. The clinical specimen submitted to the Mycobacteriology lab for culture are specimens like sputum, urine, pus which are usually contaminated. Specimens like CSF, lymph node

aspirate, biopsy material, pleural fluid are collected under sterile conditions avoiding contamination.

Lowenstein Jensens medium[29] is the most popular media for isolating human strains of Mycobacterium tuberculosis and other mycobacterial species. For culture to be positive there must be at least 10-100 organisms /ml of the sample.

Robert Koch was the first person to use Rapid slide culture technique using coagulated human serum. The growth of Mycobacteriun tuberculosis was obtained in 7 days. However the drawback was high rate of contamination [30]. RSC technique using human blood medium (HBM) with added antimicrobials has been found to be a simple, safe, sensitive method with culture available in 7 days[30]. Some of the studies have also used RSC technique for drug susceptibility. No studies have focused on the use of RSC technique in detection of extrapulmonary tuberculosis.

For detection of Mycobacterium in clinical specimens the current gold standard consist of a combination of solid and liquid media[13]. Liquid medias like Middle brook 7H9 or Dubos Tween albumin, the biphasic septichek system, the Bactec 460 TB system the most efficient and rapid technique to culture Mycobacteria for more than two decades[13].

3.17 NEWER DIAGNOSTIC TECHNIQUES FOR TUBERCULOSIS

Several rapid techniques for detection of early growth (5-14days) as compared to 2-8 weeks with conventional methods have been

described which can help in obtaining the culture and sensitivity report early.

1. BACTEC
2. MGIT
3. Septic- chek
4. MB/Bact systems
5. PCR/RFLP methods
6. Ribosomal RNA sequencing.

BACTEC SYSTEM:-

This system is developed by Becton Dickinson based on generation of radioactive CO_2 from substrate palmitic acid. It measures $^{14}CO_2$ released during metabolism of ^{14}C labelled substrate, reducing the time required for detection of Mycobacterium tuberculosis from weeks to days (12-15days). This method has been extensively used all over the world and growth can be detected in 5 to 10 days in this system. Inclusion of NAP (Beta nitro alpha acetylamine, Beta hydroxyl propiophenone) helps in distinguishing Mycobacterium tuberculosis from other Mycobacteria

MGIT:- Mycobacterial growth indicator tube: Growth is detected by a non radioactive detection system using fluorochromes for detecting and drug screening . This system helps in early detection (7-12 days) of mycobacterial growth.

MB/Bact: This system (Organon technika) is adapted from the strategy of colorimetric detection earlier tried for detection of bacterial growth in blood cultures. This has been reported to be useful for drug susceptibility testing of mycobacterium tuberculosis.

Septi-Chek:- This is a biphasic medium system (Roche) consisting of an enriched selective broth and a slide with non selective Middlebrook agar on one side and with two section on other side one with NAP and egg containing agar, second with chocolate agar for detection of contamination this system has also been found to be quite useful for rapid detection of growth of mycobacterium[26].

Reporter phages:- Mycobacterial phages and reporter genes like luciferase have been successfully used for detection of growth and for assessing the drug susceptibility to anti-TB drugs. Indication of visibility could be either emission of light from organism due to activation of Luciferase gene or production of a plaque on an indicator strain of Mycobacteria. Results can be obtained in 48hrs and such systems are commercially also available (Biotec/Medispan) [26].

Phage Assay: Recent development in tuberculosis diagnostics is the phage assay. Which utilises specific Mycobacteriophages that can infect Mycobacterium tuberculosis complex and M.smegmatis. in Mycobacterium smegmatis, the lytic cycle of Mycobacteriophage is completed within 90 minutes whereas lysis takes approximately 13 hours in Mycobacterium tuberculosis complex, There by making results available rapidly in terms of plaques[48].

3.17. Rapid identification of Mycobacterial isolates

a. Chemical methods based on lipid profile

b. Hybridization with specific gene probes.

c. Gene amplification methods.

Analysis of lipid profile: Mycobacteria have characteristic lipid profile, these can be analysed by HPLC and quick identification of Mycobacterial isolates can be done.

DNA Probes: Based on information about specific gene sequences well defined oligo nucleotide probes for identification of various clinically relevant Mycobacteria have been developed. These probes are being used in several countries for rapid confirmation of the identity of Mycobacterial isolates, when used along with newer methods of detection of the early growth (Such as Bactec, Septichek, MGIT), these are of greatest help in rapidly confirming the diagnosis as identity of isolates can be established in 1 or 2 days with gene probes as compared to much longer time required with classical biochemical tests.

Gene amplification methods: Gene amplification techniques have made a major impact on the diagnosis of Mycobacterial diseases. Gene amplification techniques are highly sensitive and under optimum conditions may detect 1-10 organisms. If systems are adequately standardized, evaluated and precautions for avoiding the contamination are taken, confirmation of diagnosis in paucibacillary extra-pulmonary forms of tuberculosis is accurately acheived[26].

IS6110 Polymerase chain reactions – restriction fragment length polymorphism(PCR-RFLP) methods such as gene for hsp 65 Kda protein, KatG and rRNA genes and sequencing of 16S rRNA have been described. The RFLP technique using IS6110 repititive sequence[31] as a probe is considered as the gold standard for typing Mycobacteriumtuberculosis complex strains.

Biomarkers: Biomarkers are measurable characteristics that indicate normal biological process, pathogenic process, or pharmacological responses to a therapeutic interventions. Some of the tuberculosis specific biomarkers are urine tuberculosis DNA,Anti alanine dehydrogenase, sputum Ag 85,sputum Ag 85 B-RNAand volatile organic compounds[52].

3.18. Non tuberculous mycobacteria

Mycobacteria other than mammalian tubercle bacilli are usually saprophytes but can cause opportunistic infections. They are refered to as atypical Mycobacterium, Non Tuberculous Mycobacteria (NTM) or Mycobacteria Other Than Tubercle bacilli (MOTT)[32]. NTM has been classified in to four groups by RUNYON based on pigment production and rate of growth

Group i: Photochromogens

Group ii: - Scotochromogens

Group iii: - Non photochromogens

Group IV: - Rapid growers

Chronic pulmonary disease, Lymphadenitis in children and infection of the skin, soft tissue and skeletal system are the common infections caused by NTM. The principal etiologic agents are Mycobacterium intra cellulare complex, Mycobacterium kansasi, Mycobacterium marinum, Mycobacterium fortuitum/chelonae complex and Mycobacterium scrofulaceum.

Disseminated disease has also become more common with the increase in the number of AIDS and cancer patients and individuals receiving immuno suppressive treatments[32].

Isolation of the Mycobacterium from representative specimens and their rapid identification as treatment strategy for tuberculosis and other mycobacteriosis is different.

3.20. IDENTIFICATION

Mycobacterium tuberculosis (Mtb)

Colony morphology

On solid media Mycobacterium tuberculosis appears as dry, rough, raised, wrinkled buff coloured colonies.

In liquid media, it grows as a pellicle which is wrinkled on top of the medium; they will grow as floccules throughout the medium. The differentiation between virulent & avirulent strains can be made out by the growth of large serpentine cords in case of virulent strains and dispersed in case of avirulent strains.

Culture smear: Positive cultures are confirmed by acid fast smear.

Rate of growth: Mtb is slow growing and takes 3 to 8 weeks to form visible colonies on solid media.

Pigment production: Mtb not produce any pigment.

Niacin test: Niacin accumulates when Mtb grows in egg containing media.

Nitrate test: Mtb reduces nitrate to nitrites.

Growth on paranitrobenzoic(PNB) acid containing medium: M.tb is sensitive to PNB and hence there is no growth in the presence of PNB, where as non-tuberculous mycobacterium grows in presence of PNB.

Mycobacterium tuberculosis is an acid fast bacilli which is slow growing, characteristically forms dry, rough, raised, irregular, buff coloured colonies on LJ medium, it does not produce pigment, fails to grow on PNB containing medium, reduces nitrate and accumulates niacin.

NTM

On the basis of rate of growth on solid media and pigment production NTM can be classified in to 4 groups, further identification can be done on the basis of additional biochemical tests.

Catalase test

Enzyme catalase forms oxygen from H_2O_2 which is indicated by the presence of effervescence. Semiquantitative catalase test quantifies

the amount of catalase produced. $68^{O}C$ catalase test determines whether catalase production is heat sensitive. Mycobacterium tuberculosis is heat stable catalase test negative, most atypical mycobacterium are positive

Arylsulfatase test:

Demonstrates the presence of the enzyme arylsulphatase in some Mycobacterial species, which splits phenolphthalein from tripotassium phenolphthalein sulphate. Free phenolphthalein in the presence of sodium bicarbonate gives pink colour to the test medium. This test is useful in differentiating rapid growers from non photo chromogenic Mycobacteriae.

TWEEN 80 Hydrolysis: Some mycobacteria have the ability to hydrolyse Tween80 to oleic acid. M.kansasi/M.gordonae hydrolyse Tween 80 while M.scrofulaceum doesn't.

Growth on MacConkey agar: This test detects ability of Mycobacterial species to grown on MacConkey agar. Rapid growers grow on MacConkey agar while other mycobacterial species doesn't grow.

Iron uptake test: Demonstrate the ability of Mycobacterium species to utilize iron from an inorganic source like ferric ammonium nitrate, useful test in differentiating M.fortuitum which is positive and M.chelonea negative.

Urease test: This test demonstrates presence of enzyme urease M.scrofulaceum positive: M.gordonae negative.

Growth on 5% Sodium chloride: M.flavescense, M.fortuitum, M.abscessus. are able to grow on 5% sodium chloride.

3.21. Serodiagnosis of tuberculosis

Antibody detection tests

Various antigens have been evaluated for detection of antibodies to Mycobacterium tuberculosis. The A60 is most extensively used Ag for both pulmonary and extra-pulmonary, adult and child TB. IgG and IgM detection has been evaluated. Tests are also available which uses purified Ag mainly 38kda and 30kda for detection of antibodies[11]. Presence of antigens may be better indicator of disease than antibody detection.

Antigen detection tests

Lipoarbinomannan urine test:

The test detects Lipoarabinomannan in urine and is a surrogate marker for Mycobacterium tuberculosis infection. This test is available in ELISA and simplified tube form. A number of antigen capture assays based on ELISA or RIA or agglutination of antibody coated latex paricles are in use[12].

Quantiferon TB gold assay:

Whole blood gamma interferon assays are promising candidates to improve the current level of diagnosis accuracy for tuberculosis infection, in particular if skin tests are equivocal. Quantiferon TB gold test is used for diagnosis of latent Mycobacterial infection and has higher specificity than tuberculin skin test when used for selected population especially in cases of tubercular meningitis[33].

4. METHODOLOGY

Study design: Cross- sectional study

4.1 Source of data

All clinical specimens from patients suspected of extra pulmonary tuberculosis inclusive of HIV infected patients at R.L.Jalappa hospital and research centre, attached to Sri Devaraj Urs Medical College, Tamaka, Kolar, were collected.

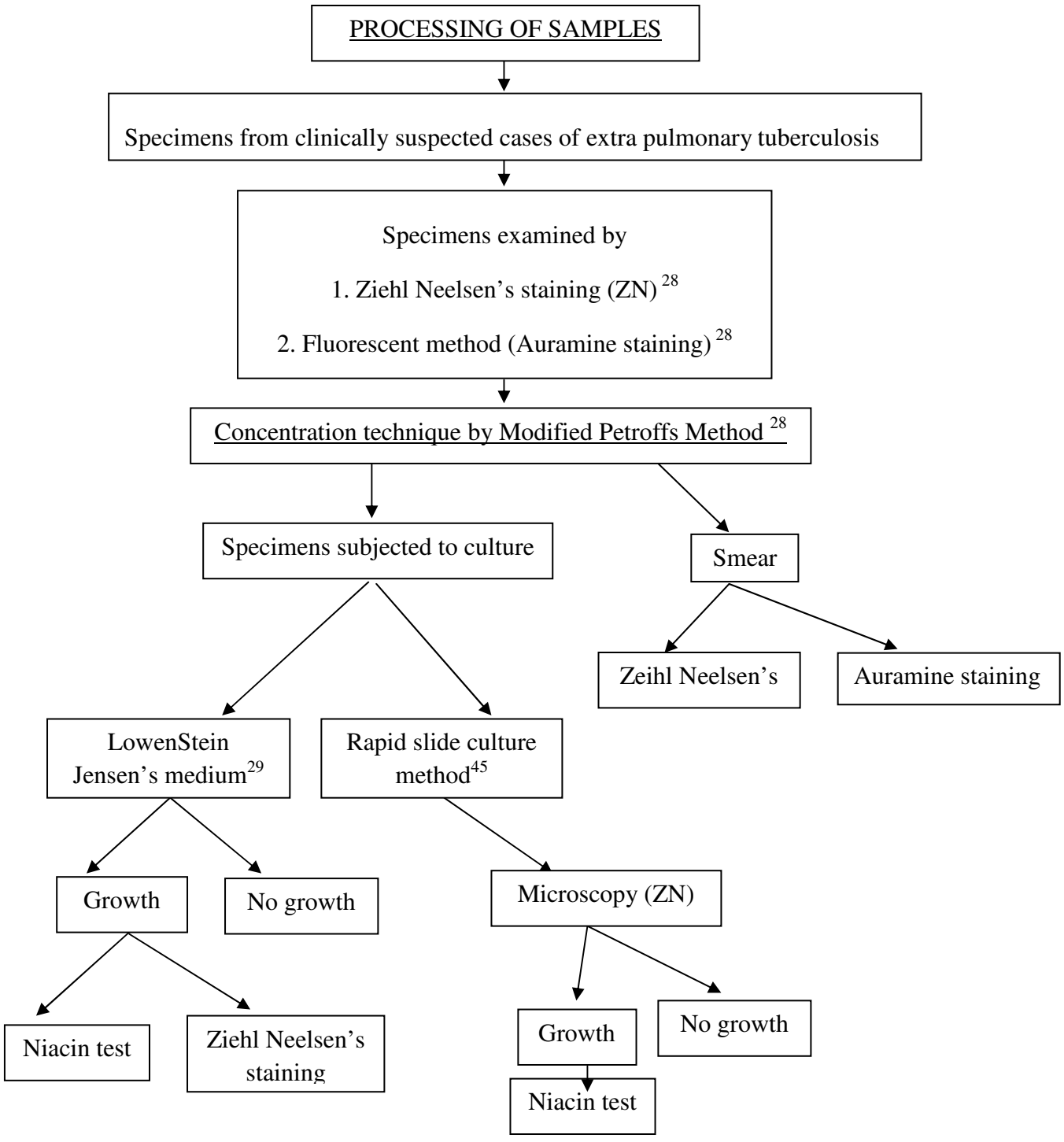
Exclusion criteria: Patients whose sputum was positive for acid fast bacilli (AFB) and diagnosed with pulmonary tuberculosis.

Sample size: 66 Samples were collected during the period of December 2008-August 2010(1 year 9 months).

The following extra pulmonary specimens were processed as per the RNTCP guidelines[28].

1. Pleural fluid -29
2. Pus samples -11
3. Lymph node aspirate-6
4. Biopsy specimens -6
5. Cerebrospinal fluid(CSF)-4
6. Synovial fluid-3
7. Ascitic fluid-3
8. Urine- 2
9. Bone marrow aspirate-2
10. Blood specimens were collected for serological study, for detection of antibodies against 38 kda, 16kda, and 6kda antigens of Mycobacterium tuberculosis.

Specimens sent in saline were accepted. Those specimens which were sent in formalin were not accepted. The specimens collected were sent to the laboratory immediately and were processed, in case of delay these specimens were kept at 4^0 c in the refrigerator. An informed consent was taken from each patient for doing the tests including serology. The tests were conducted in the following order.



Niacin test - Positive – Mycobacterium tuberculosis

Negative – Non Tuberculous Mycobacteria

If growth occurs within 1 week it was identified as Non tubercular Mycobacterium (NTM) by.

1. Ziehl Neelsen's staining.

2. Culturing on to MacConkey agar.

3. Niacin test.

Serology: All the patients evaluated for Mycobacterial microscopy and culture were also subjected to serological studies for detection of antibodies in patient suspected of extra pulmonary tuberculosis.

4.2. Procedures:

Macroscopic examination:

1) Quantity of the specimen.
2) Quality of the specimen: purulent, mucopurulent, blood stained, tissue biopsy material.

Microscopic examination:

Smear preparation: Direct smear for ZN and Fluorescent method

An appropriate portion of the specimen was transferred to a clean scratch free new decontaminated glass slide and was spread over an area of approximately 2 cm by 1cm.The slide was allowed to air dry for 20 minutes and the smear was fixed by passing the slide through the flame and then allowing it to cool, it was stained by Ziehl Neelsen method of staining and fluorescent method of staining using Auramine Phenol.

Smear preparation: Direct smear preparation from the sample before decontamination for RSC. An appropriate portion of the specimen was transferred to the lower 1/3 rd of a clean scratch free new decontaminated glass slide and air dried[45].

A) **Ziehl Neelsen's staining**

The techniques followed for staining are as per Revised National Tuberculosis Control Programme (RNTCP) guidelines[28]. The heat fixed slide was kept on a staining rack and the smear was flooded with 1% carbolfuchsin stain reagent .The slide was gently heated until the vapour rises .Carbol fuchsin was left on the slide for 5 minutes. The slide was gently rinsed in tap water and was decolourised with 25% sulphuric acid for 2-4 minutes. The slide was gently washed with tap water and the decolourisation procedure was repeated until the smear looked colorless, then the slide was counterstained with 0.1% methylene blue reagent for 30 seconds and air dried .

Examination of the slide. (Ziehl Neelsen's staining)[28]

The slides were examined under microscope using 40X to select the suitable area and then examine under 100X lens using a drop of immersion oil.

Table I Grading of smear stained by ZN method

ZN Staining grading (RNTCP)	**Reporting /Grading**
>10 AFB/field after examination of 20 fields	Positive, 3+
1-10 AFB/field after examination of 50 field	Positive, 2+
10-99 AFB/100 field	Positive, 1+
1-9 AFB/100 field	Positive, scanty
NO AFB per 100 fields	Negative

B) **Fluorescent method of staining** (Auramine staining)[28]

The heat fixed, dried smears were flooded with phenol auramine and allowed to stain for 15 minutes. The slide was rinsed with water and drained. The slide was decolorised with acid alcohol (for 2 minutes) (97ml H_2SO4 with 3ml of HCL) and washed with water and drained. The slide was counterstained with potassium permanganate for 2 minutes, washed with tap water and drained.

Examination of slides stained by fluorescent method

The slide is examined with a fluorescent microscope under low power objective 10x. A known positive slide was examined first to ensure that the microscope is correctly set. Mycobacteria were stained yellow-orange against a dark background. The morphology was confirmed with 40x. The slide is examined for a minimum of two minutes before declaring it as negative for AFB.

Table II Grading of smear stained by fluorescent method[28]

No. of bacilli	Grade
No bacillus seen after complete examination of smear for 2 minutes.	Negative
1-5 bacilli in most fields.	1+
More than 5 bacilli in most fields	2+
Masses of bacilli in most fields.	3+

The sample was then concentrated by modified petroff's method. The supernatant was discarded and the sediment was used for making smears and inoculation. Three smears were made from the sediment. 1 smear were stained by ZN method, 1 by

fluorescent method and examined for acid fast bacilli. On one slide the smear was made in the lower 1/3rd of the slide and was taken for RSC.

Modified Petroff's method

1) The whole sample was transferred to a McCartney bottle. Double the volume of sterile 4% Sodium hydroxide (NAOH) was added aseptically.
2) The caps of the Mc Cartney bottle was tightened and mixed well. The bottles were inverted to ensure that NAOH solution contacts all the sides of the bottle and the inner portion of caps.
3) The bottles were placed in Kahn shaker and kept in 37ºC incubator for 20 minutes.
4) At the end of 20 minutes, McCartney bottle were removed from the incubator and sterile distilled water was added up to the neck of the bottle.
5) This was mixed well and centrifuged at 4000 rotation per minute (rpm) for 20 minute.
6) McCartney bottles were carefully removed from the centrifuge without shaking. The supernatant fluid was discarded into 5% Lysol or phenol solution.

Note: Splashing was avoided to minimize aerosol and the whole process was carried out in a biosafety cabinet.

7) From the sediment, two slopes of LJ medium (using a sterile cool 5 mm 22 gauge nichrome wire loop) were inoculated. The bottles were labeled with the lab serial number of the specimen and named as B1 and B2.One loopful of the sediment was used for inoculating each LJ slope. All the LJ slopes were incubated at 37ºC.

8) The growth was checked weekly for eight weeks.

Procedure for Cerebro-spinal fluid, pleural, peritoneal fluids, synovial fluid,bone marrow aspirate ,ascitic fluid:

1) The decontamination with 4% NaOH was not necessary if collected with sterile precautions. The material was centrifuged .Supernatant was discarded in to 5% Lysol or phenol solution and the centrifuged deposit was inoculated on to 4 LJ slopes as well as smears were made.

Procedure for processing pus samples:

1) If Pus was obtained from an unopened abscess or a gland and is collected with sterile precautions, it was cultured without decontamination. In case of an abscess, the surface was sterilized and pus taken out in a loop through an opening made or with a sterile syringe. Equal volumes of 4% NaOH was added and mixed well. Further the steps as mentioned in modified Petroffs method were followed.

Procedure for urine specimens:

1) The entire quantity of Urine was centrifuged at 4000 rotations per minute (rpm) for 20 minutes. After centrifugation the supernatant fluid was poured into 5% Lysol or phenol solution and 2 ml was left in the test tube/container. This was mixed well by shaking vigorously and was transferred in to a sterilized McCartney bottle. 1 ml of 4% NaOH was added to the specimen in McCartney bottle. The caps of the McCartney bottle were tightened and mixed wel. Each bottle was inverted to ensure that NaOH solution contacts all the sides and inner portion of caps, after shaking the bottle it kept aside for 5 minutes. Shake and at

the end 5 minutes, sterile distilled water was added up to the neck of the bottle and mixed well and centrifuged at 4000 rpm for20 minutes.

2) McCartney bottles were carefully removed from the centrifuge machine without shaking. The supernatant fluid was discarded into 5% Lysol or phenol solution.
3) From the sediment, four slopes of LJ medium were inoculated using a sterile cool 5mm, 22gauge nichrome wire loop. The bottles were labeled with the lab serial number of the specimen as B1, B2, B3 and B4. One drop of sediment is used for each inoculation. A smear is prepared from the deposit after inoculation of each specimen. All the Mc Cartney bottles were incubated at 37ºC.
4) Growth was checked weekly for twelve weeks.

Three smears were made from the sediment for staining with Ziehl-Neelsen stain ,Auramine staining and a smear made at the lower one third of the slide for inoculation in to Human Blood Medium(HBM) for Rapid slide culture.

The sediment was inoculated on to Lowen stein Jensen's medium and incubated at 37^{o} C and followed up for 12 weeks. Growth, if any, was confirmed by biochemical reactions using Niacin test. A control strain of Mycobacterium tuberculosis H37Rv was inoculated on LJ medium. The supernatant fluid is discarded into 5% Lysol.

Niacin test was performed by Niacin test kit dtection from HI MEDIA.

Table-III Grading of positive L J Culture[46]

<19 colonies	Actual number of colonies to be written
10- 200 discrete colonies	1+
>100 colonies	2+
Confluent growth	3+

RAPID SLIDE CULTURE.

Human Blood Medium was used for Rapid slide culture technique[45]. Unused but not more than 4 weeks old citrated human blood was used to prepare the HBM. The blood is diluted with equal volumes of sterile deionized water to cause haemolysis. The medium was made selective by adding Trimethoprim (10 mg/L), Amphotericin B (10mg/L) and Ceftazidime (100mg/L). pH of the medium was adjusted between 6.5-7.5. Seven ml of this solution was dispensed in sterile screw capped McCartney bottles with antifungals and antibiotics to eliminate chances of contamination,this constituted one unit of HBM.

Smear was made on the lower one third of a clean slide and air-dried. The slide was then immersed in the HBM in such way that smear on the slide remained dipped in the medium. Inoculation was done in duplicates. The bottle was incubated at 37^{o}C for 7 days. On the seventh day slide was taken out, washed with distilled water and placed in an oven at 80^{0}C for 30 minutes. Any growth was confirmed by Zielh-Neelsen staining and microscopy under oil immersion objective for microcolonies of acid fast bacilli. Niacin test was done to confirm Mycobacterium tuberculosis. A known Mycobacterium tuberculosis strain H37Rv was used as a positive control and an uninoculated slide as negative control simultaneously.

Grading of culture by Rapid slide culture method[46]

Presence of micro colonies was demonstrated by ZN staining. Growth was graded according to the size of micro colonies.

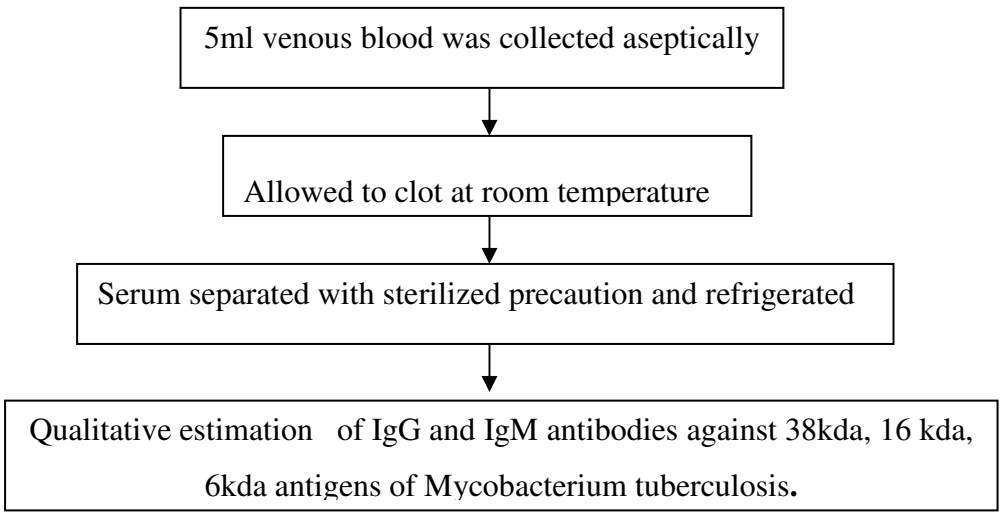
Table-IV Grading of RSC method[46]

ZN Staining	Grading
No multiplication of AFB as compared with an unincubated control	0
Small clumps of up to four bacilli	1+
Large clumps of bacilli, but no cord formation	2+
Micro colonies with some cord formation	3+
Large micro colonies with good cord formation	4+

4.3 SEROLOGY: -

A qualitative analysis of antibodies (IgG and IgM) against TB specific antigens 38kda,16kda,and 6kda using the SD Bioline TB IgG/IgM test kit supplied by Bio Standards Diagnostics.

Procedure: After obtaining an informed consent from the patient, all specimens were analysed for serological evaluation.



The SD Bioline TB IgG/IgM test is a chromatographic immunoassay for the qualitative analysis of antibodies against M .tuberculosis in human serum or plasma.

The test strip contains:

1). Conjugate pad containing recombinant Mycobacterium tuberculosis specific antigens (38kda, 16kda, 6kda) conjugated with gold.

2) A nitrocellulose membrane containing C control line, M test line (TB IgM) and G test line (TB IgG).

The M line is pre coated with monoclonal anti human IgM antibodies, G line is precoated with monoclonal anti human IgG antibodies, C line is coated with goat anti TB antibodies. The test kit is removed from the foil pouch and placed on a flat dry surface. 10µl of serum or plasma is added into the square shaped sample well with a capillary pipette. 4 drops (90-120µl) of assay diluent is added into the round shaped diluent well. As the test begins to work, a purple colour across the result window is visualized in the centre of the test device. Results to be interpreted within 15 minutes of application of serum sample.

Interpretation:

TB IgM positive: The presence of two color bands (M and C line) within the result window irrespective of which appears first indicates presence of TB IgM antibodies.

TB IgG positive: The presence of two color bands (M and C line) within the result window irrespective of which appears first indicates presence of TB IgG antibodies.

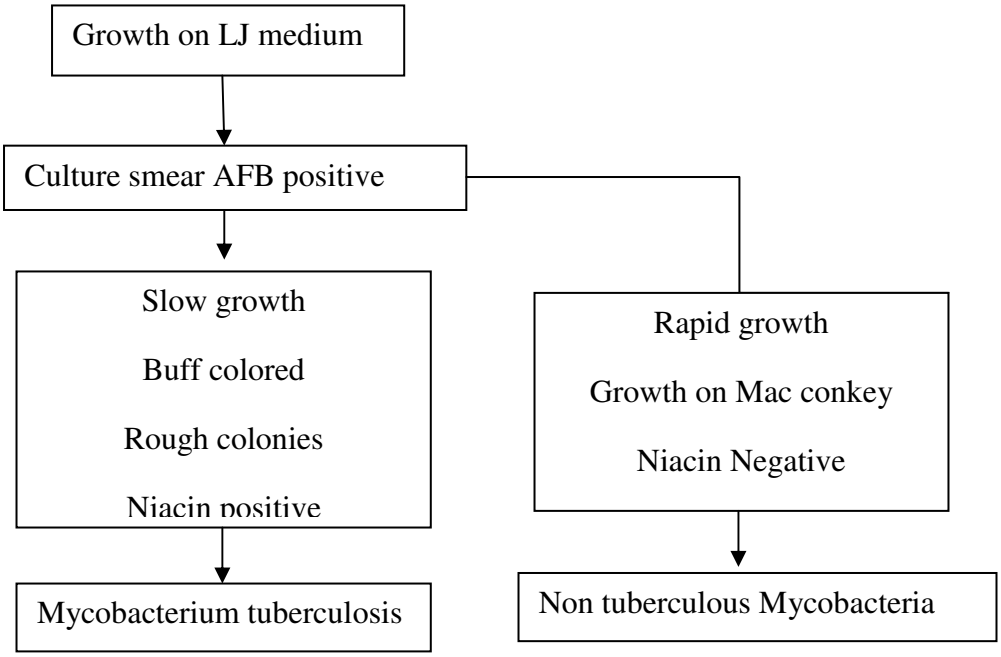
TB IgG and IgM positive : The presence of three color bands (M ,Gand C line) within the result window irrespective of which appears first indicates presence of TB IgM ,IgG antibodies.

Negative results: The presence of only control band (C line) within the result window indicates that no TB antibodies are detected.

Invalid results: If the control band is not visible within the result window after performing the test, the result is considered invalid.

The LJ bottles are incubated for a period of 12 weeks and discarded if no growth occurs. All the above mentioned procedures were done in a biosafety cabinet and universal precautions were taken. All contaminated materials were autoclaved and disposed. The cases positive by smear or culture was informed to the clinician concerned and the patients were treated as per RNTCP guidelines.

SCHEME OF IDENTIFICATION OF MYCOBACTERIUM TUBERCULOSIS.



5. Results

In the present study we included 66 patients with clinical suspicion of extra pulmonary tuberculosis, who were attending R.L Jallapa Hospital and Research Centre attached to Sri Devaraj Urs Medical College, Tamaka, Kolar.

The samples obtained from these patients were processed as per the methodology and their results were compiled and analysed.

Table – 1 Shows the sex wise distribution of the 66 patients (n=66).

Gender	Number	Percentage
Males	38	57.5%
Females	28	42.5%

Of the total 66 patients studied 38(57.5%) were males and 28(42.5%) were females.(Table-1 and Figure-1).

FIGURE -1 SEX WISE DISTRIBUTION OF PATIENTS

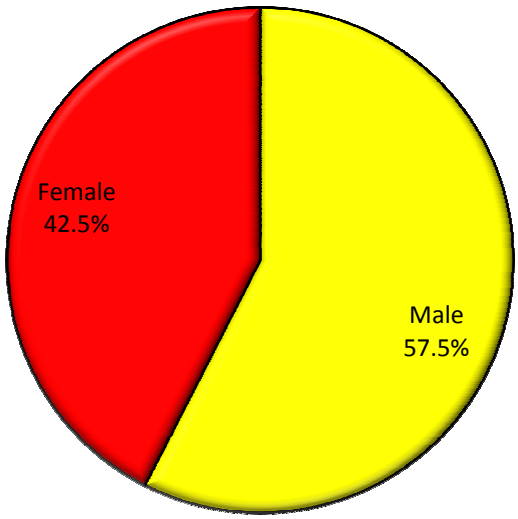


Table (2) shows the age wise distribution of patients.

Age group	Number	Percentage (%)
0 – 10	5	7.6
11 – 20	9	13.7
21 – 30	19	28.8
31 – 40	13	19.7
41 – 50	5	7.6
51 – 60	10	15.1
61 – 70	3	4.5
71 – 80	2	3

In the present study majority of the patients belonged to the age group of 21-30 years (28.85%) ,followed by 31-40 years (19.7%).The least number of patients belonged to the age group of 71-80(3%).

Figure- 2 Shows the age wise distribution of patients

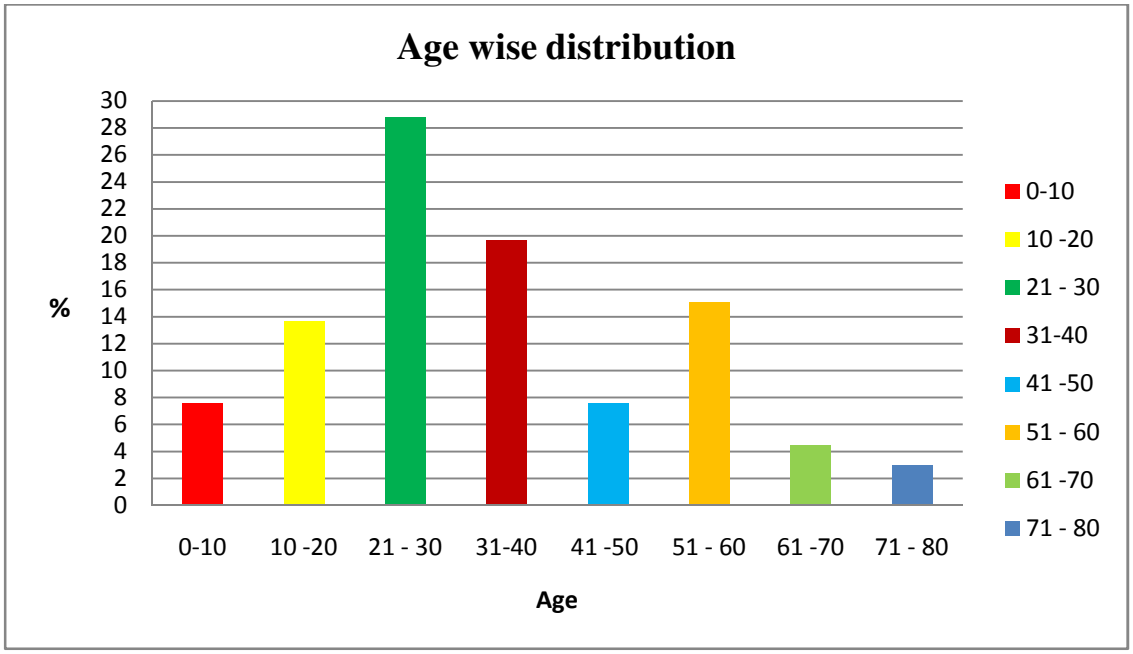


Table-3 Shows the distribution of the nature of specimens from the 66 patients processed.

Nature of specimen	Number of specimens	Percentage (%)
Pleural fluid	29	43.9
Pus	11	16.6
Lymph node aspirates	6	9.1
Biopsy sample	6	9.1
CSF	4	6.1
Synovial fluid	3	4.5
Ascitic fluid	3	4.5
Urine	2	3.1
Bone marrow aspirate	2	3.1
Total	66	100%

In the present study majority of the samples were obtained from cases of pleural effusion (43.9%), followed by pus (16.6%), lymph node aspirates (9.1%).

Figure-3 Sample distribution

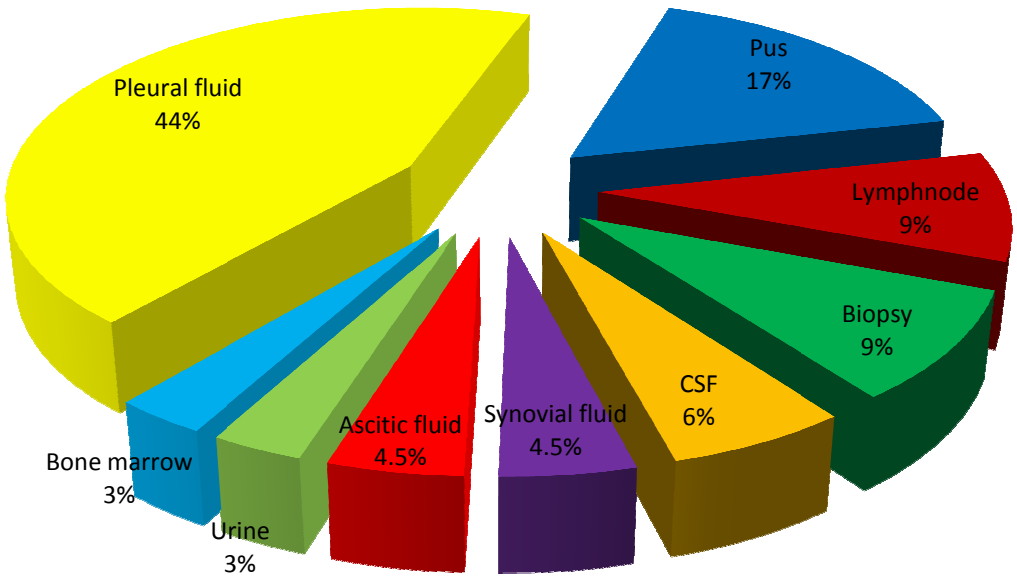


Table-4 Shows the distribution of samples which were positive by one or more methods of staining and culture.

Sl No	Age/ Sex	Specimen	Staining Methods		Culture methods	
			ZN	Fluorescent	L J	RSC
1	26/M	Lymphnode aspirate	+ve	+ve	+ve	+ve
2	11/M	Lymphnode aspirate	+ve	+ve	+ve	-ve
3	60/M	Pus potts spine	+ve	+ve	+ve	+ve
4	20/M	Pleural aspirate	+ve	+ve	+ve	+ve
5	19/F	Synovial fluid	+ve	+ve	+ve	+ve
6	60/M	Parietal wall abscess	+ve	+ve	+ve	+ve
7	53/M	Scrotal ulcer pus	+ve	+ve	+ve	+ve
8	48/F	Post Cholecystectomy pus	+ve	+ve	+ve	+ve
9	24/F	Pleural fluid	-ve	-ve	+ve	-ve
10	25/F	Pleural fluid	+ve	+ve	+ve	+ve
11	30/F	Breast abscess	+ve	+ve	+ve	+ve
12	25/F	Endometrial Biopsy tissue	-ve	-ve	+ve	+ve
13	26/F	Lymphnode aspirate	+ve	+ve	+ve	+ve
14	28/M	Lymphnode aspirate	+ve	+ve	+ve	+ve
15	40/M	FNAC ileocaecal region	+ve	+ve	-ve	-ve
16	34/M	Urine	+ve	+ve	+ve	+ve
17	30/F	Ascitic fluid	-ve	-ve	+ve	-ve
18	13/M	Csom pus	+ve	+ve	+ve	+ve
19	45/M	Shoulder Joint abscess aspirate	-ve	-ve	+ve	-ve

20	24/F	Lymphnode aspirate	+ve	+ve	+ve	+ve
21	55/M	Pleural fluid	+ve	+ve	+ve	+ve
22	75/M	Bone marrow aspirate	+ve	+ve	-ve	-ve
Total By different methods			18	18	20	16

Table–(4). Out of the total number of 66 specimens processed 22 were positive by one or more methods for detection of acid fast bacilli by (ZN microscopy, fluorescent microscopy, LJ culture and Rapid slide culture).

Table –(5) The sites of isolation of Mycobacterium tuberculosis..

Sl. No	Sample	No of sample processed	Total Positive	Percentage %
1	Lymph node aspirate	6	5	83.3
2	Pleural fluid	29	4	13.8
3	Pus	11	3	27.8
4	Synovial fluid	3	1	33.3
5	Urine	2	1	50.0
6	Ascitic fluid	3	1	33.3
7	Biopsy Specimens	6	1	16.7
8	CSF	4	0	0.0
9	Bone marrow aspirate	2	0	0.0
Total		66	16(24.2%)	

Table (5) shows the sites of isolation of Mycobacterium tuberculosis confirmed by culture on LJ medium and Niacin test .Of the total 66 specimens processed 16(24.2%) were Mycobacterium tuberculosis. Majority of the isolates (5)(83.3%) were from

lymph node aspirates , 4(13.8) from pleural fluid, 3(27.8%) from pus,1 (50%)from urine,1 (33.3%)from synovial fluid,1(33.3%) from ascitic fluid and 1(16.7%) from biopsy specimen.

Lymph Node Aspirate-Table-(6)

Sl No	Age/SEX	Specimen	Staining Methods		Culture methods	
			ZN	Fluorescent	L.J	RSC
1	26/M	Lymphnode Aspirate	+ve	+ve	+ve	+ve
2	11/M	Lymphnode Aspirate	+ve	+ve	+ve	-ve
3	4/F	Lymphnode Aspirate	-ve	-ve	-ve	-ve
4	26/F	Lymphnode Aspirate	+ve	+ve	+ve	+ve
5	28/M	Lymphnode aspirate	+ve	+ve	+ve	+ve
6	24/F	Lymphnode Aspirate	+ve	+ve	+ve	+ve

Table (6) shows the total number of lymph node aspirates processed. Out of the 6 samples processed the total number of positives was 5 (83.3%) of which 4 were positive by all the four methods. 1 was positive by ZN and fluorescent method of staining and LJ culture but negative by RSC.

Pleural effusion fluid –Table (7).

Sl no	Age/ SEX	Specimen	Staining Methods		Culture methods	
			ZN	Fluorescent	L.J	RSC
1	45/F	Pleural effusion fluid	-ve	-ve	-ve	-ve
2	4.5/M	Pleural fluid	-ve	-ve	-ve	-ve
3	58/M	Pleural fluid	-ve	-ve	-ve	-ve
4	20/M	Pleural effusion	+ve	+ve	+ve	+ve
5	35/M	Pleural effusion	-ve	-ve	-ve	-ve

6	24/F	Pleural effusion	-ve	-ve	+ve	-ve
7	70/F	Pleural fluid	-ve	-ve	-ve	-ve
8	20/M	Pleural fluid	-ve	-ve	-ve	-ve
9	2/M	Pleural fluid	-ve	-ve	-ve	-ve
10	45/F	Pleural fluid	-ve	-ve	-ve	-ve
11	25/F	Pleural fluid	+ve	+ve	+ve	+ve
12	55/M	Pleural fluid	+ve	+ve	+ve	+ve
13	75/M	Pleural fluid	-ve	-ve	-ve	-ve
14	28/M	Pleural fluid	-ve	-ve	-ve	-ve
15	19/M	Pleural fluid	-ve	-ve	-ve	-ve
16	24/M	Pleural fluid	-ve	-ve	-ve	-ve
17	40/M	Pleural fluid	-ve	-ve	-ve	-ve
18	55/F	Pleural fluid	-ve	-ve	-ve	-ve
19	58/M	Pleural fluid	-ve	-ve	-ve	-ve
20	45/F	Pleural fluid	-ve	-ve	-ve	-ve
21	40/M	Pleural fluid	-ve	-ve	-ve	-ve
22	20/M	Pleural fluid	-ve	-ve	-ve	-ve
23	30/M	Pleural fluid	-ve	-ve	-ve	-ve
24	35/M	pleural fluid	-ve	-ve	-ve	-ve
25	65/F	Pleural fluid	-ve	-ve	-ve	-ve
26	67/M	Pleural fluid	-ve	-ve	-ve	-ve
27	59/M	Pleural fluid	-ve	-ve	-ve	-ve
28	32/F	Pleural fluid	-ve	-ve	-ve	-ve
29	38/F	Pleural fluid	-ve	-ve	-ve	-ve

Table (7) shows the total number of pleural fluids processed. Out of the 29 samples processed the total number of positives were 4(13.1%) From the 4 positives ,3 samples were positive by all the four methods, 1 sample was smear negative by ZN and fluorescent method but was positive by LJ culture and negative by RSC method.

PUS –Table- (8).

Sl No	Age/ SEX	Specimen	Staining Methods		Culture methods	
			ZN	Fluorescent	L.J	RSC
1	18/M	Pus Psoas abscess	-ve	-ve	-ve	-ve
2	60/M	Parietal wall Abscess	+ve	+ve	+ve	+ve
3	37/F	Post hysterectomy wound pus	-ve	-ve	-ve	-ve
4	60/M	Potts spine pus	+ve	+ve	+ve	+ve
5	53/M	Scrotal ulcer pus	+ve	+ve	+ve	+ve
6	48/F	Post cholecystectomy wound pus	+ve	+ve	+ve	+ve
7	13/M	Pus chronic suppurative otitis media.	+ve	+ve	+ve	+ve
8	30/F	Pus breast abscess	+ve	+ve	+ve	+ve
9	28/F	Pus breast abscess	-ve	-ve	-ve	-ve
10	45/M	Pus shoulder abscess	-ve	-ve	+ve	-ve
11	9/F	Pus Multiple abscess	-ve	-ve	-ve	-ve

Table (8) shows the total number of pus samples processed. Out of the 11 samples processed the total number of positives were 7(63.60%).6 samples were positive by all the four methods.1 was positive by LJ culture but negative by ZN and Fluorescent method of staining and RSC.

Synovial fluid-Table-9

Sl No	Age/ SEX	Specimen	Staining Methods		Culture methods	
			ZN	Fluorescent	L.J	RSC
1	19/F	Synovial fluid	+ve	+ve	+ve	+ve
2	35/M	Synovial fluid	-ve	-ve	-ve	-ve
3	30/F	Synovial fluid	-ve	-ve	-ve	-ve

Table (9) shows the total number of synovial fluid samples processed. Out of the 3 samples processed 1(33.3%) was positive by all the four methods.

URINE –Table-10

Sl. No	Age/ SEX	Specimen	Staining Methods		Culture methods	
			ZN	Fluorescent	L.J	RSC
1	35/M	Urine	-ve	-ve	-ve	-ve
2	34/M	Urine	+ve	+ve	+ve	+ve

Table (10) shows the total number of urine samples processed. Out of the 2 samples processed 1(50%) was positive by all the four methods.

Ascitic fluid-Table -11

Sl No	Age/ SEX	Specimen	Staining Methods		Culture methods	
			ZN	Fluorescent	L.J	RSC
1	30/F	Ascitic fluid	-ve	-ve	+ve	-ve
2	28/M	Ascitic fluid	-ve	-ve	-ve	-ve
3	53/M	Ascitic fluid	-ve	-ve	-ve	-ve

Table (11) shows the total number of ascitic fluid samples processed. Out of the 3 samples processed 1 sample was positive by LJ culture and negative by ZN and Fluorescent method of staining and RSC.

Biopsy specimens-Table-12

Sl. No	Age/ Sex	Specimen	Staining Methods		Culture methods	
			ZN	Fluorescent	LJ	RSC
1	22/F	Endometrial biopsy	-ve	-ve	-ve	-ve
2	25/F	Endometrial biopsy	-ve	-ve	+ve	+ve
3	29/F	Endometrial sample	-ve	-ve	-ve	-ve
4	28/F	Endometrial biopsy	-ve	-ve	-ve	-ve
5	40/M	FNAC ileocecal region	+ve	+ve	-ve	-ve
6	20/F	left sided hydrosalphinx tissue	-ve	-ve	-ve	-ve

Table (12) shows the total number of biopsy specimens processed. Out of the 6 samples processed, 1 endometrial biopsy sample was positive by LJ culture and RSC and negative by ZN and fluorescent method of staining. 1 sample (FNAC tissue of ileocaecal region) was positive by ZN and Fluorescent method of staining but negative by LJ culture and RSC.

CSF samples Table-(13)

Sl No	Age/SEX	Sample	Staining Methods		Culture methods	
			ZN	Fluorescent	L.J	RSC
1	22/M	CSF	-ve	-ve	-ve	-ve
2	36/F	CSF	-ve	-ve	-ve	-ve
3	60/M	CSF	-ve	-ve	-ve	-ve
4	2/M	CSF	-ve	-ve	-ve	-ve

Table (13) shows the total number of CSF samples processed. Out of the 4 samples processed none of the samples were positive by any of the four methods.

Table-(14) Bone marrow aspirates.

Sl No	Age/SEX	Sample	Staining Methods		Culture methods	
			ZN	Fluorescent	L.J	RSC
1	75/M	Bone marrow aspirate	+ve	+ve	-ve	-ve
2	32/F	Bone marrow aspirate	-ve	-ve	-ve	-ve

Table (14) shows the total number of bone marrow aspirates processed. Out of the 2 samples processed 1sample was positive by ZN and fluorescent method of staining and negative by LJ culture and RSC method. 1 sample was negative by all the four methods.

Table (15) shows specimens which were positive by ZN and Fluorescent methods of staining. (n=66)

Methods	Total	Percentage
(Smear) ZN staining.	18	27.2%
Fluorescent staining	18	27.2%

Figure -15 Shows specimens which were positive by ZN and fluorescent staining methods.

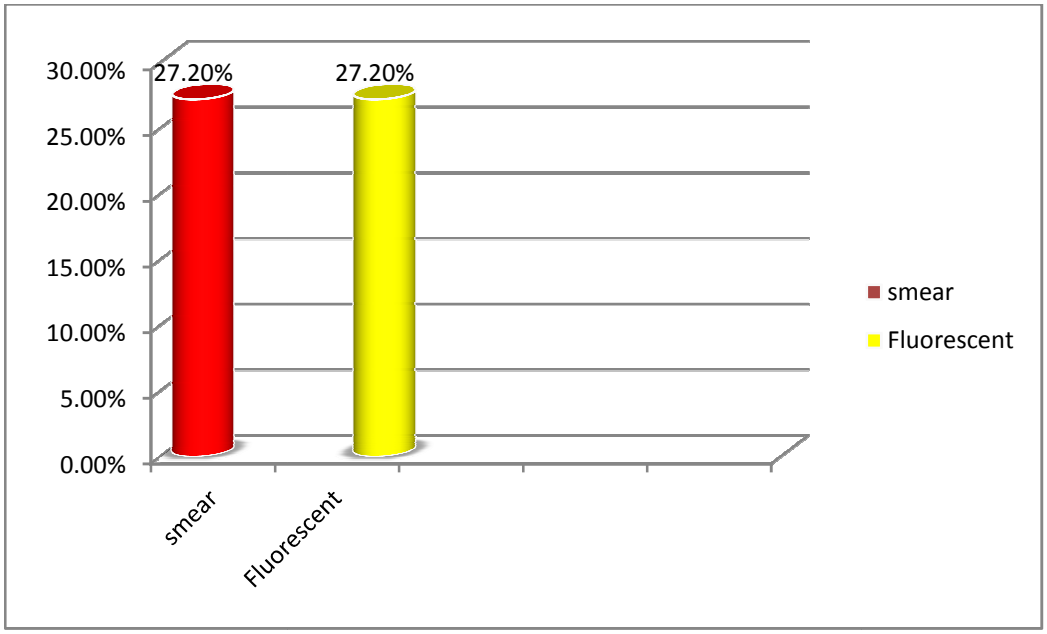


Table-(15) and figure(15) shows the specimens which were subjected to detection of acid fast bacilli, from which 18 (27.2%) specimens were positive for acid fast bacilli(AFB), when examined microscopically after staining by Ziehl Neelsens method and by fluorescent method (Auramine phenol) .

Table(16),(17) ,(19) Shows the sensitivity, specificity ,positive predictive value and negative predictive value of Ziehl Neelsen's staining, Auramine staining and Rapid slide culture technique taking culture on Lowenstein Jensen's medium as gold standard.

Table-16 – Direct smear (ZN method) and LJ method

		LJ				
		+ve	-ve	Total	Sensitivity	80%
Direct smear	+ve	16	2	18	Specificity	95.65%
	-ve	4	44	48	PPV	88.89%
		20	46	66	NPV	91.67%

Table 16 Shows a sensitivity of 80%, specificity of 95.65%, positive predictive value of 88.89% and a negative predictive value of 91.67%for ZN staining when compared with LJ culture taken as the gold standard .

Table-17 – Fluorescent staining method & LJ culture.

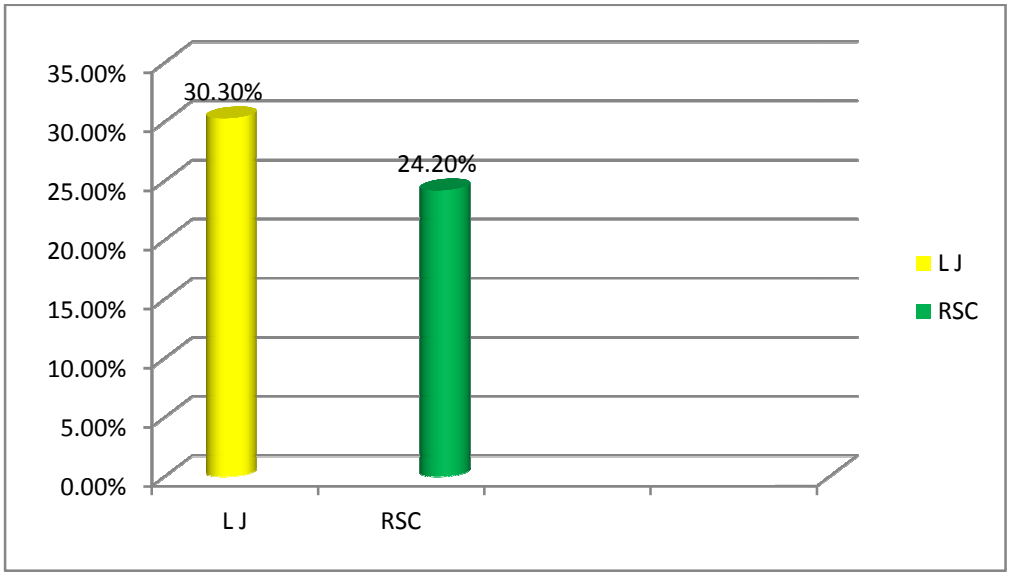
		L J Culture				
		+ve	-ve	Total	Sensitivity	80%
Fluorescent stain	+ve	16	2	18	Specificity	95.65%
	-ve	4	44	48	PPV	88.89%
		20	46	66	NPV	91.67%

Table-17 Shows a sensitivity of 80%, specificity of 95.65%, positive predictive value of 88.89% and a negative predictive value of 91.67%for fluorescent staining method when compared with LJ culture taken as the gold standard .

Table-18 Shows specimens which were positive by LJ and Rapid Slide Culture.(n=66)

Methods	Total	Percentage
L J Culture	20	30.3%
RSC	16	24.2%

Figure -18 Shows specimens which were positive by LJ and Rapid Slide Culture.



All the 66 specimens were subjected for culturing on LJ medium and Human blood medium and the results are as follows, 20(30.3%) samples were positive by LJ culture, 16(24.2%) were positive by Rapid slide culture as shown in Table (18) and shown in Figure (18).

Table19 – Rapid Slide Culture & LJ culture.

		LJ				
		+ve	-ve	Total	Sensitivity	80%
RSC	+ve	16	0	16	Specificity	100%
	-ve	4	46	50	PPV	100%
		20	46	66	NPV	92%

Table- 19 Shows a sensitivity of 80%, specificity of 100%, positive predictive value of 100% and a negative predictive value of 92 %for Rapid slide culture when compared with LJ culture taken as the gold standard .

Table –(20) Shows smear negative culture positive specimens.

Sl No	Specimens	Age	Staining Methods		Culture methods	
			ZN	Fluorescent	L.J	RSC
1	Pleural fluid	24Yrs	-ve	-ve	+ve	-ve
2	Ascitic fluid	30yrs	-ve	-ve	+ve	-ve
3	Endometrial biopsy	25Yrs	-ve	-ve	+ve	+ve
4	Pus shoulder joint	45yrs	-ve	-ve	+ve	-ve

Table –(20) Of the 66 specimens processed, 4 (6%) samples which were negative by direct smear, stained by Zeihl Neelsens and fluorescent method of staining when subjected to culturing on LJ and RSC ,proved positive by LJ culture and 1 was positive by both LJ and RSC methods.

Table - (21) Smear negative but LJ and RSC +ve

Sl No	Sample	Age	Staining Methods		Culture methods	
			ZN	Fluorescent	L.J	RSC
1	Endometrial Biopsy tissue	25yrs	-ve	-ve	+ve	+ve

Table (21) shows 1 specimen which was smear negative but positive by LJ culture and RSC.

Table-22 L J Culture +Ve RSC –VE

Sl No	Specimens	Age	L J culture	RSC
1	Lymphnode aspirates	11/M	+ve	-ve
2	Ascitic fluid	30/F	+ve	-ve
3	Pleural fluid	24/F	+ve	-ve
4	Shoulder abscess Pus	45/M	+ve	-ve

Table (22) Shows 4 specimens which were positive by LJ culture but negative by RSC.

Table -23 SMEAR +ve (Culture Negative)

Sl No	Specimen	Age	Staining Methods		Culture methods	
			ZN	Fluorescent	L.J	RSC
1	Bonemarrow aspirate	75Y/M	+ve	+ve	-ve	-ve
2	FNAC ileocecal region	40Y/M	+ve	+ve	-ve	-ve

Table (23) Shows 2 samples which were positive by ZN staining, fluorescent staining methods, but were negative by LJ culture and RSC.

Table -24 Shows specimens classified as Non tuberculous Mycobacterium (NTM).

Sl. No	Age/Sex	Sample	Staining method		Culture method	
			ZN	Fluorescent	L J	RSC
1	60/M	Parietal wall abscess	+ve	+ve	+ve	+ve
2	53/M	Scrotal ulcer pus	+ve	+ve	+ve	+ve
3	48/F	Post Cholecystectomy pus	+ve	+ve	+ve	+ve
4	13/M	Csom pus	+ve	+ve	+ve	+ve

Table (24) Shows 4 samples which were classified as non tuberculous Mycobacterium (refer to the flow chart on page 45).

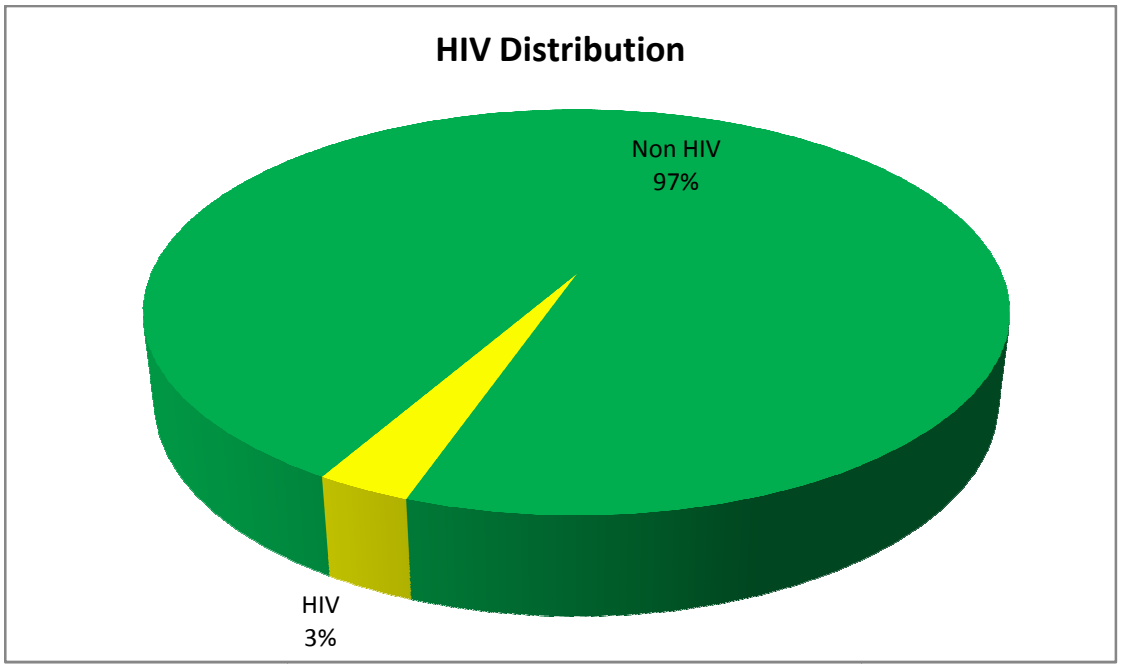
Table -25(A) shows the number of samples which were HIV positive. (n=66).

Age/Sex	Nature of Specimen	Number of specimens
75/M	Bone marrow aspirate(HIV)	1
32/F	Bone marrow aspirate(HIV)	1

Table -25(B) shows the percentage of distribution of specimens of HIV positive and negative patients.

HIV Specimen		Non HIV Specimen	
Number of specimens	Percentage	Number of specimens	Percentage
2	3%	64	97%

Figure -25(B) shows the distribution of HIV positive and negative patients.



SEROLOGY:

Qualitative analysis of antibodies against 38 kda, 16kda, 6kda antigens of Mycobacterium tuberculosis performed with the serum collected from 66 patients showed that antibodies against these antigens were not detected in any of the 66 patients.

6. DISCUSSION:

Tuberculosis is one of the leading causes of morbidity and mortality of all the infectious diseases world wide .Tuberculosis can involve any organ system. It can exist in two forms the pulmonary and extra pulmonary. The commonest being pulmonary tuberculosis, however extra pulmonary tuberculosis is also an important clinical entity[4].

Extra pulmonary tuberculosis has existed as a disease entity for centuries. It is a milder form of disease in terms of infectivity as compared to pulmonary tuberculosis. It has a broader spectrum of clinical manifestations and often presents a diagnostic challenge[5].

In the present study, clinical specimens from patients suspected of extra pulmonary tuberculosis, attending R. L Jalappa Hospital and Research Centre Kolar, during the period from December 2008 to August 2010 were processed and evaluated by different methods for establishing the diagnosis of Mycobacterium tuberculosis infection. The specimens included Pleural fluid(29), Pus(11), Lymph node aspirates(6), Biopsy specimens(6), CSF(4), Synovial fluid aspirate(3), Ascitic fluid(3), Bone marrow aspirate(2), Urine sample(2) as shown in Table -3 .

In the present study (Table-1) figure-1 shows that there was male preponderance (57.5%) in contrast to studies reported by Sharma[4], Arora V K and Gupta R[35] (57 %), which shows female preponderance in cases of extra pulmonary tuberculosis.

Majority of the patients belonged to the age group 21-30 years which constituted to 28.8%, this observation correlated well with the other studies conducted by Sharma[4]

,Musellim[53], Arora V K and GuptaR[35] et al proving that young adults are at a higher risk of tuberculosis.

In Our study we found that 43.9% of the specimens were obtained from cases of pleural effusion, followed by pus samples (16.6%), lymph node aspirates (9.1%),biopsy specimen (9.1), CSF (6.1), synovial fluid (4.5%), ascitic fluid (4.5%), urine (3.1%) and bone marrow (3.1%) as shown in (Table-3) figure-3.

The study showed a positivity of 22 (33.3%) (Table- 4) by any one or more methods of staining and culture. Narang et al showed a positivity of 35.94% considering staining technique by ZN method, LJ culture and histopathological examination in lymph node specimens[34].

The percentage of isolation of Mycobacterium tuberculosis in lymph node aspirates were 83.3% as shown in table- 5. Vanaja Kumar et al has reported 98% isolation of Mycobacterium tuberculosis from lymph nodes[6], Kishore Reddy et al has reported 45% isolation of Mycobacterium tuberculosis from lymph nodes[17] where in only lymph node specimens were considered. In the present study out of the 6 lymphnode specimens processed,5 specimens were positive by both the staining techniques (ZN and Fluorescent method) and both the culture methods(LJ culture and RSC) as shown in table -6

The percentage of isolation of Mycobacterium tuberculosis in pleural fluid was13.8 % as shown in table -5 .Epstein DM et al reported that in most of the cases of pleural effusions the culture yield is less than 30%[18].Valdes et al reported a positivity of 5.5 % by ZN staining and isolation of Mycobacterium tuberculosis on LJ culture which accounted for 36.6% in cases of pleural effusion[20] . In our study out of the 29

pleural fluids processed ,3 were positive by both the staining techniques (ZN and Fluorescent method) and both the culture methods(LJ culture and RSC) 1 sample was positive only by LJ culture and negative by ZN and fluorescent method of staining and RSC as shown in table -7

As per table- 8 out of the 11 pus samples processed total number of positives were 7 (63.6%). 6 were positive by both the staining techniques (ZN and Fluorescent method) and both the culture methods(LJ and RSC).1 sample was positive by LJ culture and negative by ZN and fluorescent method of staining and RSC which includes pus aspirated from shoulder abscess.

Out of the 6 samples which were positive by all the four methods 4 samples were identified as NTM.1 sample was pus aspirated from a case of breast abscess,1 sample was pus aspirated from a case of suspected Potts spine.

In the present study out of the 3 synovial fluid processed, 1 was positive by all the four techniques shown in table -9. Of the 2 urine samples processed, 1 sample was positive by all the four methods as shown in table-10. 1 ascitic fluid sample was positive by LJ culture and negative by ZN and Fluorescent method of staining and RSC as shown in table-11.

Out of the 6 biopsy specimens processed 1 endometrial biopsy sample was smear negative by both the staining techniques but was positive by LJ culture and RSC as shown in table 12.(1) sample (FNAC tissue) from the ileocaecal region was positive by ZN and fluorescent method of staining, but negative by both the culture methods.

In the present study out of the 4 CSF samples processed none of the samples were positive by any of these methods as shown in table 13. From the 2 bone marrow

aspirates processed 1 was positive by smear microscopy (ZN and fluorescent method of staining) negative by LJ culture and RSC as shown in table 14. .

The present study shows that , of the total 66 specimens processed 18 (27.2%) as shown in table-15 were positive for acid fast bacilli by Ziehl Neelsens method of staining and equal number 18(27.2%) too were positive by fluorescent method of staining..Narang et al has shown a positivity of 54.5% by ZN method of staining[34].Kishore Reddy et al has shown a positivity of 18% by ZN method of staining[17].

As per table (16) which shows that ZN method of staining had a sensitivity of 80%, specificity of 95.65%, positive predictive value of 88.9%, and a negative predictive value of 91.6% when compared with LJ culture taken as the gold standard. Negi SS has shown a sensitivity of 20.25% and a specificity of 100% in extra pulmonary samples[36]. In our study we had a higher percentage of sensitivity.

Taking culture on LJ medium as gold standard, Fluorescent method of staining had a sensitivity of 80%, specificity of 95.65%, positive predictive value of 88.9%, and a negative predictive value of 91.6% as shown in table (17). Narang et al has reported a sensitivity of 32.8% in lymph node specimens[34].

There was no significant difference observed between the results obtained by Zeihl Neelsens and fluorescent methods of staining in the extra pulmonary specimens. However fluorescent method of staining has offered the advantage of screening the smears under low power where large numbers of slides are screened in less time as quoted in the bulletin of ICMR[37]. There is an added advantage of reduced observers fatigue which plays a significant role in improving the sensitivity[38].

From our study we found that 20(30.3%) samples were positive by LJ culture as shown in table -18. The above finding correlates with studies conducted by Narang et al with an isolation rate of 30.3% on LJ culture[34].

In the present study as per the table-18 (24.2%) 16 were positive by RSC method. Robert Koch was the first to use RSC technique. He used coagulated human serum and was successful in obtaining the growth of Mycobacterium tuberculosis in seven days, but the problem faced by him was contamination[30] .After a gap of four decades in 1990, Dickinson and Mitchison[39] renewed interest in slide culture by employing RSC in sensitivity testing by using outdated citrated human blood as the medium. However this procedure had the limitation of usage of fluorescent microscope, which was overcome by Gupta et al by substituting the fluorescent microscope with light microscope[40].

In the present study as per table (19) which shows that RSC method of culture had a sensitivity of 80%, specificity of 100%, positive predictive value of 100%, and a negative predictive value of 92% when compared with growth on LJ culture as the gold standard. The time taken for culture results to be obtained is seven days.

To the best of our search to date there are only three studies on slide culture technique from India. Purohit et al evaluated a new medium Sheep Blood Medium and Human Blood Medium and found that the results between the two were equally good[30].

Jena et al[10] in 1995 and Nair et al[9] in 1998 used Human blood medium for drug susceptibility testing in pulmonary samples. Jena et al compared it with conventional LJ culture in fresh untreated cases of pulmonary tuberculosis. A positivity 0f 65.2% for RSC and 85.1 % for LJ culture was shown by in him his study[10].

In our study we used the RSC method as a novel method for isolation of Mycobacterium tuberculosis in extra pulmonary specimens. The isolation rate was 24.2% in RSC and 30.4% in LJ culture. Highest percentage of isolation was obtained on LJ culture. Jena et al found RSC to be more sensitive than smear microscopy[10]. Nair L et al found smear microscopy to be more sensitive than RSC[9]. In our study RSC had a sensitivity of 80% comparable with the sensitivity of smear microscopy, specificity of 100% and positive predictive value of 100% in specimens of extra pulmonary tuberculosis.

An additional advantage of RSC is that it only involved in picking up micro colonies with an ordinary bright light microscope without the need of sophisticated equipment or complicated procedures.). RSC is rapid, cheap and an effective method for obtaining culture confirmation of tuberculosis and considered most suitable in a country like India where tuberculosis is rampant. The need of the hour being rapid detection of Mycobacterium tuberculosis and treatment. However LJ medium still remains the gold standard.

Out of the total 66 samples processed,4 specimens were smear negative by Ziehl Neelsens and Fluorescent methods of staining but all the 4 were positive by LJ culture. Out of these four which were positive by L J culture, only one was positive by RSC as shown in table -20.

Extra pulmonary tuberculosis is paucibacillary in nature hence most often they are not detected by smear microscopy. Probably because of less number of organisms present in the sample as, there must be at least 10^4 organisms in the sample to be detected by smear microscopy where as the number of organisms required for culture to be positive is 10 to 100 organisms per ml[13,47].

Table (23) shows 2 specimens which were smear positive but culture negative which accounts to 3% in the present study. Kishore VC and Aparna et al has shown 12.1% to be smear positive and culture negative in lymph node aspirates[17]

This could be attributed to patients who were previously treated with anti- TB drugs[17]. Usage of broad spectrum antibiotics such as Amoxicillin, Fluoroquinolones has been reported to be inhibitory to Mycobacterium tuberculosis that might lead to negative culture and positive smear.

In the present study out of the 66 specimens processed,6 NTM was isolated as shown in table-24 It correlates well with the findings of Nataraj et al who had an isolation of 3.85% NTM in lymphnode aspirates. 3% of the patients were HIV sero positive as shown in Table 25(B).

Qualitative analysis of antibodies against 38 kda, 16kda, 6kda antigens of Mycobacterium tuberculosis performed with the serum collected from 66 patients showed that antibodies against these antigens were not detected in any of the 66 patients. However Abebe F et al has reported a sensitivity of 56% and a specificity of 99% in bone and joint tuberculosis[11]. Immune response depends on the interaction between the pathogen and infected host where the response may be weak immune or strong immune influencing the antibody response and antigen clearance[42].

Recent systematic review on immunodiagnostic assays for PTB and EPTB have analysed the data on serological test for tuberculosis and concluded that none of the assays performed well enough to replace smear microscopy and culture[42].The immune response in Mycobacterial disease appears to be associated with HLA Class II allotypes and different patients appear to recognize different antigens. It is thus

unlikely that all tuberculosis patients will recognise a single antigen and hence prove to be a handicap for the development of antibody based detection system for Mycobacteria[24].

7. Conclusion:

The present study was conducted in the Department of Microbiology, Sri Devaraj Urs Medical College. The study group included all patients with clinical suspicion of extra pulmonary tuberculosis who were attending RL Jallapa Hospital and Research Centre attached to Sri Devaraj Urs Medical College. The patients who were sputum smear positive and diagnosed with pulmonary tuberculosis were excluded.

The objectives of the present study were to evaluate the different methods in the diagnosis of EPTB and to detect the sensitivity and specificity of these tests in the diagnosis of extra pulmonary tuberculosis.

This included 2 staining techniques, Ziehl Neelsens and Fluorescent method of staining, 2 culture methods, LJ and Rapid slide culture and qualitative analysis of IgG and IgM antibodies against 38kda, 16kda, 6kda antigens of Mycobacterium tuberculosis in the serum of all the patients included in the study.

A total number of 66 specimens were processed. The specimens were subjected to ZN and Fluorescent method of staining, culturing by LJ and RSC method, and comparing growth on LJ medium as the gold standard. In the present study 57.5% were males and 42.5% were females and most of the patients belonged to the age group of 21-30 years.

Extra pulmonary specimens were Pleural fluid(29), followed by Pus(11), Lymph node aspirates(6), Biopsy specimens(6), CSF(4),Synovial fluid aspirate(3), Ascitic fluid(3), Bone marrow aspirate(2), and Urine sample(2) . The study showed a positivity of 33.3% by one or more methods of staining and culture.

Mycobacterium tuberculosis was isolated in 24.1% and Non Tuberculous Mycobacterium (NTM) was isolated in 6% of the 66 samples processed. Highest number of isolation was from lymph node aspirates (83.3%). In pleural fluid the percentage of isolation was 13.8%, pus 27.8%, synovial fluid 33.3%, urine 50%, ascitic fluid 33.3% and biopsy specimen 16.7%, 3% of the samples were HIV seropositive. 4(6%) of the samples were smear negative and culture positive. All the 4 were positive by LJ culture and RSC was positive in only one sample. 3% of the samples were smear positive and culture negative.

The percentage of positivity with ZN staining and fluorescent staining was 27.2% there was no significant difference observed between these two staining methods. The rate of positivity by LJ culture was 30.3% and RSC was 24.6%.

When compared with growth on LJ culture as the gold standard, ZN method of staining had a sensitivity of 80%, specificity of 95.65%, positive predictive value of 88.89% and negative predictive value of 91.67%. The fluorescent method of staining had a sensitivity of 80% and a specificity of 95.65% positive predictive value of 88.89% and negative predictive value of 91.67%.there was no significant difference observed in their values between the two staining methods. RSC had a sensitivity of 80%, specificity of 100%, positive predictive value of 100% and negative predictive value of 92%.

RSC had sensitivity comparable with the sensitivity of smear microscopy by ZN and Fluorescent methods. RSC had a positive predictive value of 100% which indicates that the diagnostic potential of the test is good. However from the above findings it can be concluded that with a positive predictive value of 100% and sensitivity of 80% RSC is as good as LJ culture .In the present study human blood medium was used in

RSC, as our hospital is a referral hospital; blood was screened for all the pathogens associated with bio hazard. This may not be possible in RNTCP surveillance centre hence an alternative like fetal calf serum can replace blood and RSC can be used as a diagnostic test in detection of Mycobacterium tuberculosis. An additional advantage of RSC is that it is rapid and cheap. The growth is obtained in a period of 7 days as compared to LJ culture where growth occurs in 4 weeks; hence it is useful in early confirmation of viable Mycobacterium tuberculosis which makes it an ideal diagnostic test in a country like India where tuberculosis is rampant. However since the sample size is small, a better conclusion can be derived by conducting a study with a larger number of samples.

Qualitative analysis for antibodies against 38 kda, 16kda, and 6kda antigens of Mycobacterium tuberculosis performed with the serum of 66 patients showed that antibodies against these antigens were not detected in any of these samples. From the present study it can be inferred that antibody estimation does not play an important role in diagnosis of extra pulmonary tuberculosis.

8. Summary

Diagnosis of EPTB has always been a challenge .It is a protean disease affecting virtually all the organs and has a wide spectrum of clinical presentation depending on the anatomical site involved and presents a diagnostic dilemma. The difficulty in obtaining appropriate samples from extra pulmonary sites, the paucibacillary nature of the specimens has made the diagnosis of EPTB more difficult than for pulmonary tuberculosis.

From the present study it can be concluded that on evaluating all the four methods and comparing their sensitivities and specificities , it was found that RSC is as good as LJ culture. RSC has a positive predictive value of 100% which makes it an ideal diagnostic test. It is rapid where the results are obtained in 7 days and can be recommended for the early diagnosis of extrapulmonary tuberculosis.

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10. ANEXURE

Master Chart

Sl no	Age/ SEX	Specimen	Staining Methods		Culture methods	
			ZN	Fluorescent	L.J	RSC
1	26/M	Lymphnode Aspirate	+ve	+ve	+ve	+ve
2	11/M	Lymphnode Aspirate	+ve	+ve	+ve	-ve
3	20/F	left sided hydrosalphinx tissue	-ve	-ve	-ve	-ve
4	18/M	Pus Psoas abscess	-ve	-ve	-ve	-ve
5	22/F	Endometrial biopsy	-ve	-ve	-ve	-ve
6	45/F	Pleural effusion fluid	-ve	-ve	-ve	-ve
7	60/M	Parietal wall Abscess	+ve	+ve	+ve	+ve
8	4.5/M	Pleural fluid	-ve	-ve	-ve	-ve
9	37/F	Post hysterectomy wound pus	-ve	-ve	-ve	-ve
10	58/M	Pleural fluid	-ve	-ve	-ve	-ve
11	4/F	Lymphnode Aspirate	-ve	-ve	-ve	-ve
12	28/M	Ascitic fluid	-ve	-ve	-ve	-ve
13	60/M	Potts spine pus	+ve	+ve	+ve	+ve
14	20/M	Pleural effusion	+ve	+ve	+ve	+ve
15	19/F	Synovial fluid	+ve	+ve	+ve	+ve
16	35/M	Pleural effusion	-ve	-ve	-ve	-ve
17	53/M	Scrotal ulcer pus	+ve	+ve	+ve	-ve
18	48/F	Post cholecystectomy wound pus	+ve	+ve	+ve	+ve

Sl no	Age/ SEX	Specimen	Staining Methods		Culture methods	
			ZN	Fluorescent	L.J	RSC
19	24/F	Pleural effusion	-ve	-ve	+ve	-ve
20	75/M	Bone marrow aspirate(HIV)	+ve	+ve	-ve	-ve
21	70/F	Pleural fluid	-ve	-ve	-ve	-ve
22	35/M	Urine sample	-ve	-ve	-ve	-ve
23	20/M	Pleural fluid	-ve	-ve	-ve	-ve
24	2/M	Pleural fluid	-ve	-ve	-ve	-ve
25	32/F	Bone marrow aspirate(HIV)	-ve	-ve	-ve	-ve
26	45/F	Pleural fluid	-ve	-ve	-ve	-ve
27	13/M	CSOM pus	+ve	+ve	+ve	+ve
28	25/F	Pleural fluid	+ve	+ve	+ve	+ve
29	30/F	Pus breast abscess	+ve	+ve	+ve	+ve
30	25/F	Endometrial biopsy	-ve	-ve	+ve	+ve
31	26/F	Lymphnode Aspirate	+ve	+ve	+ve	+ve
32	28/F	Pus breast abscess	-ve	-ve	-ve	-ve
33	22/M	CSF	-ve	-ve	-ve	-ve
34	45/M	Pus shoulder abscess	-ve	-ve	+ve	-ve
35	55/M	Pleural fluid	+ve	+ve	+ve	+ve
36	40/M	FNAC ileocecal region	+ve	+ve	-ve	-ve
37	34/M	Urine sample	+ve	+ve	+ve	+ve
38	36/F	CSF	-ve	-ve	-ve	-ve

Sl no	Age/ SEX	Specimen	Staining Methods		Culture methods	
			ZN	Fluorescent	LJ	RSC
39	75/M	Pleural fluid	-ve	-ve	-ve	-ve
40	29/F	Endometrial sample	-ve	-ve	-ve	-ve
41	28/M	Pus Lymphnode aspirate	+ve	+ve	+ve	+ve
42	28/M	Pleural fluid	-ve	-ve	-ve	-ve
43	35/M	Synovial fluid	-ve	-ve	-ve	-ve
44	30/F	Ascitic fluid	-ve	-ve	+ve	-ve
45	60/M	CSF	-ve	-ve	-ve	-ve
46	19/M	Pleural fluid	-ve	-ve	-ve	-ve
47	24/M	Pleural fluid	-ve	-ve	-ve	-ve
48	40/M	Pleural fluid	-ve	-ve	-ve	-ve
49	9/F	PUS Multiple abscess	-ve	-ve	-ve	-ve
50	55/F	Pleural fluid	-ve	-ve	-ve	-ve
51	58/M	Pleural fluid	-ve	-ve	-ve	-ve
52	30/F	Synovial fluid	-ve	-ve	-ve	-ve
53	45/F	Pleural fluid	-ve	-ve	-ve	-ve
54	40/M	Pleural fluid	-ve	-ve	-ve	-ve
55	20/M	Pleural fluid	-ve	-ve	-ve	-ve
56	30/M	Pleural fluid	-ve	-ve	-ve	-ve
57	24/F	Lymphnode Aspirate	+ve	+ve	+ve	+ve
58	35/M	pleural fluid	-ve	-ve	-ve	-ve

Sl no	Age/ SEX	Specimen	Staining Methods		Culture methods	
			ZN	Fluorescent staining	L.J Culture	RSC
59	2/M	CSF	-ve	-ve	-ve	-ve
60	65/F	Pleural fluid	-ve	-ve	-ve	-ve
61	53/M	Ascitic fluid	-ve	-ve	-ve	-ve
62	28/F	Endometrial biopsy	-ve	-ve	-ve	-ve
63	67/M	Pleural fluid	-ve	-ve	-ve	-ve
64	59/M	Pleural fluid	-ve	-ve	-ve	-ve
65	32/F	Pleural fluid	-ve	-ve	-ve	-ve
66	38/F	Pleural fluid	-ve	-ve	-ve	-ve

Consent

I, Mr./Miss…… attending R.L.J.H&R.C voluntarily give permission to test the samples for different tests for the diagnosis of Tuberculosis. I am made aware that the results of the test will be communicated to the treating Physician as and when the results are available.

Participant

Date

Name

Sig:

Consent in Case of Minor

I Mr. / Mrs. Guardian / parent of the child, ----------voluntarily give permission to test the samples in different specimens for the diagnosis of Tuberculosis. I am made aware that the results of the tests will be communicated to the treating physician as and when the results are available.

Guardian / Parent

Date

Name

Signature

PROFORMA

NAME:

AGE: | | |

SEX: | M | F |

ADDRESS:

CONTACT N0:

INCOME:

OCCUPATION:

BRIEF HISTORY OF PRESENTING ILLNESS:

PAST HISTORY OF TUBERCULOSIS: | YES | NO |

FAMILY HISTORY

MENSTRUAL HISTORY:

INVESTIGATIONS
NATURE OF SPECIMEN:

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

ROUTINE INVESTIGATION

LABORATORY RESULTS

I) MICROSCOPY

1) ZIEHL NEELSEN'S STAINING

2) AURAMINE STAINING

II) CULTURE

1) LOWENSTEIN JENSEN'S MEDIUM

DATE OF INOCULATION	RESULTS

2) RAPID SLIDE CULTURE TECHNIQUE

III) SEROLOGY

IV) DIAGNOSIS

DATE **EXAMINED BY**

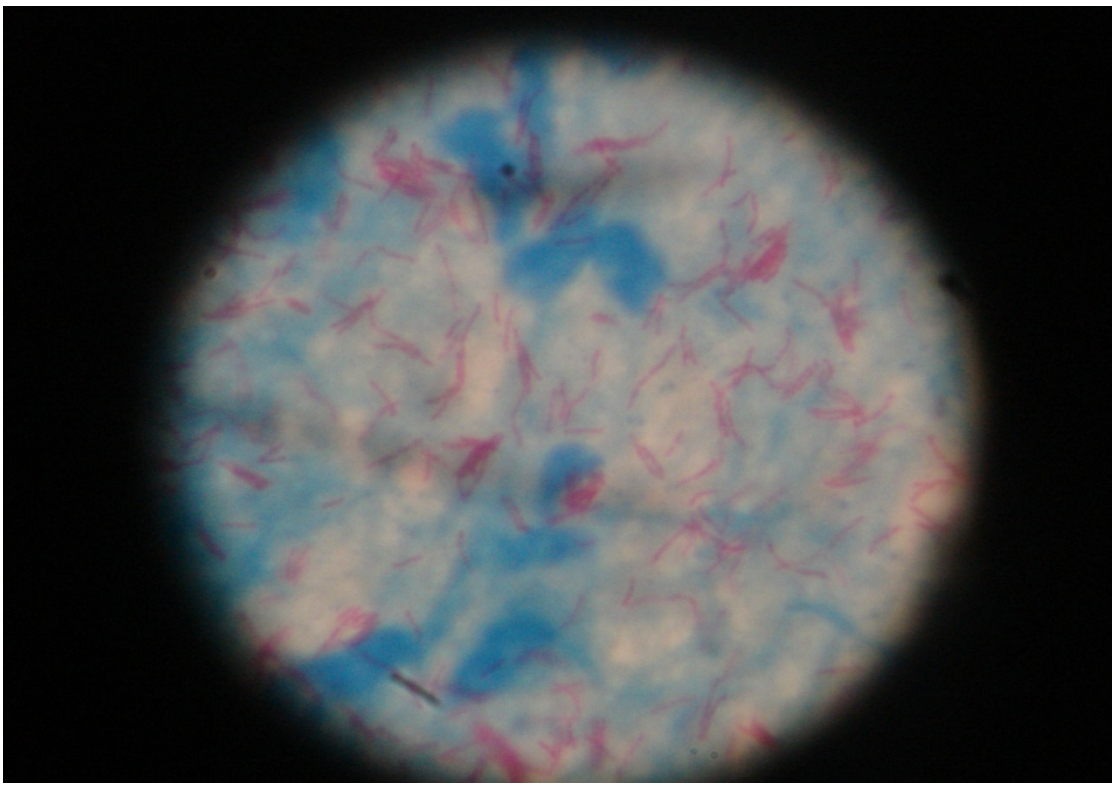

Figure-1 Ziehl Neelsens staining of Pleural fluid showing pink coloured acid fast bacilli.

Figure 2: Characteristic morphology of rough, tough, buff, coloured colonies of Mycobacterium tuberculosis

Figure 3: L J Culture Medium shows growth of Mycobacterium tuberculosis -1+ (20-100 colonies)

Figure- 4: L J Culture medium showing confluent growth- 3+

Figure- 5 Confluent growth of Non Tubercolous Mycobacterium (NTM) on L J Medium

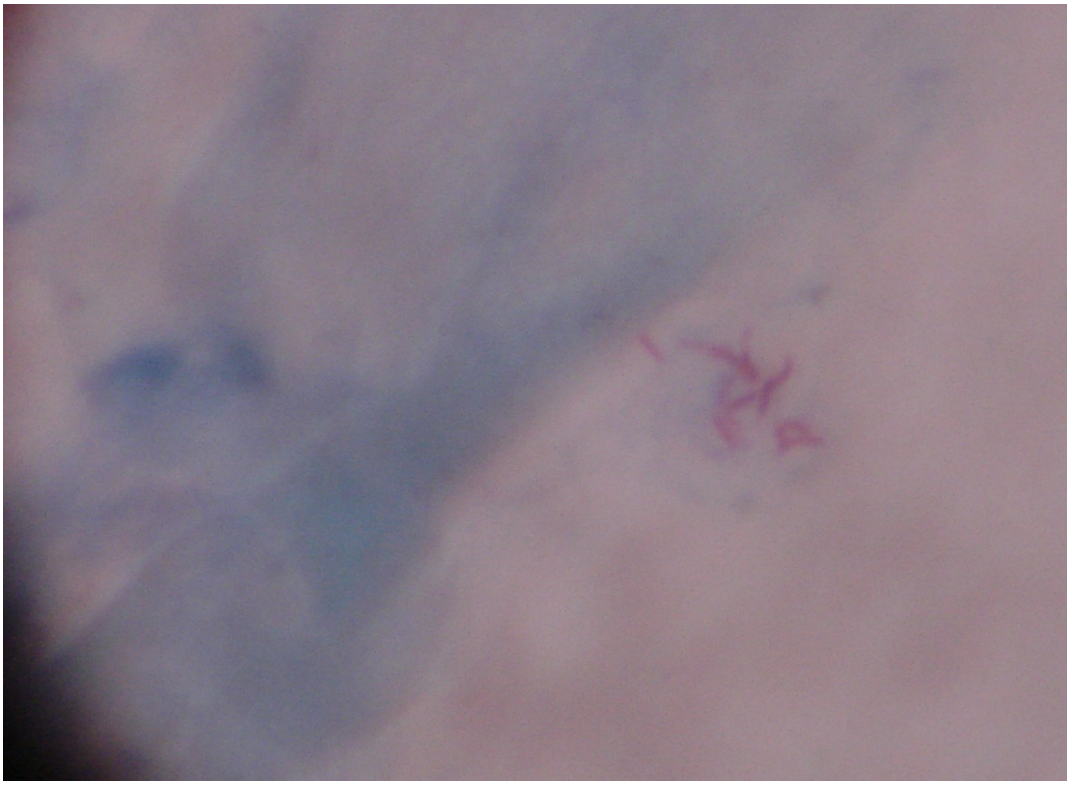

Figure 6: RSC grading (Small clumps of up to four bacilli) -1+

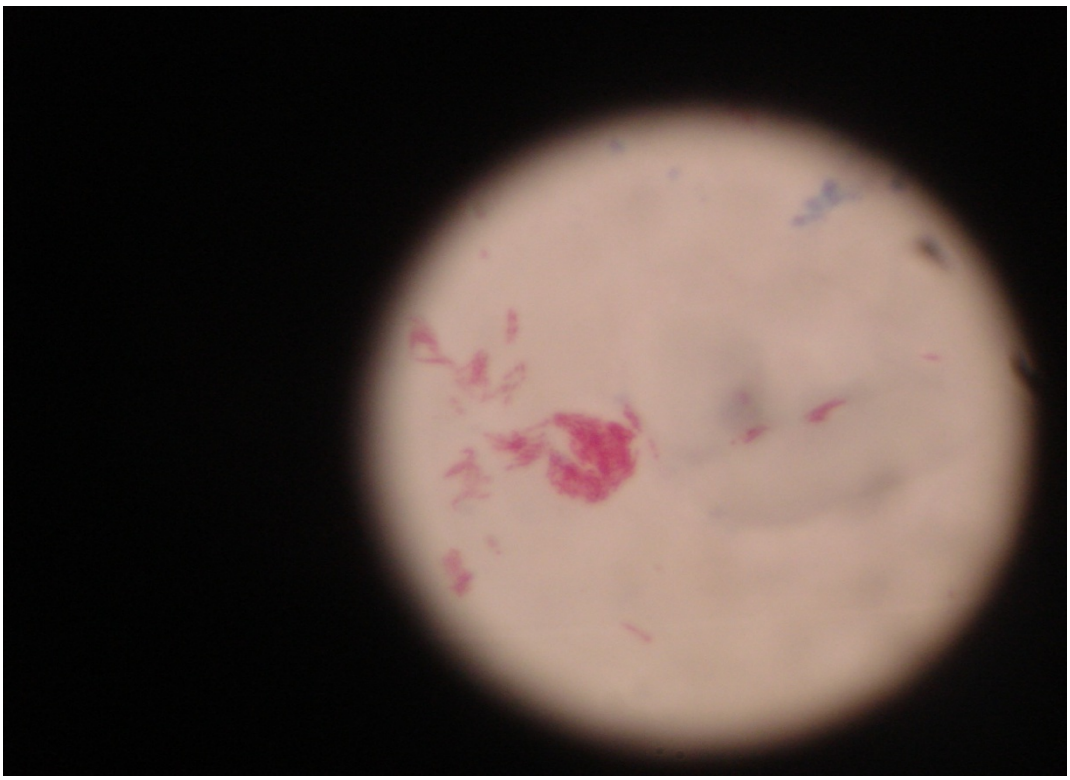

Figure-7: RSC grading small clumps of bacilli, no cord formation- 2+

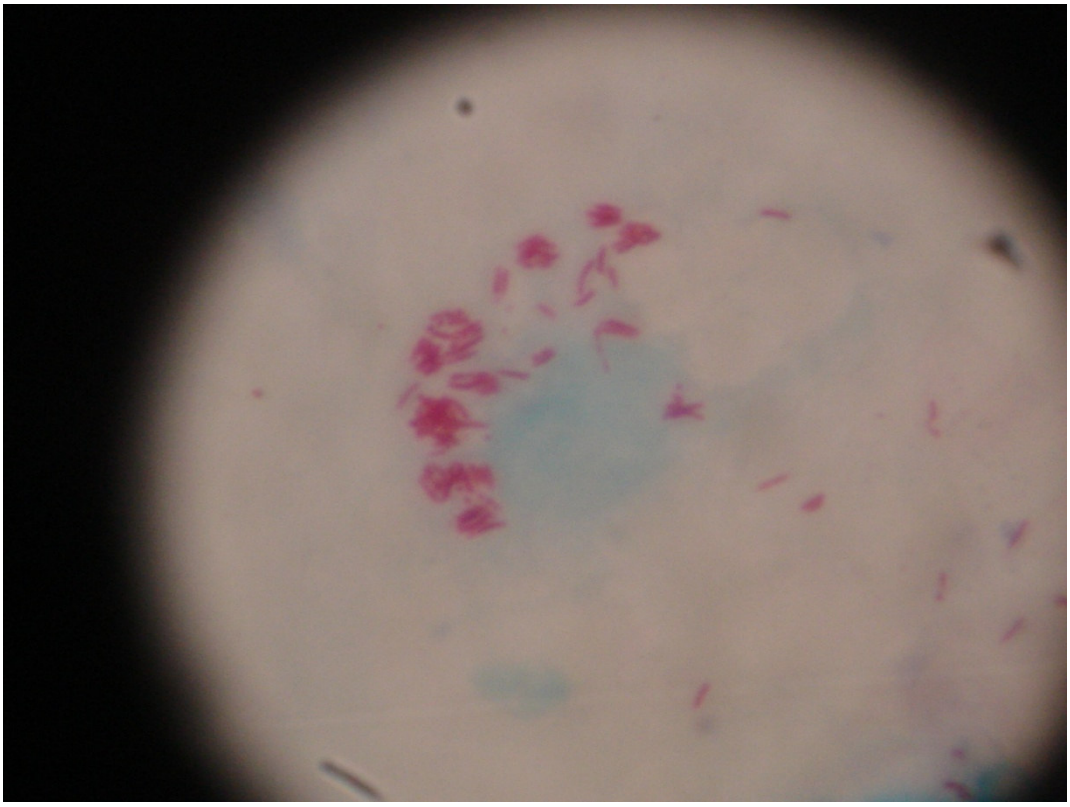

Figure: 8 RSC grading Microcolonies with some cord formation-3+

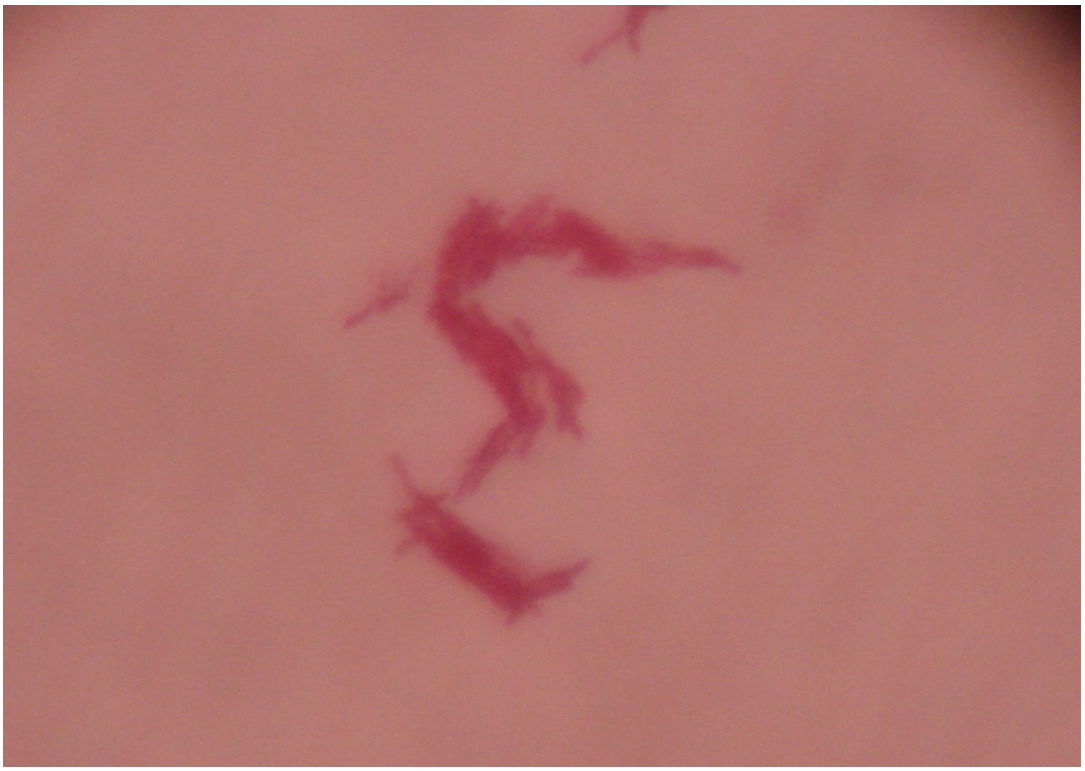

Figure: 9 RSC Grading Good cord formation -4+

Figure: 10 Niacin – test : Test sample with positive(Canary yellow) and negative controls

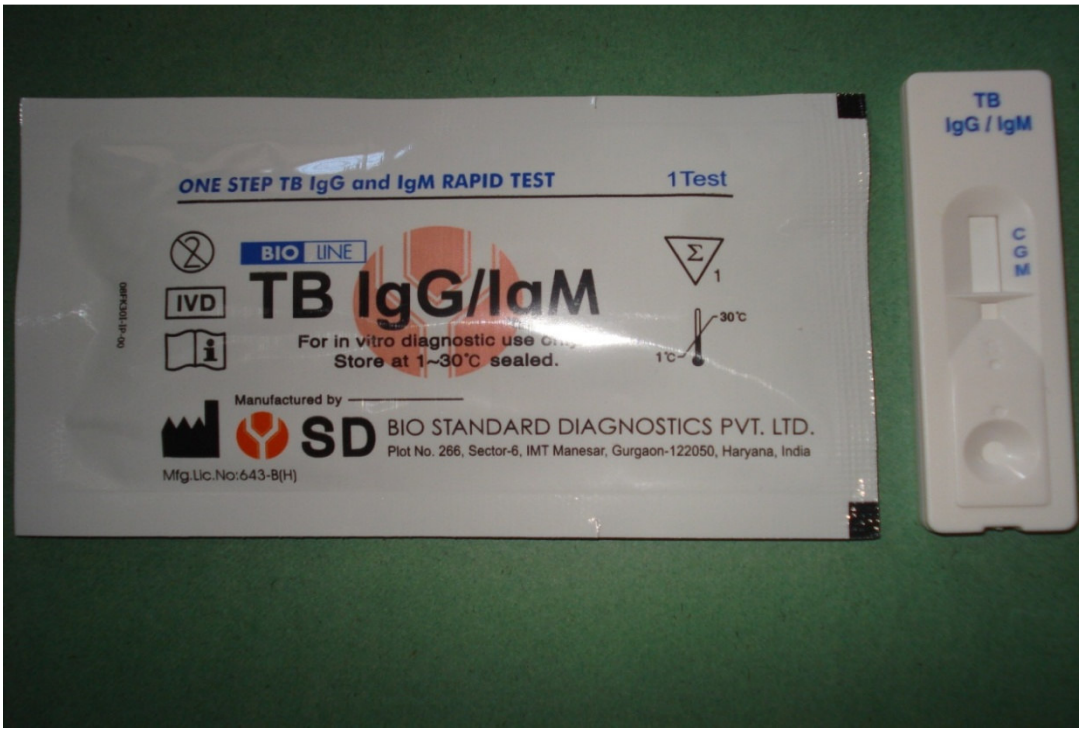

Figure: 11 shows TB IgG and IgM RAPID TEST kit

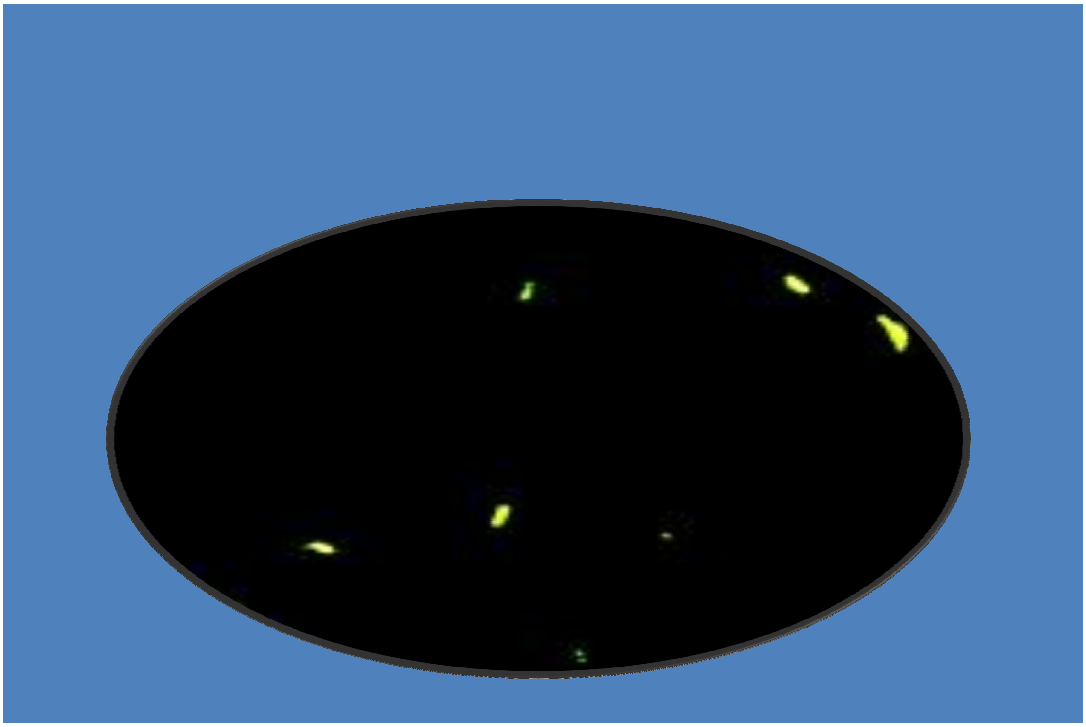

Figure: 12 showing Mycobacterium tuberculosis(bright yellowish orange rods) by Auramine staining