COMPARATIVE STUDY OF BACTERIOLOGICAL TECHNIQUES, CYTOLOGY AND MOLECULAR TECHNIQUE (PCR) USING FINE NEEDLE ASPIRATE IN THE DIAGNOSIS OF TUBERCULOUS LYMPHADENITIS

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DECLARATION

I solemnly declare that this dissertation "**COMPARATIVE STUDY OF BACTERIOLOGICAL TECHNIQUES, CYTOLOGY AND MOLECULAR TECHNIQUE (PCR) USING FINE NEEDLE ASPIRATE IN THE DIAGNOSIS OF TUBERCULOUS LYMPHADENITIS"** is the bonafide work done by me at the Department of Microbiology, Govt. Stanley Medical College and Hospital, Chennai, under the guidance and supervision of **Prof. Dr. P. R. THENMOZHI VALLI, M.D.,** Professor of Microbiology, Govt. Stanley Medical College, Chennai-600 001.

This dissertation is submitted to The Tamil Nadu Dr. M.G.R. Medical University, Chennai in partial fulfillment of the University regulations for the award of degree of M.D. Branch IV Microbiology examinations to be held in September 2006.

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CERTIFICATE

This is to certify that this dissertation entitled "**COMPARATIVE STUDY OF BACTERIOLOGICAL TECHNIQUES, CYTOLOGY AND MOLECULAR TECHNIQUE (PCR) USING FINE NEEDLE ASPIRATE IN THE DIAGNOSIS OF TUBERCULOUS LYMPHADENITIS"** is the bonafide original work done by **Dr. ANEETA ALEXIS** Post graduate in Microbiology, under my overall supervision and guidance in the department of Microbiology, Stanley Medical College, Chennai, in partial fulfillment of the regulations of The Tamil Nadu Dr. M.G.R. Medical University for the award of **M.D Degree in Microbiology (Branch IV).**

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CONTENTS

INTRODUCTION

Tuberculosis has co-existed with humanity since the days before recorded history and evidence of tuberculosis has been found in the skeletal remains of mummies[52]. Hippocrates not only described the disease but also named it 'phthisis' which means to mart or waste away. It is one of the most important diseases in the history of humanity and remains even today as an extraordinary burden to mankind. In the past tuberculosis was the greatest killer and earned the term, 'The captain of all the men of death' from John Bunyan and the 'white plaque' from Rene and Jean Dubars[1].

Tuberculosis is one of the most serious health problems in India and accounts for 30% of the global burden. About 40% of Indian population is infected with Tubercle bacilli. Every year 1.8 million people developed TB of which nearly 8 lakhs are infectious smear positive cases. Every day, more than 20,000 people become infectious with tuberculosis and about 5000 develop disease[78].

The emergence of multi drug resistant Tuberculosis (MDR - TB) and the spread of HIV / AIDS contribute to the worsening impact of the disease. This was the principal reason for WHO declaring Tuberculosis as a global emergency in 1993. Epidemics of HIV related Tuberculosis and multi-drug resistant disease have expanded in the past 5 years and global control of tuberculosis remains as a remote possibility at present.

The unique biological properties of the causative organism,

Mycobacterium tuberculosis complex allows for the long incubation period between the time of infection and the development of symptoms. Despite the availability of curative chemotherapy for more than half a century, tuberculosis continues to cause an enormous amount of suffering, disability and mortality.

Extrapulmonary tuberculosis is a condition where tuberculosis affects any organ other than the lungs. Extra pulmonary tuberculosis may be generalized or confined to a single organ. In otherwise immunocompetant adults, extra pulmonary is found in 15-20% of all the tuberculosis cases.

In the young and immunosuppressed patients rates of extra pulmonary disease are substantially higher. It is seen in more than half of patients with HIV related tuberculosis and quarter of patients with tuberculosis less than 15 years of age. Children less than 2 yrs of age have high rates of miliary and meningeal disease. Both pulmonary and extrapulmonary manifestation are found in upto 50% of patients with HIV related tuberculosis, So it is important to consider the possibility of extra pulmonary pathology when pulmonary tuberculosis is diagnosed in an HIV infected patients[28].

The unprecedented resurgence of tuberculosis in the late 1980s coincided with the growing HIV epidemic. HIV Infection now accounts for much of the recent increase in the global tuberculosis burden. World wide, an estimated 11% of new adult tuberculosis cases in 2000 were infected with HIV with wide variations among regions. Rate of HIV infection among patients with tuberculosis have so far remained below 1% in India. Rates of TB infection

among HIV patients are correspondingly high exceeding 60% in India[56]. Globally TB is a major opportunistic infection in HIV infected patients, often representing their AIDS defining illness and the first indication of immunodeficiency. The epidemiology and clinical manifestations of TB are altered in HIV infected patients.

The most common presentation of extra pulmonary tuberculosis is the tuberculous lymphadenitis, which accounts for 25-30% of the TB cases[28]. Lymph node tuberculosis is particularly frequent among HIV infected patients. Tuberculous lymphadenitis which was once caused by M. bovis is today largely due to Mycobacterium tuberculosis.

Generally, extra pulmonary tuberculosis is difficult to diagnose and in many cases diagnosis is based on clinical signs and symptoms, radiograph, tuberculin testing and history of contact with known cases of tuberculosis. Clinical features may be non-specific and do not give conclusive evidence for the disease. Though demonstration of Mycobacterium in the tissues and nodes remain the Gold Standard, it is not often possible due to the paucibacillary nature of the illness. The definitive diagnosis of extra pulmonary TB is often difficult because of lack of diagnostic facilities and at times difficulty in accessing the affected tissue for intervention. The histopathological examination of lymph node biopsy for macroscopic caseation, typical tuberculous granulation, examination of direct smear from the cut surface for AFB and culture of specimen can make the diagnosis.

Over the past decade, fine needle aspiration cytology has assumed an important role in the evaluation of peripheral lymphadenopathy as a possible non invasive alternative to excisional biopsy. The cytological criteria for the diagnosis of possible tuberculous lymphadenitis have been defined as epitheloid cell granuloma with or without multi nucleated giant cells and caseation necrosis[42]. However there are various conditions that mimic the picture of cytology such as other chronic infection, drug reaction, sarcoidosis, neoplasia etc.

Again the diagnosis of extra pulmonary tuberculosis is difficult especially when clinical condition is suggestive but bacteriological proof is lacking. The diagnosis confirmed by demonstrating AFB using conventional microscopy is specific and rapid but lacks sensitivity. While culture is more sensitive and specific it takes several weeks to get the results. The tuberculous lymphadenitis being a paucibacillary condition, the yield of positive culture has not been high. However using appropriate concentration techniques, using multiple media, and subculture it is made possible to increase the isolation rate in culture.

In paucibacillary tuberculous lesions like in extrapulmonary infections, the conventional methods such as microscopy and culture are less sensitive. For instance, microscopy detects positive smear if atleast 10000 bacilli be present per ml of sample and culture isolation is possible only if 10-100 bacilli is present.

However, recent molecular techniques such as polymerase chain reaction and restricted fragment length polymorphism (RFLP) have high sensitivity and play a major role in diagnosis of extrapulmonry tuberculosis. PCR techniques involve a number of different targets including IS6110 insertion sequences. The Insertion sequence of IS6110 is a mobile genetic element and has universal acceptance, since it is found only in mycobacterium tuberculosis complex, M.tuberculosis strain typically carrying multiple copies of the element[(64)].

In this study a correlation of the cytomorphology of the fine needle aspirate, direct smear and staining by auramine, Ziehl Neelsen Staining, isolation using multiple media such as Lowenstein- Jensen and selective liquid Kirchner media and polymerase chain reaction targeting the insertion sequence IS6110 fragment of DNA of 123 bp is done. The sensitivity and specificity of each test is evaluated statistically. Prevalence of HIV infection among the clinically suspected patients of Tuberculous lymphadenitis was also studied and analyzed.

REVIEW OF LITERATURE

TUBERCULOSIS

Definition:

Tuberculosis, one of the oldest diseases known to affect humans is caused by the bacteria, Mycobacterium tuberculosis complex. The disease usually affects the lungs, although in up to one 3rd of the cases other organs are also involved. If properly treated, virtually all cases of tuberculosis are curable. If untreated, the disease may be fatal with in five years in more than half of the cases. Transmission usually takes place through the air borne spread of droplet nuclei produced by patients with infection pulmonary tuberculosis.

History

Robert Koch first identified the tubercle bacilli in 1882. In his classic reports he defined the staining procedures for the direct observation of bacilli in clinical specimen. Culture techniques on solid medium for the in-vitro passage of bacilli isolated from clinical or experimental lesions and subsequent inoculation of guinea pigs with cultural materials to confirm its etiologic role in tuberculosis. This became the basis of 'Koch's postulates' the standard criteria for etiologic research in infectious diseases.

Tuberculous lymphadenitis is the commonest type of TB described in Hippocrates writing dating back to 460-377 BC. It was initially thought to be cured by the touch of the king (King's evil) in Europe[(60)].

Etiologic Agent

Mycobacteria belong to the Family: 'Mycobacteriaceae' and the order 'Actinomyectales' The name 'Mycobacterium' was given to this genus by

Lehmann and Neumann in 1896 on account of the mould like pellicles produced by these bacteria when grown in liquid media.

There are about 100 mycobacterial species. The term tubercle bacilli broadly include the species of mycobacteria which gives rise to tuberculosis in man. These are M. Bovis, M. africanum , M. tuberculosis, M. microti and M .carnetti[79].

The most frequent and important agent of human disease is mycobacterium tuberculosis. It is a thin rod shaped, non-capsulated non-sporing aerobic, bacteria measuring about 0.5µm/3µm in size acid fast and non-motile.

An important character of mycobacteria is their ability to resist decolorization by a weak mineral acid such as 20% sulphuric acid or 3% hydrochloric acid after being stained by an arylmethane dye such as basic fuschin with which it form stable complexes[43]. This is acid fastness and it is due to the presence of mycolic acid in their cell wall.

On culture, on Lowenstein Jensen media Mycobacterium tuberculosis form the rough, tough and buff colored colonies after an average incubation period of 2-4 weeks. On liquid kirchners media which is incubated up to a maximum period of six weeks, mycobacteria form fine granular growth.

Epidemiology

Epidemiological studies on tuberculosis are concerned with the transmission of the disease in the community and the impact of the control

measures. It is estimated that there were 8.8 million new cases of tuberculosis in 2002 of which 3.9 million were smear positive. The global incidence rates of tuberculosis is growing at approximately 1.1% / pear and the number of cases at 2.4% / year[56].

According to WHO, estimates reach 16-20 million cases of tuberculosis worldwide in 2001.

It is estimated that about one third of the current global population is infected asymptomatically with tuberculosis of whom 5-10 percent t will develop clinical disease during their lifetime. Most new cases and deaths occur and developing countries where infection in often acquired in childhood.

India accounts for nearly one – third of global burden of tuberculosis. Every year, approximately 1.8 million persons developing tuberculosis of which about 0.8 million are new smear positive highly infection cases and about 4.1 lakh people die of TB every year. One person dies every minute[78].

The incidence of TB in HIV infected persons is more than 100 times that of the general population. One of the most threatening features of TB in HIV infected patients has been the spread of multidrug - resistant (MDR) organisms. The national AIDS policy documents from Govt. of India show that > 60% of AIDS patients suffer from TB as an opportunistic infection.

Source of infection:

It is well established that, sputum positive patients i.e. positive on direct microbiological examination and thus contains at least 5000 bacilli in 1 ml and

who has either received no treatment or not being treated fully, can discharge the bacilli in their sputum for years[79]. They become the potential sources of infection.

Transmission And Development Of Disease

Almost all M. tuberculosis infection is acquired by the inhalation of aerosolized droplet nuclei (1-5μm), which reach the pulmonary alveoli. The probability that a person will become infected depends upon the duration of exposure to the source, the size of the bacillary inoculums inhaled, and the infectivity of the Mycobacterial strain. The probability of an immuno-competent host developing active TB after M. tuberculosis infection is 5-10% over the person's lifetime. Patients with extra pulmonary tuberculosis or smear negative tuberculosis constitute a minimal hazard for transmissions of infections.

The time from receipt of infection to the development of a positive tuberculin test ranges from three to six weeks. The development of disease depends upon the closeness of the contact, extent of disease, sputum positively of the source case and host parasite relationship. Thus the incubation period may be weeks, months or year[56].

Mycobacterium Tuberculosis Complex

The term M. tuberculosis complex refers to a group of very closely related species. All of them cause tuberculosis, a chronic granulomatous disease affecting man and many other mammals.

M. tuberculosis - causes human infection

M. bovis - affects cattle and other mammals

M. microti - pathogen of voles and other smaller mammals

M. africanum - intermediate between human & bovine types.

Non- Tuberculous Mycobacteria

During the early 1950s, after it had become routine practice to culture clinical specimen for M. tuberculosis, it was realized that other mycobacteria can also cause disease in human. These organisms became known collectively as non-tuberculous mycobacteria. The non-tuberculous mycobacteria, especially the Mycobacterium avium complex and M. scrofulaceum should be kept in mind in the HIV millennium.

Extrapulmonary Tuberculosis

Although virtually all organs may get affected, the order of frequency of the extrapulmonary involvement is the lymph node, pleura, genitourinary tract, bones, joints, meninges and peritoneum.

Tuberculous Lymphadenitis

Tuberculosis of the superficial lymph nodes is the commonest extra pulmonary manifestation of the disease. In India, approximately 15-20% of all TB cases is extra pulmonary and of these 2/3rd are due to lymph node disease, the commonest site being cervical lymph nodes[45].

Pathophysiology of Lymphnode Tuberculosis

Lymph node tuberculosis occurs

1) Either as a result of primary infection,

2) As reactivation of previously contained foci or

3) By extention from a contiguous focus

In primary infection, most commonly acquired by airborne infection, non-specific inflammatory reactions occur as the mycobacteria reach the alveoli, and phagocytosis and intra cellular replication takes place. A bacteremia occurs with dissemination throughout the body. Lymphatic involvement is an integral part of tuberculous infection with generalized lymphatic and hematogenous spread rather than a localized disease process. The vast majority of the primary lesion heal and reactivation as a result of decreased body defence due to various causes is the mechanism by which tuberculosis in the adult usually develops, whether it is the lungs or in lymph nodes or in other extra pulmonary sites.Supraclavicular lymph node tuberculosis is usually caused by a lymphatic spread from mediastinal disease.

HIV Infection And Extra Pulmonary Tuberculosis

Extrapulmonary disease has been reported in up to 70% of HIV related TB cases when the CD4 lymphocyte count is less than 100[36]. HIV related TB lymphadenopathy could occasionally be acute and resemble an acute pyogenic bacterial infection. .

CD4 cell mediated immunity and macrophage function are essential in the control of M.tuberculosis infection. During primary infection of an immunocompetant host, cell mediated immunity usually develops and arrests the progression of the disease. About 5% of patients, whose primary infection is controlled, have reactivation years or decades later. In another 5% patients, infection is not contained and primary, pulmonary, extrapulmonary or desseminated TB can occur.

The hallmark of HIV infection is progressive deterioration and depletion of CD4 cells, coupled with defects in macrophage and monocyte function and increasing tissue destruction. There is evidence that the immune response in patients with TB might enhance HIV viral replication and accelerate the natural progression of HIV infection. TB enhances HIV replication by secretion of TNF alpha, destruction of CD4 cells and fastened release of virions from infected cells and increased apoptosis.

The risk of TB developing in a HIV infected a patient who is latently co-infected with MTB approaches 10% per year, as opposed to a 10% lifetime risk in an immuno competent host.

Clinical Features

Persons affected with tuberculous lymphadenitis may have nonspecific constitutional symptoms[4]. Locally, there may be painless swelling with or without a discharging sinus or a scar or both. A classical presentation is multiple, matted lymph nodes with variable consistancy which are painless and slow growing.

The physical appearance of superficial tuberculous lymphadenitis has been classified into five stages by Jones & Campbell[33].

Stage 1 - Enlarged, firm, mobile, discrete nodes.

Stage 2 - Large, rubbery nodes fixed to surrounding tissue owing to periadenitis

Stage 3 - Central softening due to abscess formation

Stage 4 - Collar stud. abscess formation

Stage 5 - Sinus tract formation.

TB lymphadenitis is a common disease of children. However in the recent past, there is a peak age range of 20-40 years. Tuberculous lymphadenitis in adults show striking female preponderance.

DIAGNOSIS OF TUBERCULOUS LYMPHADENITIS

Tuberculous lymphadenitis often presents a diagnostic challenge especially when clinical presentation is suggestive but bacteriological proof is lacking.

The culture isolation of tubercle bacilli from lymphnode biopsy specimen remains the gold standard. Confirmatory test for the diagnosis of the disease.

The differential diagnoses of tuberculous lymphadenitis are many:

1. Neoplasia - Hodgkin’s and Non Hodgkin’s Lymphoma, sarcoma.

2. Other infection-acute bacterial infection, viral infection, (infectious mononucleosis), Chlamydia, toxoplasmosis, fungal infection, non-tuberculous, mycobacterial infection, BCG adenitis.
3. Drug reaction - eg. Hydantoin
4. Sarcoidosis
5. Non-lymphode swelling - Submandibular or parotid glands, bronchial cysts, cystic hygroma, carotid body tumour, thyroid swellings.

The diagnosis is often made on clinical grounds. Biopsy of the node is not always done. This may be due to the fact that either facility is not available, especially in rural areas, or reluctance on the part of patient to undergo a surgery, which needs general anesthesia.

Histological Diagnosis

Four distinct types of histological features are seen in lymphnode biopsies[33]. Most common is the 'reactive' type with typical tuberculous granuloma, with fine eosinophilic caseation necrosis, epithelioid cells, giant cells, plasma cells and lymphocytes. Next in frequency is the 'hyperplastic' type with well-differentiated epithelioid cell granuloma with very little necrosis. The third is 'hyporeactive' type with poorly organized granuloma with macrophages, immature epitheloid cells, lymphocytes, plasma cells and coarse predominantly basophilic caseation necrosis and 4th type is a non-reactive type unorganized granuloma with granuloma with macrophages, lymphocyte, plasma cells & polymorphs with non caseating necrosis[67].

Cytological Diagnosis

This simple outpatient procedure has provided an alternative and an easy method for collection of material for cytomorphologic and bacteriologic examination.

Fine needle aspiration cytology involves the study of cells obtained from virtually any part of the body. As its name itself implies, material for cytological examination is obtained by aspiration using a fine needle. Using one of the various techniques using stains such as Haematoxylin-Eosin stain, Giemsa stain, wright stain and papanicolaou stain,the cytology is studied.

The advantages of FNAC are that, it is a simple outpatient procedure that requires very little time and equipment. It causes minimum discomfort to patient. Quick diagnosis is possible and it is easy to repeat and for follow-up.

The disadvantage is that it involves interpretation of limited sample obtained by the aspiration. The aspiration technique is of at most importance. Even in the hands of an experienced person the percentage of 'Non-representative' aspirates may be up to 20% or more. It is difficult to exactly localize and aspirate the small and deep-seated lesions[42].

Diagnosis of tuberculosis can be made by demonstration of epithelioid cell granuloma with or without multinucleated giant cells and caseation. Necrotic features whether acellular or accompanied by neutrophilic infiltrate are usually misdiagnosed as suppurative abscesses, such smear however, show AFB positivity and thus the diagnosis of tuberculosis is still possible even in the absence of epithelioid granuloma[33].

FNAC is of particular use in patients presenting with multiple nodes, a common enough clinical presentation in our setting. In a patient with multiple lymphadenopathies, deciding on which node to biopsy can be difficult because some of the enlarged nodes may not reflect the true disease process. When such a lymph node is biopsied, the actual diagnosis can be delayed or even missed. FNAC is advantageous in such situations. Many if not all, of the enlarged lymph node can be sampled at one sitting. FNAC claims sensitivity and specificity of 80-90 %[67].

Bacteriological Diagnosis

Lymph node tuberculosis, like other form of extra pulmonary disease is a paucibacillary condition. The yields of positive cultures from the lymphnodes have not been high. However, using appropriate techniques it is possible to culture Mycobacterium tuberculosis. Multiple media like LJ medium, LJ medium with pyruvate, 70H, oleic acid medium and liquid Kirchner's medium are used for culturing Mycobacterium tuberculosis from extra pulmonary specimens including lymph nodes.

LABORTORY DIAGNOSIS OF TUBERCULOUS LYMPHADENITIS

This can be broadly divided into

1) Demonstration by Direct microscopy - light and Fluorescent
2) Isolation of mycobacterium tuberculosis by culture
3) Serodiagnosis of the infection
4) Molecular methods

Direct Microscopy -Staining

i) Ziehl-Neelsen Staining (ZN)

ZN staining is a highly specific technique where AFB is demonstrated after staining. It is positive only if number of AFB is more 10000 per ml of specimen[19]. The correlation of positive smear to positive cultures may be only 25% to 40%.

ii) Speical Staining For AFB

Fluorescent Microscopy

Auramine dye is a fluorochrome, which can be raised to a higher energy level after absorbing Ultra violet (Excitation) light. When the dye molecules return to their normal low energy state they release excess energy in the form of visible (fluorescent) light. Auromine requires blue excitation light, excitor filters that select light in 450-490 wavelenth range and a barrier Fluorescent filter for 515. Brightly fluorescent bacilli appear Yellow against a dark background[10]. Its sensitivity and specificity are fairly similar to ZN microscopy.

Mycobacterial Culture

Definitive diagnosis depends on the bacteriological isolation and identification of Mycobacterium species from clinical specimens.

Specimen Preparation:

Homogenization:

It is essential to release the mycobacteria from the body fluid or tissues in which they are contained. Tissue requires mechanical homogenization before decontamination. N-acetyl -L Cysteine (NALC) or 4% Sodium Hydroxide (NaOH) is used as a mucolytic agent to assist liquefaction of sputum and purulent samples[41].

The high concentration of lipids in the cell wall of most mycobacteria makes them more resistant to killing by strong acid and alkaline solutions than

other bacteria that may be present in the specimen. Consequently, specimens likely to contain a mixed bacterial flora are treated with decontaminating agent to reduce the undesirable bacterial overgrowth and to liquefy the mucus. After treatment with the decontaminating agent for a carefully controlled time period, the acid or alkali used is neutralized and the mixture is centrifuged at high speed to concentrate the mycobacteria

Digestion and decontamination:

Various agents such as 4% sodium hydroxide, trisodium phosphate alone or in combination with benzalkonium chloride (Zepheran), N-Acetyl L cysteine (NALC), cetyl pyridium chloride (CPC),4% oxalic acid and 5% sulphuric acid can be used. The exposure time to these decontaminating agents is very critical, as overexposure leads to loss of considerable number of mycobacteria. Each mycobacteriologist should select the agents to be employed in his laboratory on the basis of the number and types of specimens received[43].

Neutralization :

After the decontamination time of 15-20 minutes usually, the agents is neutralized by the addition of distilled water or phosphate buffer, in case of decontamination with NALC.

***Centrifugation* :**

Carefully controlled centrifugal force is important in the recovery of mycobacteria from clinical specimens. This centrifugation is necessary to concentrate the bacilli. The sediment is inoculated into the media. The recovery of culture is increased when the relative centrifugal force is around 3000g for 15 minutes[19].

Inoculation of specimens :

Several egg based and agar based culture media are available for recovery of mycobacteria. Some of the non-selective media available are Lowenstein Jensen medium which is most commonly used in most of the clinical diagnostic laboratories, Petragnani medium, Middle brook 7H 10 agar and 7H 9 liquid medium. American Throacic Society (ATS) medium, which contains less amount of malachite green, is recommended for use, for the

usually sterile specimen such as CSF, pleural fluid, etc[19].

Some of the selective mycobacterial isolation media are Gruft's modification of Lowenstein Jensen, selective 7H11 (Mitchinson) medium, Middle brook 7H 10 medium. For extra pulmonary tuberculosis, the selective Kirchner synthetic liquid medium containing horse or bovine serum is 213used in addition to Lowenstein Jensen Medium. It may yield high isolation from the paucibacillary samples.

Subculture from the liquid medium on to the solid medium gives isolation of organism with distinct eugonic rough, tough and buff colonies. About 4 to 8 weeks may be required before the growth is detected.

Serological Diagnosis

M. tuberculosis is the paradigm of the successful intracellular pathogen. Although the organism evokes both a humoral and a cellular immune response, it is the later that determines the outcome of an infection. A variety of immunodiagnostic tests for tuberculosis based on the recognition of specific host response to the infecting organism have been described. The first test was the tuberculin skin test. The short comings of this test include the inability to distinguish active disease from past sensitization and unknown predictive values[79]. There has been development of serological tests for tuberculosis, but no test has found widespread clinical use, because of low specificity.

Sensitivity and specificity increase if ELISA with purified antigen is done. The antigens tested in serological assays include the 38 KDa antigen, Lipoarabinomannan, Antigen-60[32], antigen 85 KDa complex and glycolipids

including phenolic glycolipid Tb1, 2y3 – diacyl trehalose and lipo-oligosaccharide.

Most patients with tuberculosis produce antibody to glycolipids and 38 KDa and 85 complex antigens and most healthy controls do not. However, a small proportion of tuberculous patients still have low levels or an absence of antibodies against any of these antigens[64].

A number of antigen capture assays based on enzyme-linked immunosorbant assay, western blot analysis of M. tuberculosis H_{37}RV culture filtrates antigen, radio immunoassay or agglutination of antibody coated latex particles have been described. There is wide variation in sensitivity and specificity of these tests and the antigen test cannot be recommended at this time.

Molecular Diagnosis

In recent times, polymerase chain reaction (PCR) has been found to be the most sensitive technique for rapid diagnosis of M. tuberculosis. This technique, capable of amplifying minute amounts of a specific DNA sequence into millions of identical copies has revolutionalised molecular biology research.

PCR is accomplished in the following manner. A specimen that may contain the organism with the DNA sequence of interest is heated to denature the double stranded DNA. The specific synthetic oligonucleotide 'Primers' (short single stranded pieces of DNA) bind to the unique DNA sequences of interest and a heat stable DNA polymerase extends the primers to create a

complete and complementary strand of DNA. This process is typically repeated sequentially 20-40 times, thereby creating million of copies of target DNA sequence. The amplified sequences can then be detected easily by gel elecrophoresis.

The DNA sequence is called as IS6110 after the insertion sequence it represents. The IS 6110 belongs to a class of molecules known as transposons, which are self-replicating stretches of DNA. This sequence has been found in the M. tuberculosis complex of organism (M. Tuberculosis, M. africanum, M. microti and M. bovis) but in no other mycobacterial species. Thus IS6110 serves as a useful amplification target in diagnosing tuberculosis because except for M. tuberculosis the members of the M. tuberculosis complex are not the usual human pathogens or colonizers.

Other amplification targets for PCR are possible. A popular choice in molecular diagnostics has been to use the ribosomal RNA (r RNA) sequence as amplification target, as these sequences are found in abundance in living organism and should allow even more sensitive PCR - based assay. Ribosomal RNA is highly specific and abundant for any particular species[(64)].

> The Restricted Fragment Length Polymorphism (RFLP) technique using the IS6110 repetitive sequence is considered for typing the M.tuberculosis complex strains. It involves the extraction of genomic DNA, restriction endonuclease digestion with PvoII, Southern blotting and probing for IS6110. Standardization of the procedure facilitates

inter-laboratory comparability of patterns. However comparision of profiles requires sophisticated software for image analysis and well-trained technical staff. Other molecular methods such as spoligotyping and whole genome finger- typing methods used for epidemiological studies[45]. Other recent methods include phage-based assays (Commercial kits-FAST Plague-TB), which diagnosis TB directly from specimens. Newer Versions are also being developed for the detection of drug resistance directly from sputum specimens[53].

Other methods for Rapid Identification:

High Performance Liquid Chromatography:

Each species of mycobacteria appears to synthesize a unique set of mycolic acid. Qualitative and quantitative differences in the spectrum of mycolic acids present in the cell wall are reliable criteria to identify mycobacterial species. Once mycobacterial cultures are available, the mycolic acids are extracted from saponified mycobactria, converted to b -bromophenacyl esters and analysed by higher performance liquid chromatography (HPLC). The resulting pattern is compared to a library of referring patterns to identify the species. This assay can provide rapid definite species identification for essentially any mycobacterium isolate in contrast to the time-consuming biochemical procedures and other conventional tests for speciation[79].

Immunohistochemistry[7]

This method lies on the principle that there are many immuno reactive substances within the cell wall and cytoplasm of mycobacteria. Polyclonal antibodies and mycobacterium specific monoclonal antibodies have been raised to these antigens. This is a simple and sensitive techniques which has been used to identify mycobacteria in cultures, sputum as well as on smear and tissue sections[13].

Automated Detection Systems:

Many Semi-automated and automated mycobacteria detection systems are currently available; they are the radiometric Bactec system, non-radiometric MB/Bac T System and ESP Myco system. Other manual systems are the mycobacteria Growth Indicator Tube (MGIT) [13] system and Septi-chek System.

DRUG SUSCEPTIBILITY TESTS

The three general methods are in use[41];

The absolute concentration method:

It involves standard inoculum on media containing graded concentration of drugs and resistance is expressed in terms of MIC - minimum inhibitory concentration.

The Resistance Radio Method:

Compares the growth of known strain in the same set and test strain is various dilutions of drug media and resistance is expressed in terms of ratio of MIC of test strain / MIC of standard strain.

Proportion method

This method enables precise estimation of the proportion of bacilli, resistant to a given drug. If >1% of strain show resistance compared to control at a critical concentration, the strain is considered as resistant. The proportion of bacilli resistant to a given drug is then determined by expressing the resistant portion as a percentage of the total population tested.

LJ Slopes with different concentrations of various drugs are prepared and a standard inoculum giving approximately 10^5 CFU/ml is inoculated in to them and incubated at 37°C. Cultures are examined for growth at 28th day[44].

Recent Methods in Detection of Drug Resistance

More recently the Bactec 460 TB instrument has allowed sensitivity test results to be produced in 1-2 weeks using a method based on 1% proportional method. A radiometric method also facilitates testing of drug combinations by using single concentration of the test agents.

The presence of drug resistant mutants in M. tuberculosis can be detected with polymerase chain reaction and RFLP methods. The resistance to drugs such as Isoniacid and Rifampicin are most often due to point mutations. Thee are several other PCR – based strategies that might make detection of point mutations possible. One such technique is single stranded conformation polymorphism (SSCP) analysis[64].

IDENTIFICTION TESTS

These include Niacin production, Aryl sulphatase, Phosphatase, Catalase and Nitrate reductase activity. Classification of Mycobacteria into different species based on biochemical tests and growth characteristics have been described and evaluated by the International working group on Mycobacterial Taxonomy. The following basic tests are routinely employed to differentiate M. tuberculosis from other mycobacterial species[39].

IDENTIFICTION OF M. TUBERCULOSIS

Test	M. Tuberculosis	Others
Growth rate	Slow	Slow/rapid
Temp. Requirement	35°C - 37°C	25°C – 37°C
Pigmentation	Absent	Present/absent
Niacin	Positive	Mostly negative
Stability of catalase at 68°C	Negative	Positive
Growth on PNB media	Negative	Positive

Species level identification of Non-tuberculous mycobacterium
Runyon's Classification

Runyon Group	Growth Rate	Pigmentation	Typical Member
I	Slow > = 5 day	Photochromogenic (Yellow or orange Pigment after exposure to light)	M. Kansasi M. marinum
II	Slow > 5 days	Scotochromogenic (Yellow or orange pigment in the dark)	M. scrofulaceum
III	Slow > 5 days	Nonchromogenic No pigment in the dark or in the light	M. avium M. intracellulare
IV	Rapid < 4 days	Variable	M.fortuitum complex M. smegmatis M. phelei

AIMS AND OBJECTIVES

- Isolation of Mycobacterium tuberculosis from the fine needle aspirate samples by employing conventional bacteriological methods in patients clinically diagnosed as tuberculous lymphadenitis.

- Sputum smear examination by Ziehl- Neelsen technique and culture isolation of Mycobacterium tuberculosis in patients with pulmonary symptoms.

- Identification of Mycobacterium species employing standard phenotypic methods.

- Performing drug susceptibility tests on the isolates identified as Mycobacterium tuberculosis.

- Detection of M. tuberculosis specific gene by performing PCR on fine needle aspirate samples.

- Screening the study patients for the presence of HIV infection.

- Correlation of the conventional techniques, such as smear & bacteriological isolation, cytomorphologic features with molecular technique in the laboratory diagnosis of tuberculous lymphadenitis.

MATERIALS AND METHODS

Study Population :

One hundred and twenty five Patients who were clinically suspected as tuberculous lymphadenitis cases were included in this study.

Study size :

125 clinically suspected tuberculous lymphadenitis cases

Study Period :

2 years from December 2003 to January 2006.

The present study was conducted in Stanley Medical College for a period of two years. Seventy five patients with strong clinical features suggestive of Tuberculous lymphadenitis from both out patient and in-patient, Department of Medicine and surgery of Government Stanley Hospital and 50 patients from Govt. Hospital of Thoracic Medicine, Tambaram Sanatorium, Chennai were included in the study.

The age of patients ranged from three years to fifty years and the female:male ratio was 1:0.95. The clinical symptoms suggestive of Tuberculous lymphadenitis were fever, anorexia or weight loss and lymphadenopathy.

Sample collection

Fine needle aspirate;

Fine needle aspirate was collected from the lymphnodes, by the pathologist in the Clinical Pathology Department of Government Stanley

Hospital and Government Hospital of Thoracic Medicine, Tambaram Sanatorium, Chennai and cytology was reported by the pathologist in Govt. Stanley Medical College.

Procedure :

Fine 23 gauze needle with disposable 10ml syringe was used. After cleaning the area with spirit swab, the lesion was fixed well and the needle is inserted into it. While moving the tip of the needle around into the lesion, maximum suction was applied on the piston of the syringe. Gradually the piston was allowed to fall back, neutralizing the negative pressure in the syringe. The needle was withdrawn and the material collected was used for the study.

The Sample was divided into 4 parts; one part was smeared on to a slide and fixed with 95% alcohol for Haematoxylin and Eosin staining. The second part was smeared on the slide for direct smear examination after staining with auramine stain and subsequently Ziehl- Neelsen stain. The third part was inoculated in to Lowenstein-Jensen & Kirchner medium after concentration technique by modified Petroff's method. One portion of the material was collected in an eppendorf tube containing 100µl of Tris EDTA buffer for PCR

Serum sample :

Under aseptic precaution 5ml of blood sample was collected by venepuncture, serum separated, aliquoted and stored at 20^{o}C till the time of test.

Laboratory Safety[44]

All the safety measures were taken during sample collection, used leak proof unbreakable containers. 5% phenol was used as disinfectant solution. A microbiological safety cabinet (Class 1) open fronted with exhaust was used. This offers adequate protection to the worker against inhalation of aerosols [49].

Cleaning of the laboratory was done with clear phenolic disinfectants and solutions of sodium hypochlorite and the room was periodically fumigated with formaldehyde.

Statistical analysis;

Taking culture positivity as gold standard, the sensitivity and specificity of different assay methods were calculated. Logistic regression analysis test was performed evaluate the diagnostic tests.

Staining Technique for the Fine Needle Aspiration Cytology

Smear Fixation :

Fixation prevents the post mortem changes and cells are preserved as close as possible to living state so as to make a proper cytologic study. Fixation was done in this study by using ascending graded concentration of alcohol beginning with 70% and going to 80%, 90% and absolute alcohol over a period of 1-15 minutes.

Haematoxylin and Eosin Staining;

Procedures

1) Slide was washed with running tap water
2) Kept dipped in a trough of haematoxylin for 10-15 min.
3) Washed with running tap water.
4) Dipped in Acid alcohol for 10 seconds.
5) Washed in running water three times.
6) Observed for the development of Blue colour (bluing).
7) Dipped in 1% eosin for 5-25 seconds
8) Washed with running water.
9) Air dried and mounted with DPX mountant and coverslip applied.
10) Viewed under high power microscopy.

Examination of Ziehl-Neelson Stained Smear of the Fine Needle Aspirate

Preparation of Smear

Marked the specimen number on the glass slide with a diamond pencil. Used one slide for each specimen. With a 5mm internal diameter 24 SWG Nichrome wire loop the specimen was smeared in an uniform layer on about two thirds of the slide.

The smears were prepared in duplicates one for Ziehl-Neelsen staining and the other for Auramine staining. Slides were air dried completely in safety cabinet and transferred to the hot plate (80^{o}C) to fix for 10 mts.

Staining for Fluorescence Microscopy[10]

1. Placed the slides on a staining rack with the smeared part upwards.
2. Covered the slides with freshly filtered auramine phenol and left it for 10 mts.
3. Washed well with running tap water in a controlled flow.
4. Decolourised by covering completely with acid alcohol for 2 mts.
5. Washed again with running tap water
6. Counter stained with 0.1% potassium permanganate for 30 seconds.
7. Washed again with water and placed in hot plate to dry.

Fluorescence Microscopy

Switched on the mercury vapour lamp10 mins before examining. Using the low power (Magnification =x250) objective first the bacillary morphology is identified and then the positive smears are graded into three degrees of positivity using the high power objective (Magnification = x 400)

Grade	**No. of Bacilli / HPF**
+	< 6
++	6 - 100
+++	> 100

Ziehl-Neelsen Staining Procedure[72]

1) Placed the slide on the staining rack with the smeared side up.
2) Flooded entire slide with strong carbol fuchsin .
3) Heated the slide slowly until it was steaming. Maintained steaming for 5 min by using intermittent heating.
4) The slide is rinsed in a gentle stream of running water until all free stain is washed away.
5) Flooded the slide with acid alcohol (3%) as a decolourising agent for 2 to 3 mins.
6) Rinsed the slide thoroughly with water. Drained excess water from the slide.
7) Flooded the slides with methylene blue counter stain for 30 seconds
8) Rinsed the slide thoroughly with water
9) Allowed the smear to air dry.
10) Viewed under oil immersion Microscope

Recording and reporting of results;

For reporting the number of AFB observed in stained smears the following method is used.

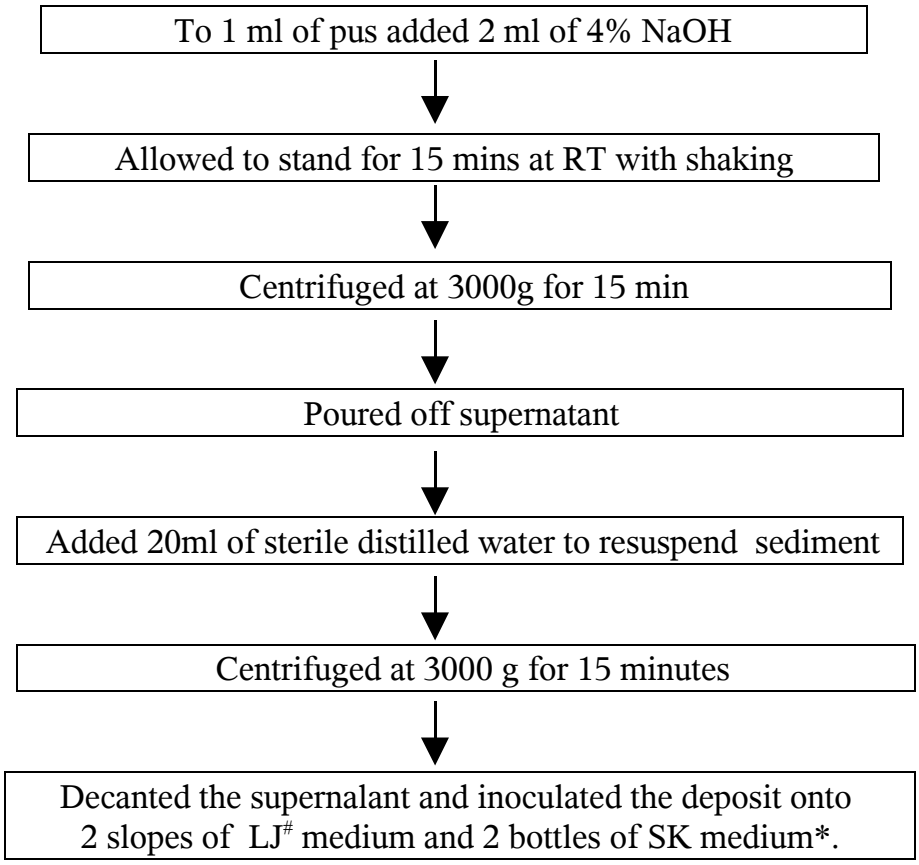
No. of Acid Fast Bacilli (AFB)	Fields (Oil Immersion)	Report
Nil	300	Negative
1-2	300	Record exact figure AFB per 300 fields
1-9	100	1+
1-9	10	2+
1-9	1 field	3+
>9	1 field	4+

CULTURE METHODS

Sample Processing for purulent fine needle aspirates

Homogenised the pus sample in vortex mixture for few minutes to free the bacilli from the mucus, cells or tissue in which they may be embedded. The sample was decontaminated by using 4% Sodium Hydroxide (Modified Petroff) method[58].

Procedure



Lowenstein Jensen Medium

* Kirchner Synthetic Medium

If fine needle aspirate contain only cells and scanty or no purulent material, it was directly inoculated in SK medium and incubated for 6 weeks, after which irrespective of whether growth is present or not, decontaminated with modified Petroff method and inoculated into 2 slopes of LJ.

Culture Media

Preparation of Selective Kirchner Synthetic Medium

The properly weighed salts are dissolved in distilled water, then added 3 ml of glycerol and 7.5ml of phenol red (0.4%) made up the volume of 250ml. Checked the pH (6.9-7.2). Then autoclaved the salt solution at 15 lbs/15 min. After being brought to RT, added antibiotic cocktail consisting of polymyxin, amphotericin B, trimetho prim and carbenicillin. Then added sterile calf serum. Distributed in 7-10 amounts into sterile Mc Cartney bottles and tightly closed. Test for sterility was done by incubating at 37^{o}C overnight.

Preparation of Lowenstein - Jensen Medium

Done in 3 steps

1) Prepared the 2% malachite green solution
2) Prepared salt solution with malachite green (SSMG)
3) Prepared the Egg fluid

Procedure

Dissolved 0.5gm of Malachite green dye in 25ml of distilled water by grinding the dye with water using a motor and pestle Dissolved the mineral salts in order in about 75ml distilled water by heating. Added 3 ml of glycerol, 5ml of malachite green solution and made upto 150ml with distilled water. Autoclaved at 121^{o}C for 30 minutes to sterilize.

Homogenisation of Whole Eggs

Fresh hens eggs not more than 7 days old with minimum air space are used. Checked for viability by candling using egg Candler. Scrubbed thoroughly with hand brush in water and soap to clean the eggs. Soaked for 30 minutes in soap solution, rinsed thoroughly in running water and then soaked them in 70% ethanol for 15 minutes. Cracked the eggs with the edge of the beaker into a sterile flask and beat them with a sterile blender after washing the hands thoroughly.

Preparation of Complete Medium

150ml of Mineral salt solution with malachite green and 250ml of homogenized eggs were mixed thoroughly. The medium was distributed in 6 to 8ml volumes in sterile universal containers and the caps tightly closed.

Coagulation of Medium

Placed the bottles in a slanted position in the inspisator (85^{o}C) and coagulated the medium for 1 hour. Re-inspisated for 30 minutes, after overnight storage at room temperature. Sterility checked at 35^{o}C to 37^{o}C for 24 hours incubation.

Inoculation

A 5mm loopful of the centrifuged sediments was inoculated over the surface of Lowenstein Jensen Medium and few drops of the sediments are inoculated into the Kirchner Medium.

Incubation of Cultures

All cultures are incubated aerobically at 35-37^{o}C

Culture Examination and Identification

All culture were examined 48 to 72 hours after inoculation to detect gross contaminations. The cultures are examined weekly upto 6 weeks in liquid Kirchner media where positive growth shows granule formation. From this it is subculture was made on to solid medium.

In case of solid media, such as Lowenstein Jensen medium, cultures were examined weekly upto 8 weeks. Positive growth produces rough, tough & buff colonies[(2)].

Culture Reporting

Culture reports were recorded qualitatively (Positive or negative) as well as quantitatively (number of colonies isolated). The following scheme is followed.

Reading	**Report**
No Growth	Negative
1-19 colonies	Positive (number of colonies)
20-100 colonies	Positive (1+)
More than 100 discrete colonies	Positive (2+)
Confluent growth	Positive (3+)
Contaminated	Contaminated

DRUG SUSCEPTIBILITY TESTS

The absolute concentration method

A standardized inoculum grown on drug free media and media containing graded concentration of the drugs were tested. Several concentrations of each drug was tested and resistance was expressed in terms of the lowest concentration of the drug that inhibits growth, i.e. minimal inhibitory concentration (MIC).

Drug Free LJ Medium

Prepared as mentioned before.

Drug incorporated medium

Preparation of Drug – containing Media :

Drug containing Lowenstein Jensen slopes are prepared by adding appropriate amounts of drugs aseptically to Lowenstein Jensen fluid before inspisation. A stock solution of the drugs is prepared to contain 10,000 mg/L in sterile distilled water for streptomycin, isoniazid and ethambutol; refampicin is dissolved in dimethyl formamide. The solutions of isomacid and ethambutol are sterilized by membrane filtration. Suitable working dilution are made in sterile distilled water and added to the Lowenstein Jensen fluid, dispensed in 6ml amounts and inspissated once at 85^{o}C for 50 minutes (See appendix)

ρ-Nitro Benzoic Acid (PNB)

Stock solution

Weighted out 1 gm of PNB and added 40ml of dimethyl formamide. Mixed to dissolve, added the entire quantity to 2 liters of LJ fluid giving a final concentration of 500 µg/ml. Distributed and insipissated.

Inoculation

A bacterial suspension was prepared by adding approximately 4 mg moist weight of a representative sample of the bacterial mass visualized as 2/3 loopful of 3mm internal diameter 24SWG wireloop into 0.2 ml of sterile distilled water in a 7ml BIJOU bottle containing twelve 2 to 3 mm glass beads. Shook the bottle for 1 minute to produce a uniform suspension, then 0.8ml of sterile water was added and mixed the bottle by hand. This suspension contains approximately 4mg/ml of the organism.

Using a 3mm external diameter 27SWG nichrome wireloop, inoculated one loopful of this suspension on each slope of the sensitivity test media. Setup 3 control drug free slopes (One for control and two for catalase, niacin tests). Setup one drug containing slope of each concentration of the drug and one slope containing PNB for each strain tested. Tested the standard sensitive strain H37Rv with each batch of test. Incubated all slopes at 37^{o}C.

Reading

Examined the inoculated slopes for growth after 28 days of incubation. Growth on a slope is defined as the presence of 20 or more colonies.

Interpretation of tests

Recorded the lowest concentration of the drug inhibiting growth (MIC). The ratio of the MIC of the test strain to the MIC of the strain H37Rv setup with each batch of tests is referred to as the resistant ratio (RR)[44].

Definition of resistance

Drug	Level of Resistance
Streptomycin	Resistance ratio of drug conc. 8µg/ml or more were resistant
Isoniazid	MIC of drug conc. 5 µg/ml or more were resistant
Rifampicin	MIC of drug conc 128 µg/ml or more can be tentatively interpreted as resistant.
Ethambutol	MIC of 8 drug conc 8 µg/ml or more can be tentatively interpretd as resistant.

Identification tests

1. Susceptibility to p-nitro benzoic acid (PNB)
2. Niacin test
3. Catalase activity at 68°C /pH 7

Para-Nitrobenzoic Acid Test

The preparation of PNB has been explained already in the section on drug susceptibility tests.

Test

Inoculated with a neat bacterial suspension 2 slopes of LJ medium without drugs and 1 slope of LJ medium containing PNB at a concentration of 500mg/liter and incubated at 37°C. Read after 28 days.

Interpretation

M. Tuberculosis does not grow on PNB medium.

Niacin Test

The positive culture tube was taken and autoclaved at 120ºC for 30 minutes for the extraction of Niacin. 0.25 ml of extract was taken and added to solution of 0.25ml of O-tolidine and 0.25ml of 10% cyanogen bromide and mixed well in a screw capped test tube. Observed the solution for the formation of pink colour (positive) within 5 minutes.

Catalase Test

Procedure

Added 0.5ml of 0.067M buffer to screw capped test tubes and emulsified with a loopful of test culture. Placed the tubes in a water bath at 68ºC for 20 minutes. After cooling to room temperature added 0.5ml of freshly prepared equal parts of 10% Tween-80 and 30% H_2O_2. Observed the formation of bubbles on the surface of the liquid. Negative tubes were kept for 20 minutes.

Interpretation

M. Tuberculosis showed catalase negative at 68ºC.

MOLECULAR TECHNIQUE

POLYMERASE CHAIN REACTION

I. Extraction of DNA

Procedure

The FNA material collected in 100µl of tris – EDTA Buffer and stored at 4°C brought to RT before performing the test.

This is centrifuge 10,000 rpm and the supernatant is discarded.

The Pellet is resuspended with 500ml TEB. This procedure is repeated 3 times to wash of blood cells and other debris.

To the pellet now,

- Added 50 µl of (10mg/ml) Lysozyme, mix and incubated 2 hr at 37°C
- Added 70 µl of 10% SDS and 6 µl of (10mg/ml) proteinase K
- Mixed and incubated 10 min at 65°C
- Added 100 µl of 5M NaCl and mix thoroughly
- Added 80 µl of CATB-NaCl solution
- Mixed thoroughly and incubated for 10 min at 65°C
- Added an approximately equal volume of chloroform/isoamyl alcohol mixture
- Mixed thoroughly, and spinned in a micro centrifuge for 5 min
- Removed the aqueous supernatant to a fresh micro centrifuge tube.
- Added 600 µl of isoporpanol
- Placed at -20°C for 60 minutes
- Spinned 15 min in a micro centrifuge at 10000 rpm at 4°C

- Washed the DNA pellet with cold 70% ethanol and respin 5 min at room temperature.
- Carefully removed the supernatant and briefly dried the pellet 15 min at 45°C
- Re-dissolved the pellet in 50 µl of TE buffer.

II. PCR :

Procedure :

PCR Primers :

TB 4 : 5' - CCT GCG AGC GTA GGC GTC GG-3'

TB : 5' CTC GTC CAG CGC CGC TTC GG - 3;

Reaction VolumePreparation

Typically 50 µl reaction volume given below will be good for almost every PCR assay.

Component	Volume	Final conc. (in 50 l)
Milli Q Water	-	
10X Reaction buffer **	5.0 µl	1x
dNTP's Mix (10mM)	1.0 µl	200µl
Primer #1*	1.0µl	20 pM/Reaction
Primer #2*	1.0µl	20 pM/Reaction
Taq DNA polymerase (1U/µl)	1.0µl	1 U / Reaction
Template	10.0µl	~ 100ng

* Prepared stock and working solution accordingly

** Magnesium ion : Mf++ form a soluble complex with dNTPs. The concentration of MgCl2 affects enzyme specificity and reaction yield. In

general lower concentration of Mg++ leads to specific amplification and the higher concentration encourages non specific amplification. The effective concentration of Mg++ is dependent on the dNTPs concentration as well as the template dNA concentration and primer concentration.

ii) Pipetting and DNA Template

To minimize the chance of primer binding to the DNA template and to prevent to polymerase from working prior to the first denaturing step, the vials were kept on ice while pipetting the ingredients of the reaction.

Pipetting was done under a laminar flow of sterile air and using acrosol - resistant pipette tip, so that, false positive results do not occur.

Amplification :

The chromosomal DNA extracted by the enzymatic methods from samples were than subjected to initial denaturation 94^{o}C for 4 min followed 35 cycles of 90^{o}C for 1 min, 60^{o}C for 1 min, 72^{o}C for 1 min, and a final extension at 72^{o}C for 10 min to complete the elongation of the PCR intermediate products.

PCR products are then run on 2% agarose gels containing ethidium bromide and examined for the presence of the 123 bp and compared with 100bp ladder.

III. Detection And Analysis Of PCR Product

Experimental Procedure

Prepared 2% Agarose gel using IX electrophoresis buffer by melting it in a microwave oven (The concentration of agarose used for gel is decided primarily based on the size of the DNA fragments to be analysed).

Added (1µl of stock / 50µl) ethidium bromide to the gel when the gelreaches 50°C. Pourd the mixture into the sealed gel-casting platform.

After the gel has hardened removed the slope from the gel-casting platform and removed the gel comb. Placed the gel into electrophoresis tank containing sufficient elctrophoresis buffer to cover the gel.

Prepared DNA sample with loading dye (18 µl of PCR product 1.2 µl gel loading buffer) and loaded samples and marker DNA into the wells with a pipette.

Attached the lids and acted the power pack at 60V/cmm/30Amp of the gel

Turned off the power supply when the bromophenol blue dye from the loading buffer has migrated a distance judged sufficient for separation of DNA fragments.

Observed for the PCR products under Transilluminator.

Detection of HIV in Study subjects

Procedure (MICROLISA)

1. The ELISA plate with 96 microwells coated with HIV recombinant proteins which has been provided was taken. The assay control wells were arranged so that well A-1 is the reagent blank. From well A-1 all controls were arranged in a horizontal configuration.
2. 100µl of the sample diluent was added to A-1 well as blank.
3. 100µl of Negative Control was added to each well No.B-1, C-1 respectively.
4. 100µl of positive control was added to D-1, E-1 & F-1 wells.
5. 100µl of sample diluent was added to each well starting from G1 will followed by addition of 10µl sample. Then added 100ml of each sample diluted in sample diluent 1:11) in each well starting from G-1well.
6. Cover seal applied and incubated at 37^{o}C for 30 min
7. After 30 min washed the wells 5 times with working wash solution.
8. 100µl of working conjugate solution was added in each well including A-1.
9. Cover seal applied and incubated for 30 min at 37^{o}C.
10. Again the wells were aspirated & wash for 5 times.
11. The 100µl of working substrate solution was added to each well including A-1.
12. Incubated at RT for 30 min in dark

13. Added 50µl of Stop solution.

14. Reading was taken by spectrophotometer with 450nm filter and cutoff value was calculated. Cut off = $\frac{NCx + PCx}{6} = \frac{0.011 + 1.584}{6}$

The HIV positive samples were confirmed by rapid spot kit test (Pareeshak).

RESULTS

A total of one hundred and twenty five clinically suspected patients of tuberculous lymphadenitis were included in the present study. The period of study lasted for two years from December 2003 to January 2006.

55% of them presented only with lymphadenopathy of variable duration. Among them 18% also had cough with expectoration and 22% had evening rise of temperature. 3 cases had pulmonary involvement whose sputa were culture positive.

The following results are obtained and tabulated.

Table - 1
Clinico-epidemiological characteristics of patients with Tuberculous lymphadenopathy (n =125)

Clinical Parameters	Observation
Age	3 years – 50 years
Male : Female ratio	1:0.95
Family H/O TB	13%
Past H/O TB	12%
Lymphonode Sites	
Cervical	65%
Supra Clavicular	13%
Submandebular	11%
Axillary	7%
Inguinal	4%
Clinical Observation	
Swelling only	55%
Swelling + fever	22%
Swelling + fever + cough	18%
Swelling + cough	5%

Table – 2
Age and Sex distribution of Cases (no:125)

Age	Male	Female	Total
< 15 years	12	10	22
15 – 40 years	45	45	90
> 40 years	7	6	13
Total	64	61	125

Of the 125 cases 22 (18%) were below 15 years of age, 90 (72%) were between 15 years and 40 years of age and 13 (10%) were over 40 years of age. Maximum cases were recorded in the age group of between 15 and 40 years. There was no significant difference in sex distribution; the male population had been slightly more than the female.

Table – 3
Results of different tests in samples from clinically suspected cases of tuberculous lymphadenitis (n=125)

Method	No. of samples Tested	Results	
		Positive (%)	Negative
Smear Microscopy	125	21 (168)	104
Culture	125	43 (34.4)	78 *
FNAC	125	59 (47.2)	66
PCR	50	28 (56.0)	22

*4 Specimens = Contamination

Of the 125 samples screened by Ziehl Neelsen staining microscopy , 21 cases (16.8%) were positive for AFB.

43 samples (34.4%) were culture positive for M.tuberculosis. 2 specimens yielded Non Tuberculous Mycobacteria

Out of 125, 59 cases (47.2%) showed granulomatous changes in cytology. 28 (56%) out of 50 samples were positive for IS6110 gene.

Table – 4
The correlation of direct smear and culture

Direct Smear (ZN)	Culture	
	Positive	**Negative**
Positive	14	7
Negative	29	70

Of the 21 smear positive cases 14 samples were culture positive also. 6 samples were smear positive but culture negative. The sensitivity of direct smear as compared to the gold standard technique, the culture was only 32.6% but the specificity was 92.1%.

Table – 5
Comparison of Fluorescent staining and Ziehl Neelsen staining (n= 50)

ZN Staining	Aunamine Staining	
	Positive	**Negative**
Positive	12	0
Negative	3	35

Ziehl Neelsen stained smear was positive in 12 samples compared to fluorescent stained smear positive in 15 samples fluorescent staining detected 3 more cases which were negative by Ziehl Neelsen.

Table – 6
Comparison of Ziehl Neelsen with culture (no: 50)

Direct smear (Ziehl Neelsen)	Culture	
	Positive	**Negative**
Positive	8	3
Negative	14	22*

* 3 samples = contamination

Out of 50 samples, 10 samples were both culture and Ziehl Neelsen staining postive. The sensitivity of Ziehl Neelsen microscopy was 36% and specificity was 88%.

Table – 7
Comparison of fluorescent staining with culture (no: 50)

Direct smear (Fluorescent staining)	Culture	
	Positive	**Negative**
Positive	10	4
Negative	14	22

Out of 50 samples is which co-relation of culture and smear microscopy by Auramine staining was done, 10 samples were culture and fluorescent staining positive. The sensitivity of fluorescent staining was 41.6% and specificity was 84.6%.

Table – 8
Correlation of FNAC with culture isolation of M.tuberculosis (n=125)

FNAC	Culture	
	Positive	Negative
Positive	31	24
Negative	12	54

Out of 43 culture positive samples 31 samples showed cytomorphological features of tuberculous lymphadenitis. 24 samples though were suggestive of tuberculous lymphadenitis by cytology was culture negative. The sensitivity of FNAC is against culture was 72.1% and the specificity was 69.2%.

Table – 9
Comparison of Ziehl Neelsen Stained Direct Smear and FNAC (n = 125)

FNAC	Direct smear	
	Positive	Negative
Positive	17	42
Negative	4	62

Out of 21 direct smear positive samples, 17 samples were positive by cytology.

Table – 10
Comparison of the conventional bacteriological diagnostic methods (direct smear and culture) with cytomorphological diagnosis.
(n = 125)

FNAC	Either culture or direct Smear (ZN)	
	Positive	**Negative**
Positive	37	17
Negative	13	53

Of the 50 samples which were bacteriologically positive (either ZN or culture) cytomorphological evidence of tuberculosis lymphadenitis were consistent with in 37 cases. The sensitivity of FNAC was 73.5% and specificity was 75.7%

Table - 11
Results of the different tests (n = 50)

Method	No of Positives	Positivity
PCR	28	56%
Culture	22	44%
Direct Smear	15	30%
FNAC	20	40%

Table - 12
Comparison of PCR with FNAC
(n = 50)

PCR	FNAC	
	Positive	Negative
Positive	14	14
Negative	6	16

Out of 28 samples which were positive for M.tuberculosis by polymerase chain reaction, 14 samples showed cytomorphological positivity for M.tuberculosis.

Table – 13
Correlation of PCR with direct smear
(n=50)

PCR	Smear (Ziehl Neelsen + Fluorescent Staining)	
	Positive	Negative
Positive	9	19
Negative	3	19

Out of 12 direct smear positive samples 9 were positive by PCR. also PCR was able to detect M.tb genome in 19 samples which were negative on smear examination

Table – 14
Correlation of culture with PCR
(n= 50)

PCR	Culture	
	Positive	**Negative**
Positive	19	7
Negative	3	18

Out of 21 culture positive samples PCR was positive in 19 samples, sensitivity was 86.4% and specificity was 72.0%.

Table – 15
Correlation of the bacteriological diagnosis (either culture or direct smear) with molecular diagnostic technique.

PCR	Either culture or smear	
	Positive	**Negative**
Positive	21	6
Negative	5	16

Out of 28 samples that were positive by PCR, 21 samples were also either smear or culture positive for M.tuberculosis. One sample showed contaminated growth. The sensitivity of PCR when compared with bacteriological methods was 80% and specificity was 72.7%.

Table – 16
Comparison of PCR with conventional technique
(FNAC smear, or culture)

PCR	Direct smear, FNAC or culture	
	Positive	Negative
Positive	23	5
Negative	8	14

Out of 31 samples positive by any one conventional method (i.e. direct smear,FNAC or culture) PCR was found to be positive in 23 specimens. The sensitivity of PCR as against any conventional method was 74.2% and specificity was 73.7%.

Table-17
Culture Vs other diagnostic methods (n = 50)

Either PCR, Smear, or FNAC	Culture	
	Positive	Negative
Positive	21	11
Negative	1	14

Out of 22 culture positive samples, other diagnostic modalities showed 21 positives, the sensitivity of the tests was 95.5% and specificity 56.5%.

Table – 18
Drug susceptibility patterns in the culture positive cases.
(n=43)

No. of Culture Positives	Drug conc. for std. Sensitivity tests				PNB	Response to tests
	Streptomycin	INH	Rifam-picin.	Eth.		
43	< 2µg	< 0.2µg	< 32	< 2µg	Sensitive	Sensitive to all drugs

All the culture isolates were sensitive to all first line drugs indicated by no growth on the medium containing the required and minimum concentration of drugs.

Table – 19
Prevalence of HIV infection in the clinically suspected Tuberculous Lymphadenitis Patients (n=100)

No. of clinical Tuberculous lymphadenitis cases	No. of positives	% of positivity
100	25	25%

Table – 20

Results of Diagnostic tests for Mycobacterium tuberculosis in HIV patients (n = 20)

Test	No of positives	Positivity
Smear	8	40%
FNAC	12	60%
Culture	14	70%
PCR	17	85%

Out of 20 patients diagnosed by any of the laboratory tests, 8(40%) showed smear positivity for AFB, 12 (60%) were positive cytologically, 15 (70%) were culture positive and PCR detected Mycobacterium tuberculosis specific gene in 17 samples (85%). These figures depict the efficacy of the various diagnostic tests of tuberculous lymphadenitis in HIV patients.

DISCUSSION

Despite the discovery of the tubercle bacillus more than a hundred years ago and all the advances in our knowledge of the disease made then, tuberculosis still remains one of the major health problems facing mankind particularly in developing countries. Early diagnosis of tuberculosis and initiation of optimal treatment would not only enable a cure of an individual patient but will also curb the transmission of infection and disease to others in the community. To add to the existing burden, the situation is compounded by the large-scale increase of new TB cases associated with increasing HIV infection. The diagnosis of TB with HIV positive patients is more difficult than in those without HIV infection[65].

> Tuberculous lymphadenitis being the most common extra pulmonary tuberculous infection is often diagnosed on clinical evidence only. Over the past decade fine needle aspiration cytology has assumed an important role in the evaluation as a possible alternative to excision biopsy. This is a simple outpatient diagnostic procedure well accepted by the patients and has practically no complications. This has been found to be as efficient as biopsy, particularly in cases of tuberculous lymphadenitis[25].

There are various other alternate diagnostic modalities to biopsy and cytomorphological study. These include smear microscopy, which consist of various staining methods, and culture isolation of Mycobacterium tuberculosis. Recently, more rapid and sensitive molecular technique, like polymerase chain reaction (PCR) is found to be an efficient alternate modality in the diagnosis of tuberculous lymphadenitis.

In the present study, various diagnostic techniques, including PCR, were done from fine needle aspirates to confirm the diagnosis of tuberculous

lymphadenitis and the results are compared, to find out which of the techniques are more sensitive and specific and gives earlier results.

The sex distribution in this study showed that among the clinically suspected tuberculous lymphadenitis cases, there was a slight male preponderance, the ratio with female being 1:0.95. However, there is no striking discrepancy in the sex distribution in the present study. This is similar to Madurai Study conducted by Tuberculosis Research Centre (TRC) where there were equal number of male and female[35].

This study showed that tuberculous lymphadenitis is most frequently found in the age group between 15 and 40 years, which consists of the productive age group. The next prevalent age group suffering from tuberculous lymphadenitis is the children. In the recent past, numerous studies have shown a peak age range of 20-40 years. This shift in age probably reflects the falling incidence of childhood tuberculosis in the developed countries. In India the disease is still common in children and young adults[38,74]. In a large clinical trial on lymphonode tuberculosis conducted by TRC in Madurai, 35% of patients were aged 12 years or less and 87% were aged 30 years or below. More than 50% of patients were still in the age group of 13-30 years. This correlates with our study.

In the present study in 125 samples, the direct smears by Ziehl Neelsen method was positive in 21 cases. On comparing with culture as gold standard, its sensitivity was 32.6% and specificity was 92.1% .The detection rate of AFB

from aspirated materials in extra pulmonary tuberculosis is usually low because of the paucibacillary nature of the disease and direct smear could be positive only if the number of AFB is more than 10^4/ml in the specimen[19].

There is convincing evidence of increased effectiveness of using fluorescent staining technique such as Auramine Staining to demonstrate AFB as compared to the Ziehl Neelsen method[30,31]. In this study, the Ziehl Neelsen staining and fluorescent staining methods were compared for 50 samples and the positivity of each was correlated with the culture positivity which was taken as gold standard. The sensitivity and specificity of Ziehl Neelsen staining was 36% and 88% respectively and that of fluorescent staining technique was 41.6% and 84.6% respectively. This shows that fluorescent stained smear improved the detection of AFB in paucibacillary smears. The time required to screen fluorescent stained smears was similar to Ziehl Neelsen stained smear for, the bacilli had to be viewed under high power for confirmation. In necrotic smears, the fluorescence of the necrotic material interfered with the visibility of bacilli[6].

It is interesting to note that among the three exclusively fluorescent stained positive samples one was culture negative but PCR positive indicating that it may be non-viable bacilli.

In further analysis of the results, it was found that 7 samples were smear positive but culture negative. This perhaps could be attributed to several factors such as paucibacillary situation, varied replicate nature, prior

antituberculous treatment etc. For example, M. tuberculosis exists as district bacterial population in the host, each with different rates of metabolic activity and replications[75].

The other possible explanation for this smear positive but culture negative could be the contamination of the culture, which was 4(3.2%) in this present study, which led to uncertainty of growth.

Isolation of Mycobacterium from any clinical sample by culture still represents the corner stone on which definite diagnosis of tuberculosis and other mycobacteriosis relies. In this study, culture of mycobacteria from the fine needle aspirate samples was done. There are very many studies on culture from excision biopsy specimens, showing a varied range of 18.7% - 63% positivity[55].

A few study have shown that the culture from fine needle aspirate samples have been made possible, this being an easy and widely used outpatient procedure to obtain the sample[25]. In this study, fine needle aspirates from 125 samples were cultured on multiple media like selective Kirchner's Synthetic liquid medium and Lowenstein Jensen's Medium. Out of them, 43 samples (34.4%) showed growth of M.tuberculosis.

In a study of 390 cases in which needle biopsies were obtained to diagnose the suspected TB lymphadenitis, the overall rate of direct smear positivity was 23.6% and culture positivity was 35%. Caseating lesions were more likely to be positive by cultures (40%) than the non-caseating ones (9%)

[19]. In another study by Ruma Patwa et al, 22% were positive by culture from the fine needle aspirates [68].

Cytomorphological diagnosis of tuberculosis depends on demonstration of epithelioid cells and Langerhan's giant cells in smears. However epithelioid granulomas can be seen in non-tuberculous lesions such as Sarcoidosis, Brucellosis, Cat Scratch disease, Leprosy and occasionally malignancies such s Hodgkin's disease and metastatic lesions also. Presence of epithelioid cells is the first feature suggestive of diagnosis of tuberculous lymphadenitis while further data on morphological, microbiological and clinical features can be of additional help.

In the present study, out of 125 fine needle aspirates, cytomorphological diagnosis of tuberculous lymphadenitis was made on 59 samples (47.2%). In a study conducted by Arora and Arora et al., at Department of Pathology and Microbiology, Medical College, Rohtak, fine needle aspiration cytology out of 200 clinically suspected tuberculous lymphyadenitis cases showed positivity of 62%[1]. Similar results were obtained by Nataraj, Kurup et al., at Mumbai [51].

When compared the number of FNAC positives with direct smear positives as in Table 9 is found that out of 59 cytologically positive samples, direct smear demonstrated AFB in only 17 samples. Though these two tests are the initial screening tests the paucibacillary nature of tuberculous lymphadenitis may show poor smear positivity whereas adequate inflammatory response may be responsible for the higher cytomorphologically positive

samples.

Necrotic features whether acellular or accomplished by neutrophilic infiltrates are usually misdiagnosed as suppurative abscesses. Such smears however, show AFB positivity and thus the diagnosis of tuberculosis is still possible even in the absence of epithelioid granuloma[43]. This is evident in the present study where four samples, which were cytologically negative for tuberculous lymphadenitis, showed AFB positivity. This was noted in purulent aspirates, which showed necrosis, polymorpho nuclear leukocytic infiltrates and lymphocytes only [46].

The culture isolation of M. tuberculosis and the FNAC reports are compared in Table 6. 31 samples out of 59 with tuberculous cytomorphology grew M. tuberculosis. Failure to obtain growth of tubercle bacilli is certainly not a conclusive evidence of their absence in the lesions (Middle Brook 1965). The natural healing process, previous antituberculous treatment and un-representative specimens of lymph nodes used for culture can all account for negative cultures (Braunstien and Adriaro: 1961, Kubica and Diji: 1967). Otherwise the culture negative cytology positive samples may be due to smears, which are richly cellular with occasional clusters of epithelioid cells but no necrosis. In such cases other granulomatous conditions have to be taken into consideration [63].

In this study, it is also noted that from 12 samples, M.tuberculosis is isolated by culture despite the FNAC results being negative. This correlates

with a study by Nataraj et al[51], where 28 FNAC negative samples were culture positive. This is possibly due to more of necrotic features, which led to misdiagnosis or inadequate smear preparation. In this study it is found that FNAC when combined with microscopy and culture improved the diagnostic accuracy. 59 cases out of 125 were diagnosed tuberculosis by FNAC alone as against 67 when combined with microscopy and culture.

As the next phase of this study, it was planned to include the highly sensitive and recent molecular technique such as polymerase chain reaction targeting the insertion sequence 1S6110 fragments coding the DNA of 123 bp. along with the other conventional modalities for the laboratory diagnosis of tuberculous lymphadenitis. Studies on PCR in FNA aspirates of tuberculous lymphdenitis are limited This study was conducted in 50 clinically suspected cases of tuberculous lymphdenitis from whom adequate sample could be aspirated.

In this study PCR was positive in 19 out of culture positive samples(90.4%)in the culture positive cases and7 out of 25 culture negative cases(40%) with overall positivity of 65.2%. In a study of cervical lymph node biopsies, PCR was positive in 5 out of 7 specimen while histology and culture were positive in 4 cases and 1 case respectively (Fusegawa,Mutachi et al.,).

This study showed the ability of the PCR test to detect even very low number of bacteria in the minimal sample. The sensitivity of PCR when compared with culture was 86.4% and specificity was 72.0%. PCR detected

M.tuberculosis in one day compared to an average of 24days required to detect by culture. This is supported by earlier studies by Ruma Pahma, Suresh Hedau et al[68].

The false negativity of PCR in 3 samples that were culture positive for M. tuberculosis (table 13) could have resulted from one of the following factors. 1) The presence of PCR inhibitors and not detected by the controlled amplication, 2) due to non-homogenous distribution of bacteria in the specimens so that the fraction tested does not contain mycobacteria. This is the reason for which some studies recommend that pre –enrichment in culture medium increases PCR sensitivity [63]

In this study, PCR was positive in 9 out 12 smear positive samples the detection rate being 75%.In the smear negative samples PCR was positive in 19 out of 38 (50%). The overall detection rate being 62.5%, which was very high, compared to the sensitivity of direct smear in this study, which was only 32.6%. As is evident, from the present study, the PCR test was found to be more sensitive than smear examination.

In this study, the cytomorphological reports were compared with PCR results and it was observed that among the cytology positive fine needle aspirate samples (20/50) PCR detected the M.tb gene in 14 samples (70% positivity) and also among the cytology negative samples (30/50) PCR detected 14 samples as positive (46.6% positivity). The overall positivity is 58.3%. The positive rate varies from 61 to 94% but it is found to be a most sensitive

technique (Kim et. al., 1996, Ersoz et. al, 1998, Goel et. al 2001, Kidane et. al, 2002). The high frequency of FNAC negative PCR positive samples (14/50) in this study may be due to inadequate sample aspirated, due to other factors such unrepresentative samples, or more necrotic samples which gave negative cytological report or due to the high sensitivity nature of PCR. The limitation of the FNAC is that it is an invasive technique yielding minimal, inadequate samples. Hence, a high sensitivity technique like PCR need to be incorporated along with conventional technique. This correlated with the study by Singh K.K., Muralidhar M. et al.,which showed the sensitivity of 55%[71].

On comparing the bacteriological diagnosis, that is, either culture or direct smear with PCR, as in table: 15, it was found that out of 50 samples, 21 were bacteriologically positive, either by direct smear or culture whereas, 28 samples were positive by PCR. The sensitivity of PCR was 80% and specificity was 72.7%. On comparing with individual or combination of conventional test the specificity of PCR was found to be unaltered. It was 72% to 73.2%.

The logistic regression analysis reveals that FNAC and direct smear are significantly associated with culture diagnosis. Among FNAC, direct smear and PCR, PCR ($p \leq 0.001$) is most significantly associated with culture positivity and other tests FNAC ($P \leq 0.155$), direct smear ($p \leq 0.421$) are insignificant. When FNAC ($p \leq 0.001$) and direct smear ($p \leq 0.421$) were compared, FNAC is found to be more significantly associated with culture than direct smear.

As a part of analysis in this study, PCR results were compared with the results of any other conventional method such as FNAC, Direct Smear or Culture (table: 16) and it was observed that, out of 31 samples positive by any method, PCR was positive in 23 samples. The sensitivity of PCR against any other method was 74.2% and specificity was 73.7%. This finding suggest that PCR can be used as an effective screening and confirmative test in the diagnosis of tuberculous lymphadenopathy from the fine needle aspirate samples, which can be obtained with ease from the clinically suspected patients.

This shows that, PCR is the most sensitive single technique available to date for the demonstration of M. tuberculosis in specimen derived from patients with a clinical suspicion of tuberculous lymphadenitis. Also, the value of PCR lies in its use as a supplementary test in the diagnosis of tuberculous lymphadenitis, particularly in those patients where conventional methods fail.

PCR has more significant statistical association with culture ($p \leq 0.001$), in comparison with other conventional test like FNAC and direct smear and also it is found that PCR is a high sensitive tool. For the above reasons PCR has to be considered as a ideal test alone or along with other conventional techniques.

Although conventional diagnostic techniques remain the method of choice in regions with low resource settings, PCR may be employed in cases

with strong clinical suspicion and with equivocal results, especially at an early stage of the disease for better diagnosis and management.

The patients included in the present study were screened for the presence of HIV / AIDS infection, as they belonged to the high risk group. Serological test was done in 100 samples obtained from the clinically suspected tuberculous lymphadenitis patients who were included in this study. 25 were positive for HIV antibodies-1&2 by ELISA (25%) In them tuberculous lymphadenitis was diagnosed by any one modality in 20 patients.

The sex distribution of the 20 seropositive tuberculous patients showed that 14 (70%) were male and 6 (30%) were female. This is similar to a study by S. Rajasakeran, A. Uma, et. al., in rural south India [62]. The study of HIV seroprevalence among tuberculous patients in India so far have reported rates varying from less than 1% to around 30%[73]. From Pondichery there is report of increase in HIV seroprevalence among TB patients from 2.65% in 1991 to 10.4% in 1993. It is estimated that 60% of HIV infected patients break down with active tuberculosis in their life time [77]. The study also indicated that most of the increase in HIV in tuberculous cases is being contributed by male patients in the age group of 21 to 45 years which is supposed to be the most economically productive age group in any society. This information does have an economic and public health implication for the country.

SUMMARY

Different methods of laboratory diagnosis of tuberculous lymphadenitis like, bacteriological, cytological and molecular biological tests were performed using fine needle aspirate samples from Government Stanley Hospital and Govt. Hospital of Thoracic Medicine, Tambaram Sanatorium, Chennai.

Out of 125 samples tested, Mycobacterium tuberculosis was isolated in forty three (34.4%) patients, cytopathological features of tuberculous lymphadenitis was reported in 59 (47.2%) samples and AFB was demonstrated in direct smear in 21 (16.8%). Out of 50 samples subjected to PCR targeting IS6110 fragment of DNA of 123 bp of Mycobacterium tuberculosis, 28(56.0%) samples were positive.

The lab diagnosis of tuberculous lymphadenitis by PCR is completed in one day compared to minimum of 24 days required for detection by conventional culture method.

All the culture isolates of M. tuberculosis were susceptible to first line anti-tuberculous drugs.

This study proved that by using multiple culture media it is possible to get a good yield of M. tuberculosis from the paucibacillary extrapulmonary tuberculosis.

The logistic regression analysis revealed that FNAC ($p \leq 0.001$) and direct smear $P \leq 0.034$ are significantly associated with culture diagnosis. FNAC is more significantly associated with culture than direct smear. Among

FNAC, direct smear and PCR, PCR is most significantly associated with culture ($p \leq 0.001$).

20% of the tuberculous lymphadenitis patients were co-infected with HIV 70% of them were male ranging between 25-50 years of age, the most economically productive age group.

CONCLUSION

- Diagnosis of tuberculous lymphadenitis based on clinical finding alone gives false positive results.
- Single diagnostic parameter alone is not sufficient for correct diagnosis.
- Fluorescent staining found to be more sensitive than Ziehl Neelsen staining for the demonstration of AFB in direct smear.
- Cytological positivity ($p \leq 0.001$) and demonstration of AFB in direct smear ($P \leq 0.034$) of the fine needle aspirate has significant statistical association with culture. FNAC is more significantly associated.
- Among FNAC, Direct smear and PCR, PCR ($P \leq 0.001$) is most significantly associated with culture.
- PCR is the most sensitive technique.
- 20% of tuberculous lymphadenitis patients were co-infected with HIV.

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