"A COMPARATIVE STUDY ON ZIEHL NEELSEN STAINING AND IMMUNO HISTOCHEMICAL ANALYSIS IN SUSPECTED TUBERCULOUS LESIONS"

Dissertation submitted in partial fulfilment of the requirements for the degree of

M.D., (PATHOLOGY)

BRANCH-III

INSTITUTE OF PATHOLOGY
MADRAS MEDICAL COLLEGE
CHENNAI- 600003

THE TAMIL NADU DR. M.G.R MEDICAL UNIVERSITY
CHENNAI

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CERTIFICATE

This is to certify that this dissertation entitled **"A COMPARATIVE STUDY ON ZIEHL NEELSEN STAINING AND IMMUNO HISTOCHEMICAL ANALYSIS IN SUSPECTED TUBERCULOUS LESIONS"** is the original work of **Dr. G. VEERA RAGHAVAN,** in partial fulfilment of the requirement for M.D., (Branch III) in Pathology examination of the Tamilnadu Dr.M.G.R. Medical University to be held in May 2018.

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DECLARATION

I, **Dr. G. VEERA RAGHAVAN,** solemnly declare that the dissertation titled **"A COMPARATIVE STUDY ON ZIEHL NEELSEN STAINING AND IMMUNOHISTOCHEMICAL ANALYSIS IN SUSPECTED TUBERCULOUS LESIONS"** is the bonafide work done by me at the Institute of pathology, Madras Medical College under the expert guidance and supervision of **Prof. Dr. BHARATHI VIDHYA JAYANTHI** M.D., Director & Professor of Pathology, Institute of pathology, Madras Medical College. The dissertation is submitted to the Tamilnadu Dr. M.G.R Medical University towards partial fulfilment of requirement for the award of M.D., Degree (Branch III) in Pathology.

Place: Chennai

Date: **Dr. G. VEERA RAGHAVAN**

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CERTIFICATE OF APPROVAL

To
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Dear ,

The Institutional Ethics Committee has considered your request and approved your study titled **"A COMPARATIVE STUDY ON ZIEH L-NEELSEN STAINING & IMMUNOHISTOCHEMICAL ANALYSIS IN SUSPECTED TUBERCULOUS LESIONS " NO.25092016 .**

The following members of Ethics Committee were present in the meeting hold on **06.09.2016** conducted at Madras Medical College, Chennai 3

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We approve the proposal to be conducted in its presented form.

The Institutional Ethics Committee expects to be informed about the progress of the study and SAE occurring in the course of the study, any changes in the protocol and patients information/informed consent and asks to be provided a copy of the final report.

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ABBREVIATIONS

AFB	:	Acid Fast Bacilli
AIDS	:	Acquired Immunodeficiency Syndrome
EDTA	:	Ethylene Diamine Tetra Acetic acid
EPTB	:	Extra Pulmonary Tuberculosis
H&E	:	Haematoxylin& Eosin
HIV	:	Human Immunodeficiency Virus
IHC	:	ImmunoHistoChemistry
MTB	:	Mycobacterium tuberculosis
NAAT	:	Nucleic Acid Amplification Test
PCR	:	Polymerase Chain Reaction
PTB	:	Pulmonary Tuberculosis
RNTCP	:	Revised National Tuberculosis Control Programme
WHO	:	World Health Organisation

CONTENTS

INTRODUCTION

INTRODUCTION

Tuberculosis remains a major global health problem, despite the identification of the causative agent 135 years ago and the advent of effective anti-tuberculosis therapy[1]. This is attributable to many factors like emergence of virulent strains, multi drug resistance, and rise of HIV pandemic among others[2]. In India, tuberculosis remains one of the leading causes of mortality and morbidity[3].

Mycobacterium tuberculosis, the causative agent of tuberculosis is an intracellular microbe, capable of evading the host immune system and persisting within the macrophages even in the face of host immune response[4]. Granuloma formation with or without caseation necrosis, the hallmark of tuberculosis is a delayed type hypersensitivity or Type IV hypersensitivity reaction[5]. Granulomas, however are not limited to tuberculous infections, but can also occur in variety of settings like Leprosy, Atypical Mycobacterial Infections, Schistosomiasis, Histoplasmosis, Cryptococcosis and Cat Scratch Disease, and in non-infectious conditions like Crohn's Disease, Sarcoidosis, Primary Biliary Cirrhosis, Rheumatoid Arthritis, Foreign Body Granulomas and even in Neoplasms[6,7,8,9]. Thus a definitive diagnosis needs to be made in the setting of granulomas, because in developing countries where there is dearth of availability of resources, treatment of tuberculosis has largely been modelled on presumptive diagnosis based on the imaging findings and clinical symptoms, which carries a high false positive rate[10].

Although few classification systems exist for tuberculosis, it has been conventionally classified as Pulmonary Tuberculosis (PTB) and Extra Pulmonary Tuberculosis (EPTB), based on which treatment strategies also differ[11].

Diagnosis of pulmonary tuberculosis had always relied on sputum microscopy, acid fast staining of the bacilli and culture until the advent of molecular diagnostic tests like polymerase chain reaction (PCR), Xpert MTB/RIF (Nested PCR), although the definitive diagnosis of TB requires culture of Mycobacterium from respiratory secretions with sensitivity testing[12]. Culture, however takes several weeks, before a result is obtained[13]. World Health Organisation recommends the use of Xpert MTB/RIF as the initial diagnostic test to diagnose PTB, based on systematic reviews[14]. The efficacy of Xpert MTB/RIF in diagnosing PTB in sputum positive, culture positive cases, as well as in sputum negative, culture positive cases has been well documented[15,16].

Unlike PTB, where sputum samples and even Broncho alveolar lavage are easily obtained and sent for analysis, sample collection in EPTB is always invasive[17,18]. WHO policy update in 2013 recommended that Xpert MTB/RIF be used as initial diagnostic test in EPTB as well based on the review of published studies[19,20,21]. Although Xpert MTB/RIF has proved its efficacy and reliability as an initial rapid diagnostic test in EPTB, it is expensive and sensitive to contamination[22]. Such tests are still out of reach for patients owing to the cost factor in developing countries[23].

ROLE OF HISTOPATHOLOGY:

Histopathology of formalin fixed paraffin embedded tissue sections can provide a presumptive diagnosis of tuberculosis based on the morphology of granulomas and the presence of caseation necrosis & Langhans giant cells, although non-necrotizing granulomas also occur[24,25]. However as mentioned earlier, it cannot reliably distinguish from other causes of granulomas[6,7,8,9]. This can result in under-treatment and sometimes in erroneous over-treatment[26].

ROLE OF SPECIAL STAINS:

Mycobacterium tuberculosis is acid fast, meaning that they resist decolourization by acids, which can be utilized for their identification by special stains[27]. Some of the stains which can be used include but not limited to Ziehl-Neelsen stain, Kinyoun stain and fluorochrome based stains like auramine-rhodamine stain, of which Ziehl-Neelsen stain is the most widely used[28]. The tubercle bacilli appear as red curved rods which can be easily identified against the blue background[29]. Ziehl-Neelsen stain, although is rapid and inexpensive has very low and variable sensitivity which depends on the bacterial load[30].

ROLE OF IMMUNOHISTOCHEMISTRY (IHC):

IHC can localize the bacilli or their components in the tissue sections, for which both monoclonal and polyclonal antibodies are available[31,32]. IHC offers a significant advantage over Ziehl-Neelsen in that, it can detect tubercle bacilli even when there is low bacterial load and even in those who were

partially treated[33]. In areas where Xpert MTB/RIF is not available or not affordable by the patients, IHC could be an effective alternative which could reduce the time to treatment[33,34].

AIMS AND OBJECTIVES

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1. To evaluate the diagnostic value of immunohistochemical analysis using rabbit polyclonal mycobacterium tuberculosis antibody in suspected tuberculous lesions
2. To compare the immunohistochemical analysis with Ziehl-Neelsen (ZN) staining in tissue sections of suspected tuberculous lesions

REVIEW OF LITERATURE

REVIEW OF LITERATURE

INTRODUCTION:

Tuberculosis is a highly infectious disease caused by the bacterium Mycobacterium tuberculosis[35]. It is primarily an infection of the lungs (pulmonary tuberculosis), though it can affect virtually any organ system including but not limited to lymph nodes, gastrointestinal tract, genitourinary tract, bone & joints, meninges and skin (extra pulmonary tuberculosis)[36,37].

HISTORICAL PERSPECTIVE:

The name tuberculosis derives its origin from the Latin word tuberculum meaning "small swelling or pimple", so named in reference to the tubercles found in the lungs[38]. Tuberculosis has been referred to by a multitude of names over time, which include white plague, consumption, Pott's disease & phthisis[39,40,41]. Tuberculosis has plagued mankind for much of known history, though the exact origin is debatable[42].

Gutierrex et al via DNA sequencing and phylogenetic analyses, estimated that the progenitor species of mycobacterium tuberculosis may be 3 million years old and may have infected our hominid ancestors[43]. The earliest documentation of tuberculosis dates back to circa 3500 B.C. inferred from the Egyptian tomb paintings and examination of mummies which showed tubercular lesions[44]. In ancient India, dating back to circa 1500 B.C., the Rig Veda describes a disease, 'Yaksma' which translates to 'wasting disease', closely resembling that of tuberculosis[45].

For millennia, the aetiology of tuberculosis remained unknown. But in 1882, Robert Heinrich Hermann Koch identified the causative agent of tuberculosis as tubercle bacillus, later came to be known as Mycobacterium tuberculosis[46]. Koch also demonstrated the tuberculin reaction, which later formed the basis for Mantoux test[47]. The identification of the offending agent was followed by the development of Bacillus Calmette-Guerin vaccine and the discovery of streptomycin and other anti-tubercular drugs[48]. Despite the advancement, tuberculosis remains unconquered and is one of the leading infectious causes of mortality & morbidity[49].

EPIDEMIOLOGY:

Tuberculosis has become an epidemic of vast proportion, so much so that World Health Organization (WHO) declared it as a global emergency in 1993[50]. According to WHO, it has been estimated that 2-3 billion of the world's population have been infected with M.tuberculosis and about 5%-15% of them will develop tuberculosis in their lifetime[51].

India has the highest tuberculosis burden in the world accounting for 27% of global incident tuberculosis cases[51]. The estimated incidence of tuberculosis in India for the year 2015 was 28, 40,000. Among the notified cases, 82% were pulmonary tuberculosis and 18% were extra pulmonary tuberculosis. The mortality rate was 36 per 100,000 population[52].

RISK FACTORS:

An untreated sputum positive patient can infect approximately 10 persons per year[53]. Hence close contacts of infectious TB cases are at a higher risk for acquiring infection and developing active disease. The risk of developing active disease after exposure is governed by a multitude of factors[54].

Factors like HIV co-infection, malnutrition, younger age, diabetes, immunosuppressive drugs, tobacco smoke, silicosis, alcohol, air pollution, and low socioeconomic status play a significant role in the progression to active disease[54,55,56,57]. Specific groups such as health care workers are also at an increased risk of TB infection and disease[58].

ETIOLOGICAL AGENT:

Tuberculosis is caused by the bacillus Mycobacterium tuberculosis. Mycobacterium are aerobic, non-sporing, non-motile rods[59]. Mycobacteria possess two types of antigens viz. cell wall antigens & cytoplasmic antigens[60].

The thick lipid rich cell envelope of mycobacteria is composed of three major constituents, the plasma membrane, the cell-wall core, and the extractable non-covalently linked glycans, lipids and proteins[60]. The major lipids in the mycobacterium tuberculosis cell wall are mycolic acids, cord factor, and wax-D[61].

External to the plasma membrane is the peptidoglycan layer which is covalently linked to the arabinogalactan layer, which in turn is attached to the mycolic acids with their long meromycolate and shorter alkyl-chains[62]. This portion is termed the cell-wall core, the mycolyl arabinogalactan-peptidoglycan complex (MAPc)[60,62].

The mycolic acids unique to mycobacteria are long-chain fatty acids that are covalently bound to the arabinogalactan peptidoglycan co-polymer; they are implicated in the formation of the inner layer of an asymmetric outer membrane while other lipids constitute the outer leaflet[63]. The mycolic acids extend perpendicular to the arabinogalactan / peptidoglycan while other cell wall-associated glycolipids intercalate into the mycolic acid layer to form a 'pseudo' lipid bilayer[60,62,63].

When the cell wall is subjected to treatment with various solvents, the free lipids and proteins are solubilised and the MAPc remains as an insoluble residue. Thus it was hypothesised that these lipids, proteins, and lipoglycans which were soluble act as the signalling effector molecules in the disease process, whereas the insoluble MAPc core is necessary for the viability of the cell[60].

Cytoplasmic antigens are proteins which include antigen 5, antigen 6, antigen 14, antigen 19, antigen 32, and antigen 38. Antigen 60 is also a cytoplasmic antigen which is a protein lipopolysaccharide complex[59].

Mycolic acids which are strongly hydrophobic form a lipid shell and are considered to be a significant determinant of virulence in Mycobacterium tuberculosis. They are thought to resist the attack of the mycobacteria by cationic proteins, lysozyme, and oxygen radicals in the macrophages[64]. They also protect extracellular mycobacteria from complement deposition in serum[65].

PATHOPHYSIOLOGY:

Tuberculosis is a highly communicable disease and the most important mode of transmission is by aerosol[66]. When patients with active tuberculosis cough, droplet nuclei of size 1–5 μm in diameter containing the bacteria are released into the air[67]. The relatively very small droplet nuclei can continue to persist in the air for several hours[68].

The risk of infection is dependent on a multitude of factors like the infectiousness of the source case, the closeness of contact, the bacillary load inhaled, and the immune status of the potential host[69]. The primary path of infection affects the lungs and the droplet nuclei because of their relatively small size can escape the defence mechanisms of the bronchial tree and reach the terminal part of the alveoli where they get engulfed by the macrophages and dendritic cells[70].

M. tuberculosis also infects cells other than phagocytic cells in the alveolar space such as the specialized epithelial M cells overlying the lymphoid tissue, alveolar endothelial cells, and type 1 & type 2 pneumocytes[71].

In the early stage of infection, M. tuberculosis after internalization by the phagocytes, replicates intracellularly and the bacteria laden immune cells can cross the alveolar barrier to cause systemic dissemination[72]. The intracellular replication of the pathogen and its subsequent dissemination to the pulmonary lymph nodes and other extra pulmonary sites occurs before the development of immune mediated adaptive responses[73]. This illustrates that M. tuberculosis carves for itself a protected niche, where it can evade the host immune system, thereby continuing to persist for an indefinite length of time[74].

In majority of the infected persons, the immune system mounts an effective T cell-mediated immune response 2–3 weeks after infection which then activate the macrophages to kill the intracellular mycobacteria[75]. The blood-derived macrophages, epithelioid cells (differentiated macrophages) and multinucleated giant cells (also known as Langhans giant cells) form granulomas surrounded by T lymphocytes, that wall off the bacteria limiting further replication and spread of the tubercle bacilli[76].

Most of the M. tuberculosis are killed in the caseating granulomas, typical of the tuberculosis infection, which are formed by the epithelioid macrophages surrounding a cellular necrotic region with a rim of lymphocytes and the disease progression is arrested[77]. In some individuals, the tubercle bacilli is not completely eradicated, as the bacteria has developed effective mechanisms to escape the host immune response resulting in the survival and perseverance of some bacilli in a non-replicating state in the host[78].

Literature has showed that the tubercle bacilli has been cultured from lung tissue samples of persons who died from other causes and did not exhibit any signs & symptoms of tuberculosis, supporting the hypothesis of latent tuberculosis infection[79]. Surviving bacilli may remain dormant for a long time even lasting up to a lifetime, and a subsequent defect in cell-mediated immunity may result in reactivation of dormant bacilli causing active disease many years after the infection (reactivation TB)[80]. Hence exposure may lead to clearance of M. tuberculosis, persistent latent infection, or progression to primary disease[81].

IMMUNE REPSONSE – "THE GRANULOMATOUS INFLAMMATION":

Granulomatous inflammation (delayed type hypersensitivity or Type IV hypersensitivity), the hallmark of tuberculosis, is rather an unsuccessful attempt to limit the infection, because it also provides a fertile microenvironment for the proliferation and survival of the tubercle bacilli, by modulating the immune response[82]. The balance between the pro-inflammatory cytokines and anti-inflammatory cytokines which are produced to control the microbial proliferation determines the establishment of infection[83].

Cytokines like TNF-α and Interferon gamma promote the formation of granuloma whereas Interleukin-10 acts as a negative regulator[84]. The production of chemokines by the epithelial cells of the respiratory tract and the immune cells themselves, is also vital for the recruitment of inflammatory cells to the site of infection, particularly, the chemokines binding to the CCR2

receptor (CCL2/MCP-1, CCL12, and CCL13) which play an essential role for the early recruitment of macrophages[85].

The tubercle bacilli are able to withstand the bactericidal mechanisms of the macrophage by inhibiting the fusion of phagosome and lysosome, multiply within the phagosome, ultimately resulting in macrophage necrosis[86]. The released bacilli also multiplies extracellularly and are then phagocytosed by another macrophage which also fails to control the growth of the tubercle bacilli and is similarly destroyed.

Meanwhile, dendritic cells with the phagocytosed bacilli migrate to the regional lymph nodes and sensitise the T lymphocytes (both CD4+ and CD8+) against the mycobacterial antigens[87].The resulting specific immune response creates sensitised T cells which then transmigrate to the focus of infection, guided by the chemokines produced by the infected cells. The accumulation of the macrophages including the activated macrophages called the epithelioid cells, T lymphocytes, and other host cells like the dendritic cells, fibroblasts, endothelial cells, and stromal cells lead to the formation of granuloma at the site of infection[88].

PULMONARY TUBERCULOSIS:

Pulmonary tuberculosis accounts for about 85% of tuberculosis[51].The classical initial site of infection in the lungs, known as the "Ghon focus", which is a sub pleural focus, is generally located in either the upper part of the lower lobe, or the lower part of the upper lobe, and together with the regional

draining lymph nodes constitute the “Ghon complex”, named after Anton Ghon, an Austrian pathologist[89]. This apical localisation of infection is attributed to several factors like low blood flow, low ventilation and high oxygen tension[90].

Haematogenous spread from a primary site of infection elsewhere in the body to lungs can result in a lesion which is known as a Simon focus, named after Georg Simon, a German paediatrician and is classically found in the apex of the lung[91]. This haematogenous transmission can also disseminate infection to more distant sites, like the peripheral lymph nodes, the kidneys, the brain, and the bones[92]. In many situations, the infection ebbs & tides with tissue destruction, necrosis which are often followed by healing and fibrosis[93].

Affected tissue is replaced by scarring or may result in the formation of cavities filled with caseous necrotic material. During the active phase of the disease, some of these cavities may be joined to the air passages like bronchi & bronchioles and this material can be coughed up which contains live tubercle bacilli, that can result in the spread of infection[94]. The clinical symptoms include productive cough, purulent sputum, haemoptysis, breathlessness, weight loss, anorexia, fever associated with night sweats, wasting and terminal cachexia[95].

EXTRA PULMONARY TUBERCULOSIS:

Extra pulmonary tuberculosis (EPTB), as the name implies, refers to the tuberculous infection of any organ except lungs (e.g., lymph nodes, pleura,

abdomen, genitourinary tract, skin, joints and bones, or central nervous system) [96].It has been observed that EPTB constituted about 15 to 20 per cent of all cases of TB and its incidence has been on the rise in the past decade due to a multitude of factors[97]. The arrival of Human Immunodeficiency Virus (HIV) pandemic has made matters worse, with EPTB accounting for more than 50 per cent of all cases of tuberculosis in HIV positive individuals[98].

Lymph node tuberculosis:

Tuberculous lymphadenitis or as is known from ancient times as King'evil was believed to be cured by the Royal Touch up until the eighteenth century[99]. The Latin term 'scrofula' is also used to refer lymph node tuberculosis, which translates to 'full-neck sow' meaning the full appearance of fat around a pig's neck[100]. Worldwide and in India, lymph node tuberculosis represents the most common form of EPTB[101]. Peripheral lymph nodes are most commonly affected and among the peripheral nodes, cervical group of lymph nodes frequently involved[102].

Involvement of lymph node by tuberculosis is often the result of entry of pathogen through the tonsils or as a manifestation of systemic involvement[103,104]. The bacilli after gaining access to the respiratory tract, involves the hilar and mediastinal lymph nodes, which may occur as a primary infection or activation of a latent infection and may later disseminate via lymphatic system to involve peripheral nodes[105]. When the tonsil acts as a point of entry, the draining lymph nodes are affected and clinically presents with

enlarged neck nodes, which are discrete initially and then coalesce later to form matted nodes and break open due to the formation of pus[106].

Thus tuberculous lymphadenopathy progresses through certain stages viz. mobile discrete nodes, matted nodes, abscess formation, pus discharge and sinus tract formation[107]. Tuberculous lymphadenitis commonly affects young adults and children[108]. Patients typically present with enlarging neck mass, fever with night sweats, loss of appetite, loss of weight and fatigue[109]. Patients with hilar and mediastinal lymphadenopathy typically present with cough and breathlessness[110,111]. Besides cervical and mediastinal lymph nodes, other less common group of nodes affected include axillary, mesenteric, perihepatic and inguinal lymphnodes[112]. Before the diagnosis of tuberculosis is arrived at, other causes of granulomas in lymph nodes must be excluded[113].

Pleural Tuberculosis:

Pleural tuberculosis is the second most common form of extra pulmonary tuberculosis and in spite of the proximity & intricate anatomic relationship to lung, it is considered as extra pulmonary form of tuberculosis[114]. It is one of the commonest causes of pleural effusion, affecting young adults with a male predominance[115,116]. It is hypothesized that a sub pleural tubercular focus rupturing into the pleural space, a few weeks after the primary infection, is behind the pathogenesis of tuberculous pleural effusion[117].

The mycobacterial antigens elicit a delayed type hypersensitivity response, which leads to accumulation of fluid secondary to increased

permeability of pleural capillaries to serum proteins which caused increased oncotic pressure[118]. Other forms of presentation include empyema, calcification, pleural thickening, and pleural nodules[119]. Empyema is a rare manifestation of tuberculosis characterised by the presence of thick pus with thickening and calcification of the visceral pleura, for which decortication is often required[120].

Increased incidence of pleural involvement in tuberculosis is seen in association with Acquired Immunodeficiency Syndrome (AIDS)[121]. The clinical manifestations include non-productive cough, chest pain, breathlessness, fever with night sweats, loss of weight, loss of appetite, and fatigue[122]. Rarely, pleural tuberculosis can present as a persistent chest wall mass or with chest wall sinus tract formation[123, 124].

Gastrointestinal & peritoneal tuberculosis:

Abdominal tuberculosis is the sixth most common form of extra pulmonary tuberculosis and can manifest as tuberculous lymphadenopathy, gastrointestinal tuberculosis, peritoneal tuberculosis and visceral tuberculosis[125, 126]. In the Gastrointestinal tract (GIT), tuberculosis can occur in any site from mouth to anus, with the most common site being ileocecal junction[127, 128].

The reasons for increased incidence of tuberculosis in the terminal part of ileum include abundance of lymphoid tissue, increased stasis, increased absorptive activity and closer contact of tubercle bacilli with the mucosa[129, 130].

The mechanisms by which the tubercle bacilli reach the GIT and cause infection include haematogenous spread, ingestion of infected sputum, contiguous spread from adjacent involved organs and through lymphatics[131,132].

Oesophageal tuberculosis is a rare entity accounting for about 0.3% of GIT tuberculosis[133]. It usually involves the middle one third of the oesophagus and frequently presents with dysphagia[134, 135]. It usually occurs a part of systemic involvement and isolated involvement of oesophagus is extremely rare[136]. Gastroduodenal tuberculosis is also rare with reported incidence of around 0.5% of abdominal tuberculosis[137]. Symptoms are non-specific like pain, vomiting, dyspepsia and can mimic peptic ulcer or carcinomas[138].

Duodenal tuberculosis can sometimes present with strictures and fistulas[139]. Ileocecal tuberculosis, the commonest form of GIT tuberculosis can present grossly as ulcerative, hypertrophic or a combination of both[140]. The symptoms include colicky or dull aching abdominal pain, altered bowel habits, vomiting, loss of weight and loss of appetite[141].

Complications include strictures or adhesions, malabsorption and perforation[128, 142]. Grossly and microscopically, GIT tuberculosis needs to be distinguished from inflammatory bowel disease[143]. Colorectal tuberculosis can be multifocal in up to 40% of cases[144]. The most common finding is the presence of ulcers and can mimic Crohn's disease[145].

Peritoneal tuberculosis can present with any of the three forms viz. the wet ascitic type with increased amount of free fluid or loculated effusion, the

fixed fibrotic type with omental and mesenteric involvement with adherent bowel, the dry plastic type with peritoneal nodules and adhesions or as a combination of these[146]. The symptoms include abdominal distension, diffusely tender doughy abdomen with other classical symptoms of tuberculosis[147].

Hepatobiliary tuberculosis:

Hepatobiliary tuberculosis is rare and usually occurs in the setting of military tuberculosis[148]. The bacilli reach the hepatobiliary system by haematogenous route either via hepatic artery or portal vein[149, 150]. Tuberculosis of hepatobiliary system can occur in three forms viz. military tuberculosis which is the most common form, tuberculous hepatitis and localized hepatic tuberculosis with or without bile duct involvement[151].

The clinical manifestations include hepatomegaly, fever, weight loss, abdominal pain, and jaundice[152]. Tuberculosis of gall bladder is extremely rare with less than 120 cases being reported in literature till date[153]. Most cases occur as part of disseminated tuberculosis[154].

Pancreatic tuberculosis:

Contrary to popular belief, tuberculosis can occur in pancreas and less than hundred cases have been reported in literature[155]. The rarity of the infection at this particular site has been attributed to the destructive nature of the pancreatic enzymes[156]. It occurs as part of disseminated tuberculosis and it is hypothesised that the infection is spread by haematogenous route[157]. The

clinical manifestations are non-specific and can mimic pancreatic adenocarcinoma[158].

Splenic tuberculosis:

Splenic tuberculosis can occur in two forms viz. either as part of military tuberculosis or primary isolated involvement of spleen[159, 160]. Spleen is the third most common organ to be involved in disseminated tuberculosis after lung and liver[161]. It has no specific symptoms and thus can be mistaken for a mass lesion, splenic abscess or lymphoma[162].

Neurological tuberculosis:

Neurological tuberculosis accounts for around 10% of extra pulmonary tuberculosis and 1% of all tuberculosis cases[163]. The pathogenesis involves the development of tuberculous foci called as 'Rich Foci' in any part of the central nervous system (CNS) viz. brain, spinal cord or meninges[164]. The disease can take the form of tuberculous meningitis which is the most common manifestation, or in the form of tuberculous encephalitis, intra cranial tuberculomas and tuberculous brain abscess[164].

CNS involvement by tuberculosis is more common in HIV infected patients[165]. The release of tubercle bacilli into the subarachnoid space results in the formation of gelatinous exudate, which may extend and envelop the spinal cord, cranial arteries and cranial nerves, sometimes resulting in the obstruction of normal flow of cerebrospinal fluid causing hydrocephalus[166].

The dreaded complication of tuberculous meningitis includes the development of vasculitis of circle of Willis resulting in infarction[166]. Tuberculomas are small masses or tubercles ranging from few mm size to 4 cm, which develop within the brain parenchyma without rupturing[167]. They account for about 10% of space occupying lesions in the brain and may or may not be associated with tuberculous meningitis[167]. Tuberculous brain abscess may be a complication of either tuberculous meningitis or tuberculomas, characterised by encapsulated collection of pus[168].

Tuberculous meningitis presents with fever, headache, seizures, stiff neck, altered consciousness or focal neurological deficits[169]. Tuberculomas or brain abscess elicit symptoms depending on their location which include fever, headache, seizures, signs of increased intracranial tension, and papilledema[169]. Tuberculomas can mimic neoplastic lesion clinically and radiologically and thus needs to be distinguished[169]. CSF analysis may reveal increased lymphocytes and increase in proteins and low sugar[170].

Pericardial tuberculosis:

Involvement of pericardium by tuberculosis can occur in the form of pericarditis, pericardial effusion and cardiac tamponade[171]. Tuberculosis is the leading cause of pericardial effusion, which has a very high mortality rate and occurs commonly in association with HIC infection[37, 172]. The involvement can progress through phases of dry stage, effusive stage, absorptive stage and constrictive stage[37].

Patients present with fever, cough, dyspnoea, chest pain, and weight loss and when presenting with tamponade, they can exhibit severe distress, retrosternal compression, tachycardia, cardiomegaly and pericardial rub which then becomes an emergency, necessitating the need for pericardiocentesis[37]. A definitive diagnosis is possible only by evaluation of pericardial fluid for tubercle bacilli or by biopsy[37].

Bone and joint tuberculosis:

Skeletal tuberculosis occurs due to haematogenous spread and spine is the most commonly affected part which is commonly known as Pott's spine, followed by hip joint, knee joint, bones of hand & feet[173]. Thoracolumbar spine is the commonest site involved, with the disease process eventually causing bony destruction resulting in the collapse of vertebra[173].

When involving the cervical vertebra, the exudate can track along the path of least resistance, and collect behind the prevertebral fascia and can present as retropharyngeal abscess, which extend down into the mediastinum or laterally to present as neck abscess[173]. When involving the thoracic vertebra, can extend into mediastinum and present as abscess and when involving the lumbar spine, can track along psoas sheath and present as psoas abscess, which can extend further down to the scarpa's triangle and gluteal region[173].

Tuberculosis of hip and knee joint can present with pathological fractures. The clinical manifestations depending on the site involved include breathlessness, difficulty in swallowing or hoarseness of voice in cases of

retropharyngeal abscess; fixed flexion deformity of hip in cases of psoas abscess; pain, reduced range of movement, night cries due to muscle spasm in cases of hip & joint tuberculosis[173]. Paraplegia is the most dreaded complication of spinal tuberculosis which most commonly occurs secondary to compressive neuropathy[173].

Genitourinary tuberculosis:

Genitourinary system is one of the most commonly affected sites by haematogenous spread of the bacilli and like all other forms of extra pulmonary tuberculosis, it shows an increased incidence in HIV infection[174]. The histomorphological changes vary ranging from parenchymal changes, pelvicalyceal system changes, and tuberculous interstitial nephritis to chronic renal failure[174].

Clinical manifestations include flank pain, fever, dysuria and sterile pyuria[174]. Involvement of female genital tract by tuberculosis is one of the commonest causes of infertility[175]. The disease process can involve endometrium, fallopian tubes causing extensive adhesions; the symptoms include fever, pelvic pain, and altered menstrual pattern[175]. In male genital tract, tuberculosis can affect epididymis, testicular parenchyma, spermatic cord, and accessory sex glands with the manifestations including painless or painful scrotal mass, sterile pyuria and fever[176].

Cutaneous tuberculosis:

Cutaneous tuberculosis can occur due to exogenous, endogenous or autoinoculation of the tubercle bacilli[177]. The various cutaneous forms of tuberculosis include lupus vulgaris, scrofuloderma erythema induratum of Bazin, erythema nodosum, lichen scrofulosorum, papulonecrotic tuberculids and tuberculosis verrucosa cutis[37, 177]. It is very difficult to diagnose cutaneous tuberculosis clinically, unless biopsy or culture is done[177].

Tuberculosis of Breast:

The incidence of tuberculous mastitis is very high in endemic countries like India[178]. Clinically and radiologically, it mimics carcinoma and typically affects young, lactating women and the risk factors include multiparity, HIV infection and trauma[178]. The infection can be the result of haematogenous, lymphatic or contiguous spread. The manifestations include tender breast mass, ulcer, abscess formation with or without discharging sinus, and fever[178].

Ocular Tuberculosis:

Ocular tuberculosis occurs in the setting of military tuberculosis. It can affect any part of the eye, with choroid being the most common site[179].It can present as uveitis, choroidal tubercles, or as iridocyclitis[179]. The clinical manifestations include conjunctival congestion, blurred vision and eye pain[179].

Otorhinolaryngology related Tuberculosis:

The commonest site affected by tuberculosis in otorhinolaryngological system is larynx, other than cervical lymphadenitis[180]. IT can however involve any site including oral cavity, nasal cavity, nasopharynx, oropharynx, salivary glands and middle ear[180]. Laryngeal tuberculosis typically presents with odynophagia, dysphagia and hoarseness of voice and biopsy or culture is essential to differentiate it from malignancy[180].

Disseminated/miliary tuberculosis:

Miliary tuberculosis, which refers to the disease involvement in two or more non-contiguous sites, is a highly lethal form of tuberculosis, where widespread lymphatic or haematogenous dissemination of the tuberculous foci results in the formation of tiny tubercles on the organs involved, which resemble millet seeds in their appearance and hence the name (miliarius in Latin means 'relating to millet seeds') [181]. It accounts for about 20% of all extra pulmonary tuberculosis cases and is more common in HIV infected patients[181].

The factors which predispose to miliary tuberculosis include HIV infection, diabetes, smoking, immunosuppressant drugs, transplant recipients, chronic renal failure, and pregnancy[181]. The pathogenesis behind the development of miliary tuberculosis is believed to be due to inadequate effector T cell response which fails to contain the bacilli, eventually resulting in widespread dissemination[181]. No single symptom or sign is diagnostic of miliary tuberculosis; a myriad of manifestations such as peripheral

lymphadenopathy, cold abscesses, skin lesions, hepatomegaly, splenomegaly, serous cavity effusions and choroid tubercles could be a clue to the diagnosis[181].

Various diagnostic modalities need to be put in place for proper work up and even with treatment, it carries a high mortality rate[181]. Miliary tuberculosis has now been classified as pulmonary tuberculosis, because if a person has both pulmonary & extra pulmonary tuberculosis, he should be classified as having pulmonary tuberculosis[36].

DIAGNOSTIC MODALITIES:

IMAGING:

Conventional radiography (X-ray) and ultrasound remain thepreferred initial forms of imaging modalities. Though imaging cannot accurately pinpoint the diagnosis, it does give some clues to the nature of the lesions, which provides the basis for next line of investigation[182]. Computerised Tomography (CT) and Magnetic Resonance Imaging (MRI) are also used depending on the part to be imaged[182]. Positron emission tomography/computed tomography with the use of ^{18}F-fluorodeoxyglucose (^{18}F-FDG PET/CT) is another imaging method used principally to distinguish benign from malignant lesions[182]. Increased uptake of ^{18}F-FDG is seen in tuberculous lesions which can be helpful in identifying them[182].

Pulmonary tuberculosis:

Imaging in primary pulmonary tuberculosis reveals consolidation, which can be either unilobar or multilobar along with enlarged hilar or paratracheal lymph nodes with or without calcification[182]. In post-primary tuberculosis, cavity formation is the radiological hallmark, which may progress via endobronchial spread classically referred to as the 'tree-in-bud' sign[182]. CT is more sensitive than conventional radiography in detecting the parenchymal lesions and lymphadenopathy. CT can also differentiate active tuberculosis from healed lesions[182].

Lymph node tuberculosis:

Imaging cannot reliably differentiate between the causes of lymphadenopathy. However, CT & MRI can identify matted lymph nodes, necrosis and calcification, which are suggestive of tuberculosis[183].

Pleural & pericardial tuberculosis:

Ultrasound & conventional radiography can identify pleural effusion & pericardial effusion, while CT & MRI can pick up pleural nodules, thickening, pericardial thickening and calcification[182].

Abdominal tuberculosis:

CT & MRI can detect circumferential wall thickening and mesenteric lymphadenopathy which can mimic carcinomas and inflammatory bowel disease and can also detect omental adhesions and mesenteric thickening[183].

Barium studies are also helpful which can demonstrate ‘pulled up caecum’, commonly seen in tuberculosis[183]. CT & MRI can detect miliary or macronodular involvement in hepatosplenic tuberculosis[183].

Neurological tuberculosis:

Tuberculous meningitis demonstrates abnormal meningeal enhancement on MRI and tuberculomas appear on CT as homogeneous ring-enhancing lesions with irregular walls of varying thickness[183].

Bone & joint tuberculosis:

MRI is superior to other modalities in imaging of tuberculous spondylitis. Involvement of two or more contiguous vertebra with or without paravertebral abscess and calcification is diagnostic of Pott’s spine[183]. CT helps in identifying the bony destruction in tubercular arthritis[183].

Genitourinary tuberculosis:

Putty kidney (ground glass-like calcification) is the end stage tuberculous kidney disease seen on x-ray; Intravenous urography and ultrasound can also reliably identify ureteric tuberculosis apart from renal tuberculosis[183]. CT is the most sensitive of all imaging modalities in identifying all stages of renal tuberculosis, as well as in detecting the tuberculous lesion of male genital tract[183]. Hysterosalpingography detects the uterine synechiae[183].

Miliary tuberculosis:

Conventional radiography can reliably identify the miliary pattern in pulmonary tuberculosis and ultrasound can identify the serous cavity effusions, focal lesions in liver, spleen and lymphadenopathy[181]. 18 FDG PET-CT is a potentially useful tool in miliary tuberculosis[181].

SPUTUM MICROSCOPY:

For long, sputum microscopy with demonstration of acid fast bacilli had been the primary modality for diagnosing pulmonary tuberculosis and is still the principal method used in many parts of the world and in India, especially in resource constrained settings, because it is rapid and inexpensive[184]. Early morning sputum samples, which are high in bacterial load are collected for three consecutive days and examined; swallowed sputum in children aspirated by gastric lavage and bronchoalveolar lavage fluid can also be used as samples for microscopy[184].

Ziehl-Neelsen staining which is the most commonly employed technique among others, utilises the acid fast property of the tubercle bacilli to stain them, meaning that the carbol fuchsin dye which is used in the staining process stains the bacterial cell wall when heat is applied and after cooling, the cell wall resists decolourisation with acid and thus retains the stain while the background stain disappears, thus enabling the identification of the rod shaped bacili[184].

As simple as it sounds, this test is not sensitive, with sensitivity ranging from 0%-45% and depends on the bacillary load with a minimum of 10^4 bacilli per ml of specimen required for the stain to demonstrate the bacilli.[185]. Fluorescent staining with Auramine-rhodamine is a better alternative given its better sensitivity and superiority over conventional acid fast stains like Ziehl-Neelsen, but requires the use of fluorescent microscopes[184].

TISSUE MICROSCOPY:

For easily accessible tuberculous lesions, affecting areas like peripheral lymph nodes, breast, and abscesses and even in deep seated tuberculous lesions with the help of imaging modalities, Fine Needle Aspiration Cytology is a minimally invasive procedure with reasonable accuracy and is helpful in identifying the granulomas[185]. The tissue thus obtained can be used for Ziehl-Neelsen staining or can be sent for culture, for testing by Nucleic acid Amplification Test and for Immunocytocehmistry[186].

Immunocytochemistry is a relatively cheap method with high sensitivity & specificity and a wide range of antibodies are available which are directed against the mycobacterial antigens like BCG, MPT64, Antigen 5, LAM, ESAT6, and HspX[186]. Histopathological examination of biopsy specimens is the preferred method for diagnosing extra pulmonary tuberculosis, though it can identify pulmonary tuberculosis also with equal efficacy[186]. The main limitation is the occurrence of granulomas in varied number of causes, which can be confusing and in such settings ancillary techniques like Ziehl-Neelsen staining and immunohistochemistry can be employed[186].

IMMUNOHISTOCHEMISTRY:

Immunohistochemistry (IHC) has high sensitivity and specificity and fares better when compared to acid fast stains, and can identify the antigens even in densely necrotic tissues[186]. IHC refers to the process of detecting antigens in the cells using specific antibodies[33]. The procedure was conceptualized and initialized by Dr. Albert Coons in 1941. A number of ways are present to visualize the antigen-antibody interaction[33].

The most common method in routine use is where the antibody is conjugated to an enzyme like peroxidase which catalyses a colour producing reaction. Other methods include use of antibody tagged to a fluorophore like fluorescein or rhodamine[33].

Steps:

1. Tissue processing and antigen or epitope retrieval

    a. 10% neutral buffered formalin is the preferred fixative.

    b. These fixatives cause certain reversible changes in tertiary and quaternary structure of proteins.

    c. Formalin fixed paraffin embedded tissue sections are cut 4 to 5 microns thick and mounted on to glass slides.

    d. Trypsin or protease enzyme digestion

    e. Heating in buffered solutions like citrate or ethylene diamine tetra acetic acid (EDTA) buffer in either a microwave oven or pressure cooker to retrieve or unmask the antigens that have been altered by formalin fixation

2. Antigen-antibody interaction

- Either the direct or indirect method is used

3. Visualizing with detection systems

- Antibody molecules can be labelled with either fluorescent compounds or active enzymes. Horse radish peroxidase (HRP) enzyme is commonly used. The chromogens are oxidized by HRP enzyme, giving a resultant brown or red coloured staining[33].

CULTURE:

Isolation of mycobacteria by culture is the gold standard for diagnosis of tuberculosis, based on which the definitive diagnosis is made[185]. The main drawback is that it is time consuming and can take anywhere between up to 6 weeks, before a conclusive result is arrived at, which affects the time to treatment[186]. This can be overcome with newer culture systems like Myco/F Lytic and the BacT/Alert culture systems, which provide results in as little as 16 days[184].

The major advantage of culturing is that apart from the definitive diagnosis, drug susceptibility testing can also be done[185]. Any type of specimen viz. sputum, gastric lavage, blood, urine, body fluids, and stools can be used for culture, as also tissues and tissue aspirates, provided they are sent in normal saline[184]. All of the specimens need decontaminating procedures with N-acetyl-L-cysteine and sodium hydroxide, to kill the contaminating bacteria which may overgrow the mycobacteria when present and also various antibiotics which prevent the growth of the contaminating bacteria[184].

Culturing can be done using solid media or liquid media. Traditionally Lowenstein-Jensen medium, which is an egg based solid medium, is suitable in most conditions[184]. Various semi-automated broth-based culture systems like BACTEC MGIT 960 system, the VersaTREK system, and the MB/BacT are available which improve the yield by 10% over solid media and also improves the turnaround time[184]. These new systems can also detect atypical mycobacterial colonies as well, and positive cultures are subcultured to solid media to detect mixed cultures and for correlation with any colony morphologies present[184,186].

SEROLOGICAL TESTS:

Though a wide number of serological tests are available which detect the antibodies (IgM, IgG, IgA) in the serum against various mycobacterial antigens, WHO does not recommend the use of these tests for routine diagnostic purposes, as it has very low specificity and very low accuracy, found out on a Meta analysis[186]. These tests had been in routine use previously, because it was simple, rapid and cheap, however the government of India has banned the manufacture and use of serodiagnostic test kits in an order dated 07.06.2012 based on the WHO recommendations[187].

IMMUNOLOGICAL TESTS:

Tuberculin skin test popularly known as the Mantoux test or Pirquet test or Purified Protein Derivative test has been in vogue for more than 100 years, used for screening of tuberculosis infection[186]. It is an example of delayed type

hypersensitivity reaction (Type IV), where the previously sensitised T-cells due to previous infection are recruited to the skin site where they release lymphokines causing a measurable response i.e., induration[186].

A small amount of purified protein derivative tuberculin is injected into the volar aspect of forearm and the result is read at 48 hours. Induration develops if the individual has been exposed to the bacilli[186]. The test does not differentiate between active tuberculosis, prior infection, BCG vaccination, or sensitization by Non tuberculous mycobacteria[186]. However, it is still widely used as the initial screening test[186].

Interferon gamma (IFN-γ) is a macrophage activating cytokine which plays an important role in regulating the cell-mediated immune response to M. tuberculosis infection IFN-γ-release assays are in vitro tests which stimulate the T-cells in peripheral blood to release IFN-γ in response in response to mycobacterial antigens such as ESAT-6 and CFP-10, which are then measured[186]. There are two commercially available Interferon gamma release assays namely Quantiferon TB Gold, & T-SPOT.TB[186].

Studies have shown that these assays have better sensitivity (75%) than Mantoux test and very high specificity (94%) [186]. BCG vaccination does not alter the results, however the assays cannot differentiate between active tuberculosis and latent infection[186]. Nevertheless, it is a better alternative to the Mantoux test[186].

Adenosine deaminase is an enzyme involved in the conversion of adenosine to inosine, in the purine catabolism, and is known to exist as two isoenzymes viz. ADA-1 & ADA-2[186]. While ADA-1 is produced by most cells, ADA-2 is believed to be produced by lymphocytes & monocytes and occurs in negligible quantities[186]. The interaction of mycobacterium with the immune system results in the production of ADA by the activated T lymphocytes & monocytes and ADA-2 levels have been found to occur in high quantities in tuberculosis and thus the measurement of ADA activity has been found to be useful in the diagnosis of extra pulmonary tuberculosis especially in the setting of tuberculous serous cavity effusions like pleural fluid, pericardial fluid, and ascitic fluid[186].

NUCLEIC ACID AMPLIFICATION TESTS:

Molecular methods like Nucleic acid amplification tests amplifies the DNA/RNA of the pathogen, and is a diverse group of tests that includes Polymerase Chain Reaction (PCR), Ligase Chain Reaction (LCR), Transcription Mediated Amplification (TMA) and Nuclei Acid Sequence Based Amplification (NASBA)[186]. The tests are highly sensitive and highly specific with a positive predictive value approaching 99%, and the results are available on the same day & can detect as few as 10 mycobacteria[186].

PCR technique is commonly used which includes conventional PCR, Nested PCR and Real-time PCR[186]. Besides the diagnosis, drug susceptibility testing can also be performed by NAAT[186].World Health Organisation has approved the use of Xpert MTB/RIF, a cartridge based nested PCR technique,

as the initial diagnostic technique for the diagnosis of pulmonary tuberculosis as well as extra pulmonary tuberculosis, based on meta-analysis[186].

Various studies have demonstrated the efficacy of Xpert MTB/RIF in diagnosing extra pulmonary tuberculosis, with sensitivity ranging from 50% - 100% and specificity of more than 80%[188]. The major drawbacks of these methods are their high cost, low sensitivity in smear negative cases and extreme sensitivity to contamination which may result in high false positive rates[186].

TREATMENT:

The Revised National Tuberculosis Control Programme (RNTCP), in its 2016 guidelines, has recommended the use of multi drug regimen in the intensive phase (IP) and consolidation phase (CP) [189]. For all new cases of tuberculosis, irrespective of pulmonary or extra pulmonary, the use of Isoniazid (H), Rifampicin (R), Pyrazinamide (Z), Ethambutol (E), in daily or intermittent doses for a period of 2 months in IP (2HRZE) and the use of HRE for 4 months in the CP (4HRE) [189]. For all previously treated tuberculosis cases, the addition of Streptomycin (S) injection for 1 month in the IP is advised, which is as follows: 2HRZES+1HRZE in the IP and 5HRE in the CP[189].

For multidrug resistant tuberculosis (MDR-TB), the patients are to be treated with a standard regimen of Kanamycin, Levofloxacin, Ethambutol, Pyrazinamide, Ethionamide and Cycloserine for 6 to 9 months in the IP and 18 months of CP with Levofloxacin, Ethambutol, Ethionamide and Cycloserine[189].

For extremely drug resistant tuberculosis (XDR-TB), 6 to 12 months of IP with Capreomycin, Para Amino Salicylic acid, Moxifloxacin, High dose Isoniazid, Clofazimine, Linezolid and Co-Amoxyclav, followed by 18 months of CP with Para Amino Salicylic acid, Moxifloxacin, High dose Isoniazid, Clofazimine, Linezolid and Co-Amoxyclav[189].

MATERIALS AND METHODS

MATERIALS AND METHODS

This is a prospective and retrospective study conducted at the Institute of Pathology, Madras Medical College & Rajiv Gandhi Government General Hospital, Chennai for a 2 year study period from August 2015 to July 2017. Out of the 24617 cases of histopathological specimens received, 349 cases were granulomatous lesions in extra pulmonary sites, out of which 198 cases were of suspected tuberculous etiology based on the clinical suspicion, presence of necrosis with or without caseation or Langhans giant cells. The remaining 151 cases were of different aetiologies viz. fungal infection, foreign body induced, inflammatory bowel disease and vasculitis.

DATA COLLECTION:

Case details especially age, sex, presenting complaints, details of relevant investigations, procedure done and histopathological diagnosis of the tissues were obtained from pathology registers. Relevant details like clinical diagnosis, imaging findings, prior history of anti-tuberculous therapy, history of Human Immunodeficiency Virus (HIV) infection, immunocompromised status were also noted. Haematoxylin and Eosin sections of the paraffin tissue blocks were reviewed. Out of the 198 granulomatous lesions of suspected tuberculous etiology in extra pulmonary sites, 50 cases were selected by proportional representation of the organ systems involved and their corresponding tissue blocks were obtained for immunohistochemical analysis of Mycobacterium tuberculosis using polyclonal antibody and Ziehl- Neelsen staining for the visualisation of the acid fast bacilli.

CONTROLS:

Positive Control:

Formalin fixed, paraffin embedded tissue section of Mycobacterium tuberculosis infected lung tissue which were positive for AFB by Ziehl-Neelsen staining was used as positive control

Negative Control:

Formalin fixed, paraffin embedded tissue sections of foreign body granuloma was used as negative control.

TABLE 1: PROCEDURE OF IMMUNOHISTOCHEMISTRY

Antigen	Vendor	Species (clone)	Positive Control	Negative Control
Mycobacterium tuberculosis	Biocare Medical	Rabbit polyclonal	M. Tuberculosis Infected Lung Tissue	Foreign body granuloma

IMMUNOHISTOCHEMISTRY (IHC):

1. 4 micron thick sections were cut from formalin fixed paraffin embedded tissue blocks and transferred onto positively charged glass slides.
2. The glass slides were kept in an incubator at 58 degree Celsius overnight.
3. Deparaffinisation in xylene for 15 minutes x 2 changes
4. Dehydration with absolute alcohol for 5 minutes x 2 changes
5. Washing of sections in tap water for 10 minutes

6. Washing of sections in distilled water for 5 minutes
7. Retrieval of antigens done with microwave oven with sections immersed in Tris-EDTA buffer (preheated at 800 watts for 4 minutes) for 20 minutes
    A. 800 watts – 5 minutes
    B. 640 watts – 10 minutes
    C. 480 watts – 5 minutes
8. Cool the slides to room temperature and then wash with distilled water for 10 minutes
9. Then wash in phosphate wash buffer for 5 minutes x 2 changes
10. Application of peroxide block over the sections for 5 minutes
11. Slides are washed with phosphate wash buffer for 5 minutes
12. Primary antibody was applied over the sections and incubated for 45 minutes
13. After washing in wash buffer for 5 minutes, HRP-conjugated polymer applied to the sections and incubated for 30 minutes
14. Slides were washed with 2 changes of wash buffer for 2 minutes each
15. Sections are then covered with Di-amino benzidine (DAB) chromogen (prepared by diluting 1 drop of DAB chromogen to 1ml of DAB buffer) for 5 minutes.
16. Counterstaining was done with haematoxylin, washed in running tap water, air dried, cleared with xylene and mounted.

INTERPRETATION AND SCORING OF IHC:

The IHC slides were analysed for the presence of reaction, cellular localisation of the staining – mycobacterium tuberculosis shows fine or coarse granular cytoplasmic dust-like positive staining within the epithelioid histiocytes, giant cells and in extracellular locations like areas of necrosis. Percentage of cells taking up the stain and the intensity with which they stain were also analysed. Scores of 1, 2 and 3 were assigned to mild, moderate and strong intensity staining respectively.

TABLE 2: PROCEDURE FOR ZIEHL- NEELSEN STAINING

Vendor	Positive Control	Negative Control
Fisher Scientific	M. Tuberculosis Infected Lung Tissue	Foreign body granuloma

ZIEHL – NEELSEN STAINING:

1. Deparaffinise the sections in 2 changes of Xylene for 15 minutes each
2. Immerse the slides in 2 changes of absolute alcohol for 5 minutes each. Air dry the slides.
3. Flood the slide with carbol fuchsin solution, and allow to stand at 37°C for 1 hour
4. Rinse in tap water until no colour washes off.
5. Dip in 1% acid-alcohol until the sections are light pink.
6. Counter stain with methylene blue for 5 minutes

7. Rinse well with tap water.
8. Dehydrate & clear with xylene and mount with coverslip.

INTERPRETATION OF ZIEHL-NEELSEN STAINING:

When stained with strong stains (carbol fuchsin), acid acid-fast bacilli retain their colour even after treatment with strong decolourizing solutions due to the presence of the mycolic acids which takes up the stain and remains resistant to decolourization with 1% acid-alcohol or 20% sulphuric acid as compared to other tissues. They remain red after counterstaining with methylene blue, whereas the other tissues and microorganisms which are susceptible to acid take on the blue colour. Acid fast bacilli appear bright red curved rods and background appears blue.

STATISTICAL ANALYSIS:

Statistical analysis was performed with IBM Statistical Package for the Social Sciences software version 20.0. The immunohistochemical expression of MTB antibody and Ziehl-Neelsen staining were correlated and studied using McNemar test and chi square test.

OBSERVATION AND RESULTS

OBSERVATION AND RESULTS

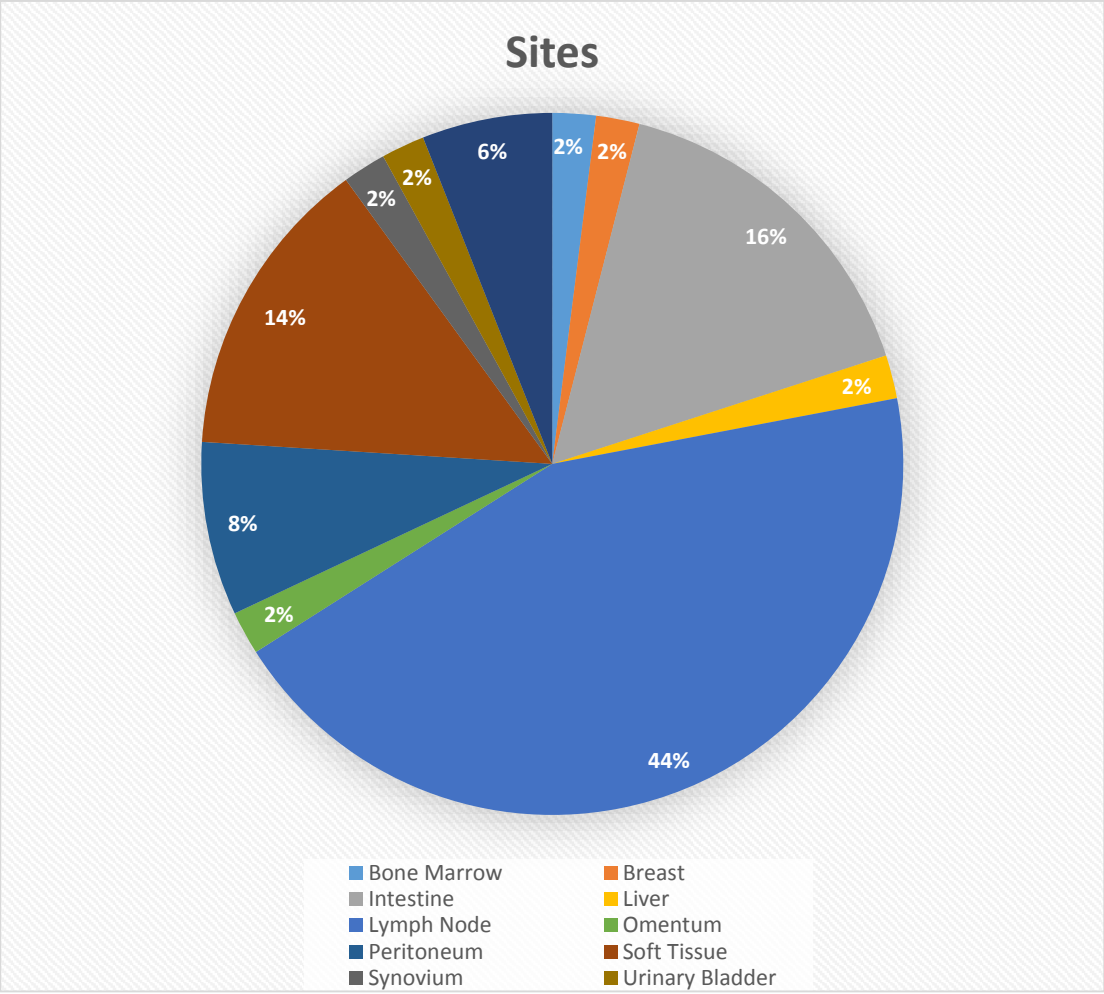
In the 24 month study period performed from August 2015 to July 2017, a total of 24617 specimens were received at the Institute of Pathology, Madras Medical College & Rajiv Gandhi Government General Hospital for histopathological examination. Out of the total 24617 specimens, 50 cases which were selected by proportional representation were taken up for study with immunohistochemical analysis and Ziehl-Neelsen staining.

The sites of the selected lesions are listed below (Table 3, Chart 1)

TABLE 3: FREQUENCY OF GRANULOMATOUS LESIONS IN SPECIFIC SITES

SITE	FREQUENCY	PERCENT	CUMULATIVE %
Bone Marrow	**1**	**2%**	**2%**
Breast	**1**	**2%**	**4%**
Intestine	**8**	**16%**	**20%**
Liver	**1**	**2%**	**22%**
Lymph Node	**22**	**44%**	**66%**
Omentum	**1**	**2%**	**68%**
Peritoneum	**4**	**8%**	**76%**
Soft Tissue	**7**	**14%**	**90%**
Synovium	**1**	**2%**	**92%**
Urinary Bladder	**1**	**2%**	**94%**
Vertebra	**3**	**6%**	**100%**

CHART 1: FREQUENCY OF GRANULOMATOUS LESIONS IN SPECIFIC SITES



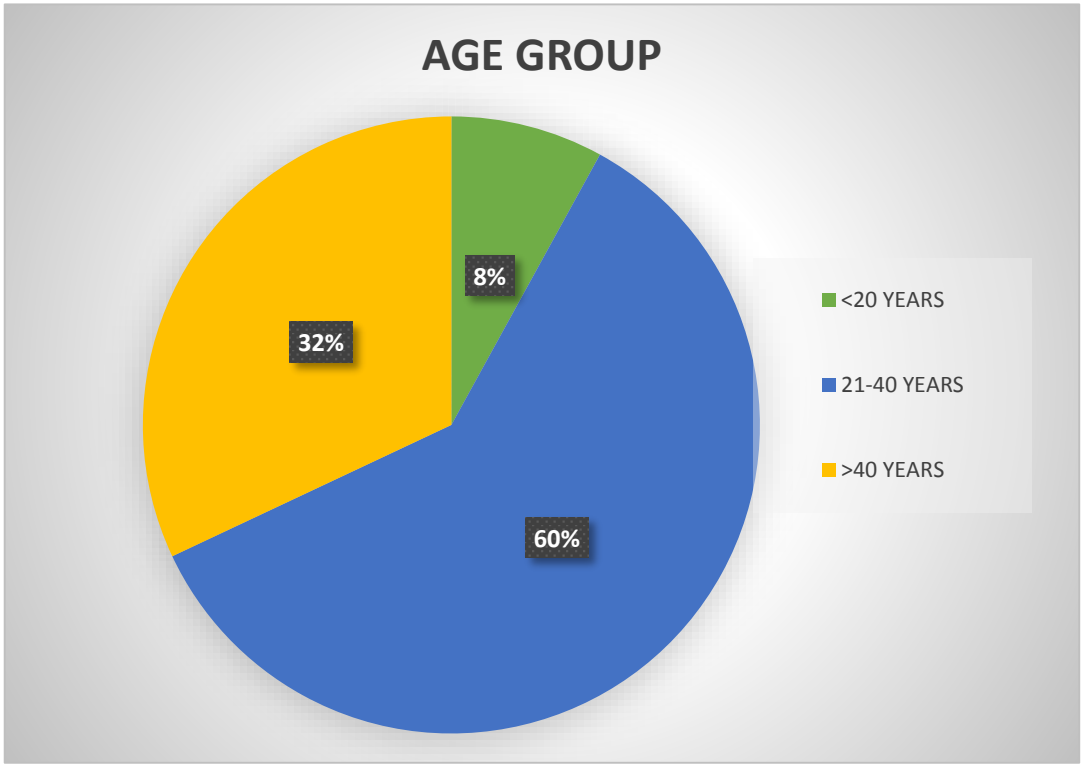
Lymph nodes accounted for the majority of extra pulmonary granulomatous lesions, constituting about 44% of all lesions, followed by Intestine (16%), soft tissues (14%), peritoneum (8%) and vertebra (6%). Other rare sites of occurrence included bone marrow, breast, liver, omentum, synovium and urinary bladder which all accounted for 2% each.

Majority of the cases had a peak incidence in the age group 21-40 years which constituted about 60% followed by the age group of above 40 years which formed about 32%. Mean age is about 34.56 years (Table 4, Chart 2)

TABLE 4: AGE WISE DISTRIBUTION OF THE LESION

AGE GROUP	FREQUENCY	PERCENT
<20 YEARS	4	8%
21-40 YEARS	30	60%
ABOVE 40 YEARS	16	32%
TOTAL	50	100%

CHART 2: AGE WISE DISTRIBUTION OF THE LESION

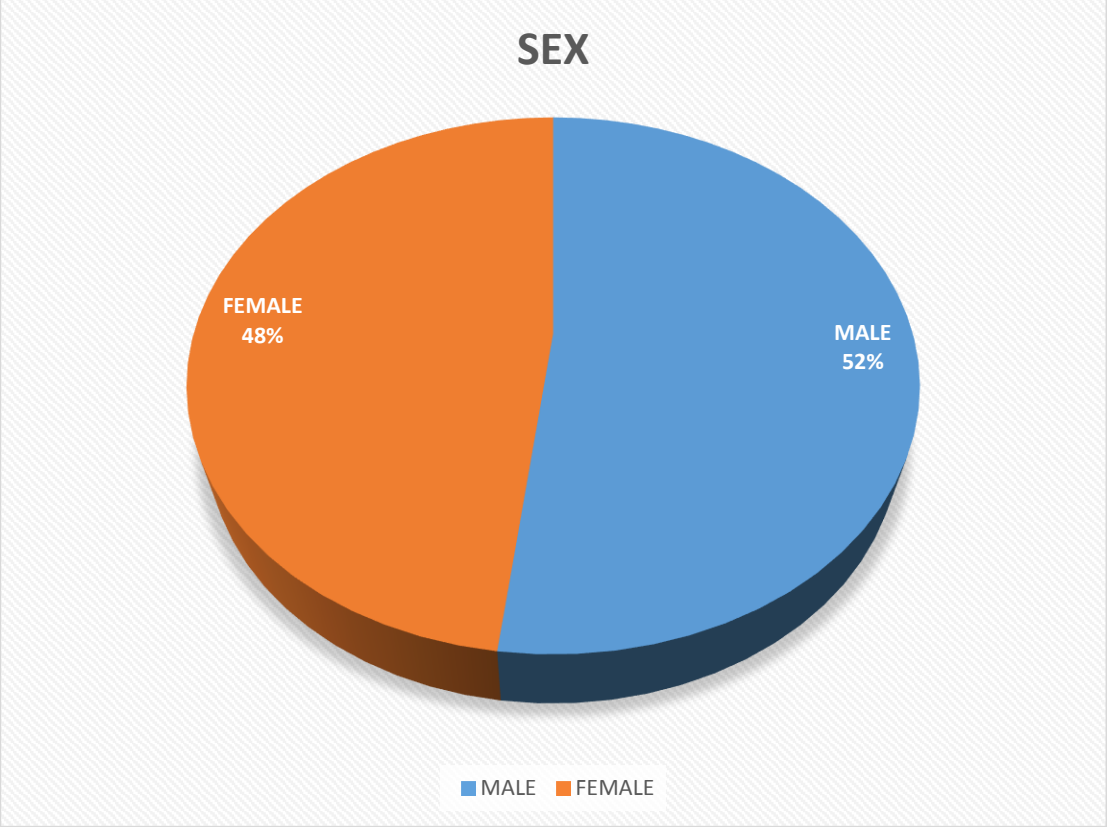


There was not much significant difference in the sex with males accounting for 52% and females accounting for 48% of cases. (Table 5, Chart 3)

TABLE 5: SEX WISE DISTRIBUTION

SEX	FREQUENCY	PERCENT
MALE	26	52%
FEMALE	24	48%
Total	50	100%

CHART 3: SEX WISE DISTRIBUTION

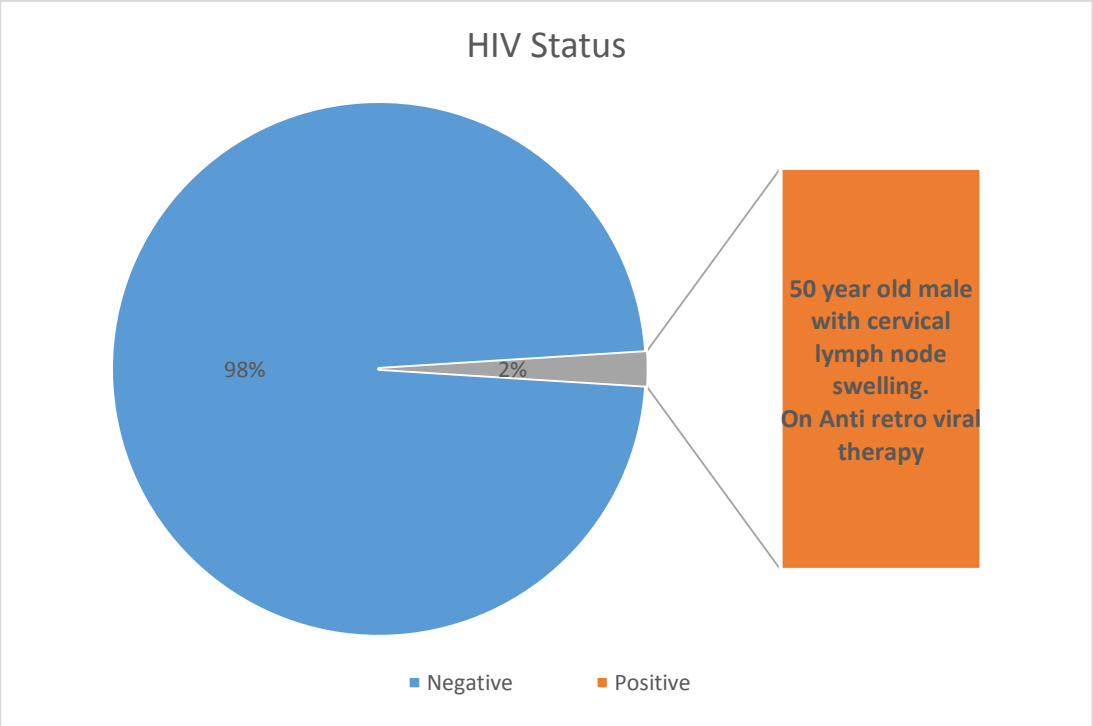


Among the 50 cases, only one patient was positive for Human Immunodeficiency Virus (HIV), who presented with cervical lymphadenopathy. (Table 6, Chart 4)

TABLE 6: HIV STATUS

HIV STATUS	FREQUENCY	PERCENT
Negative	**49**	**98%**
Positive	**1**	**2%**
Total	**50**	**100%**

CHART 4: HIV STATUS

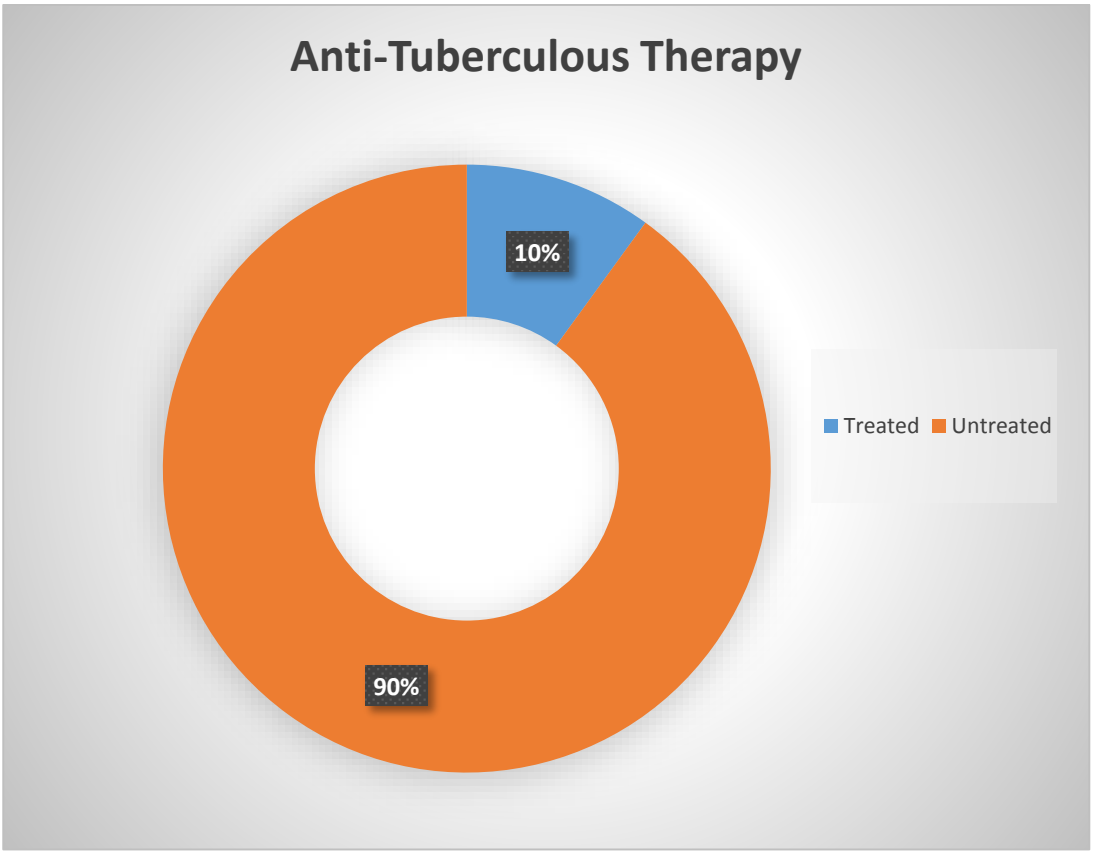


Among the 50 cases, 5 of the patients has previously undergone anti-tuberculous therapy. (Table 7, Chart 5)

TABLE 7: ANTI-TUBERCULOUS THERAPY HISTORY

ANTI-TUBERCULOUS THERAPY HISTORY	FREQUENCY	PERCENT
Treated	**5**	**10%**
Untreated	**45**	**90%**
Total	**50**	**100%**

CHART 5: ANTI-TUBERCULOUS THERAPY HISTORY

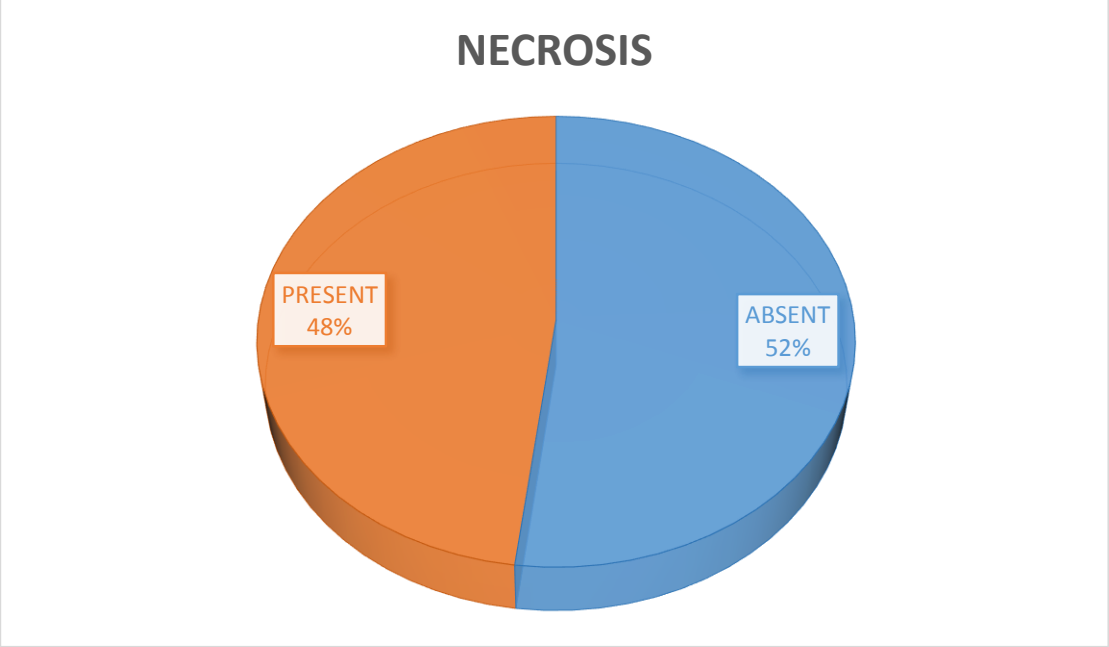


Among the 50 granulomatous lesions, on histopathological examination (H&E), necrosis was present in 24 cases which constituted about 48% of cases. (Table 8, Chart 6)

TABLE 8: PRESENCE OF NECROSIS ON HISTOPATHOLOGICAL EXAMINATION

NECROSIS	FREQUENCY	PERCENT
ABSENT	26	52.0
PRESENT	24	48.0
Total	50	100.0

CHART 6: PRESENCE OF NECROSIS ON HISTOPATHOLOGICAL EXAMINATION

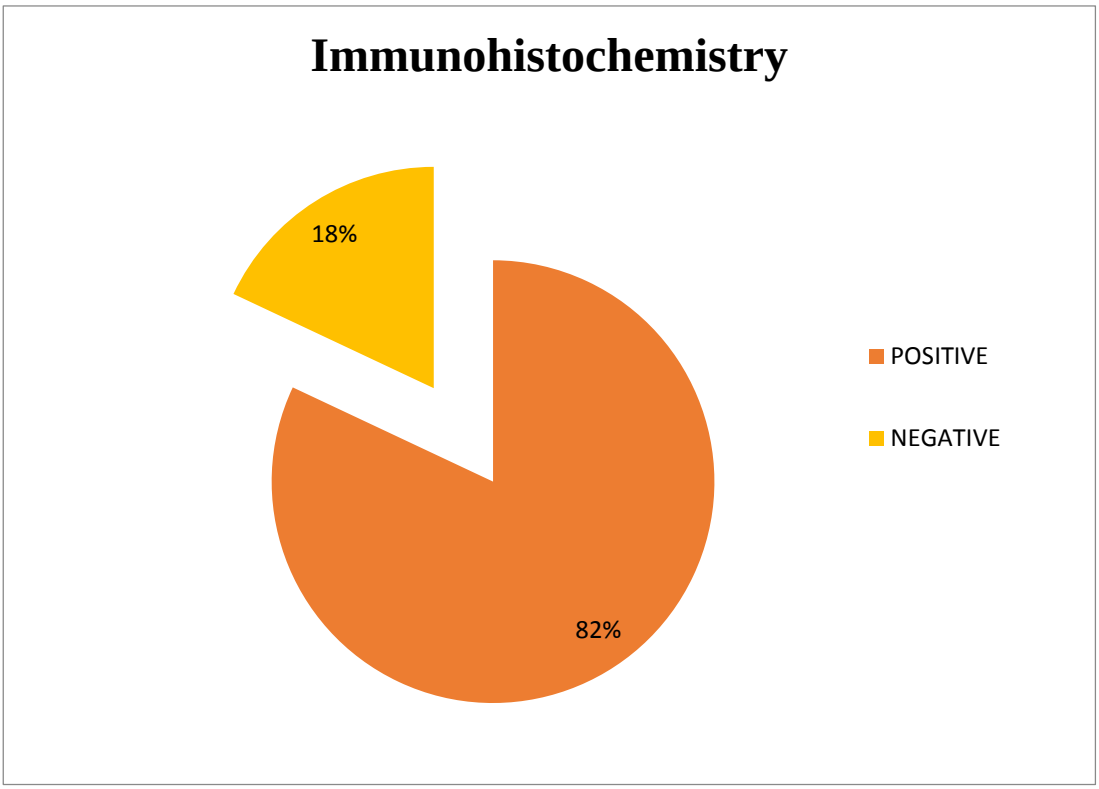


All 50 cases were subjected to immunohistochemical analysis using polyclonal anti mycobacterium tuberculosis antibody. 41 cases showed cytoplasmic (antigenic dust) positivity (Table 9, Chart 7)

TABLE 9: IMMUNOHISTOCHEMICAL ANALYSIS

IMMUNOHISTOCHEMISTRY	FREQUENCY	PERCENT
POSITIVE	41	82%
NEGATIVE	9	18%
Total	50	100%

CHART 7: IMMUNOHISTOCHEMICAL ANALYSIS

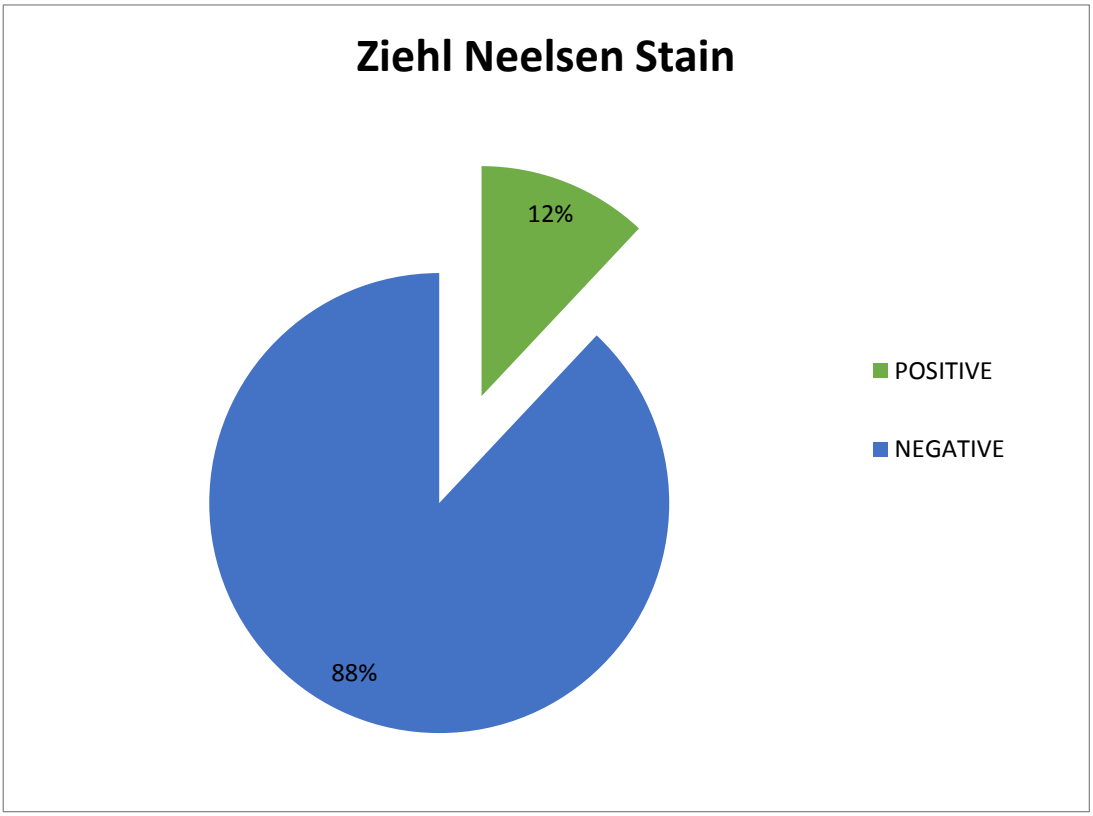


All 50 cases were subjected to Ziehl-Neelsen staining as well. 6 cases turned positive for the tubercle bacilli. (Table 10, Chart 8)

TABLE 10: ZIEHL-NEELSEN STAIN

ZIEHL-NEELSEN STAIN	FREQUENCY	PERCENT
POSITIVE	**6**	**12%**
NEGATIVE	**44**	**88%**
Total	**50**	**100%**

CHART 8: ZIEHL-NEELSEN STAIN

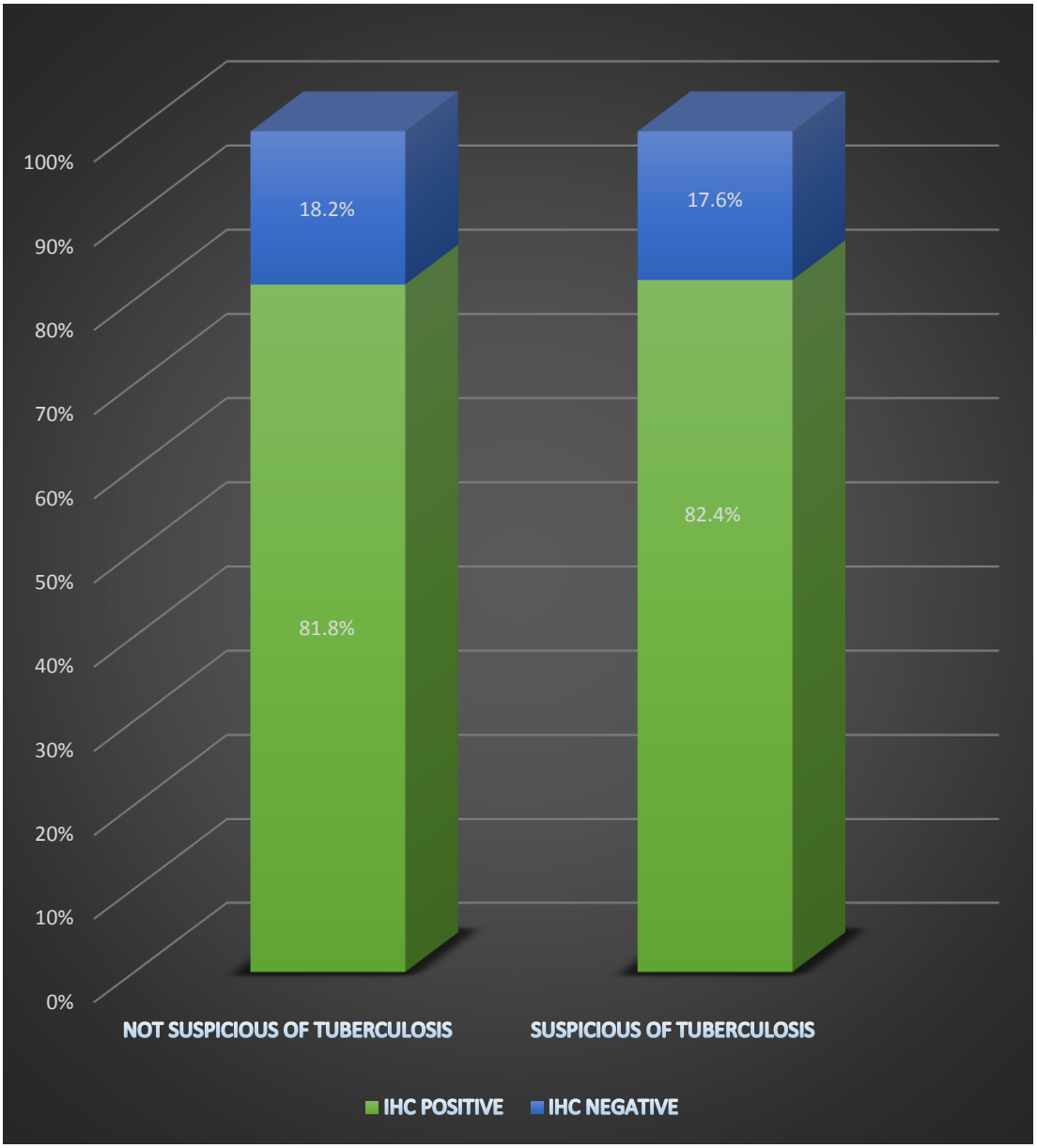


14 out of 17 cases which had a clinical suspicion of tuberculosis were positive by immunohistochemical staining, whereas 27 out of the remaining 33 cases were positive by immunohistochemical staining. (Table 11, Chart 9)

TABLE 11: COMPARISON OF CLINICAL SUSPICION OF TUBERCULOSIS WITH IMMUNOHISTOCHEMISTRY

Clinical suspicion of tuberculosis * Immunohistochemistry Cross tabulation					
			Immunohistochemistry		Total
			Positive	Negative	
Clinical Suspicion of Tuberculosis	No	Count	27	6	33
		% within IHC	65.9%	66.7%	66.0%
	Yes	Count	14	3	17
		% within IHC	34.1%	33.3%	34.0%
Total		Count	41	9	50
		% within IHC	100.0%	100.0%	100.0%

CHART 9: COMPARISON OF CLINICAL SUSPICION OF TUBERCULOSIS WITH IMMUNOHISTOCHEMISTRY

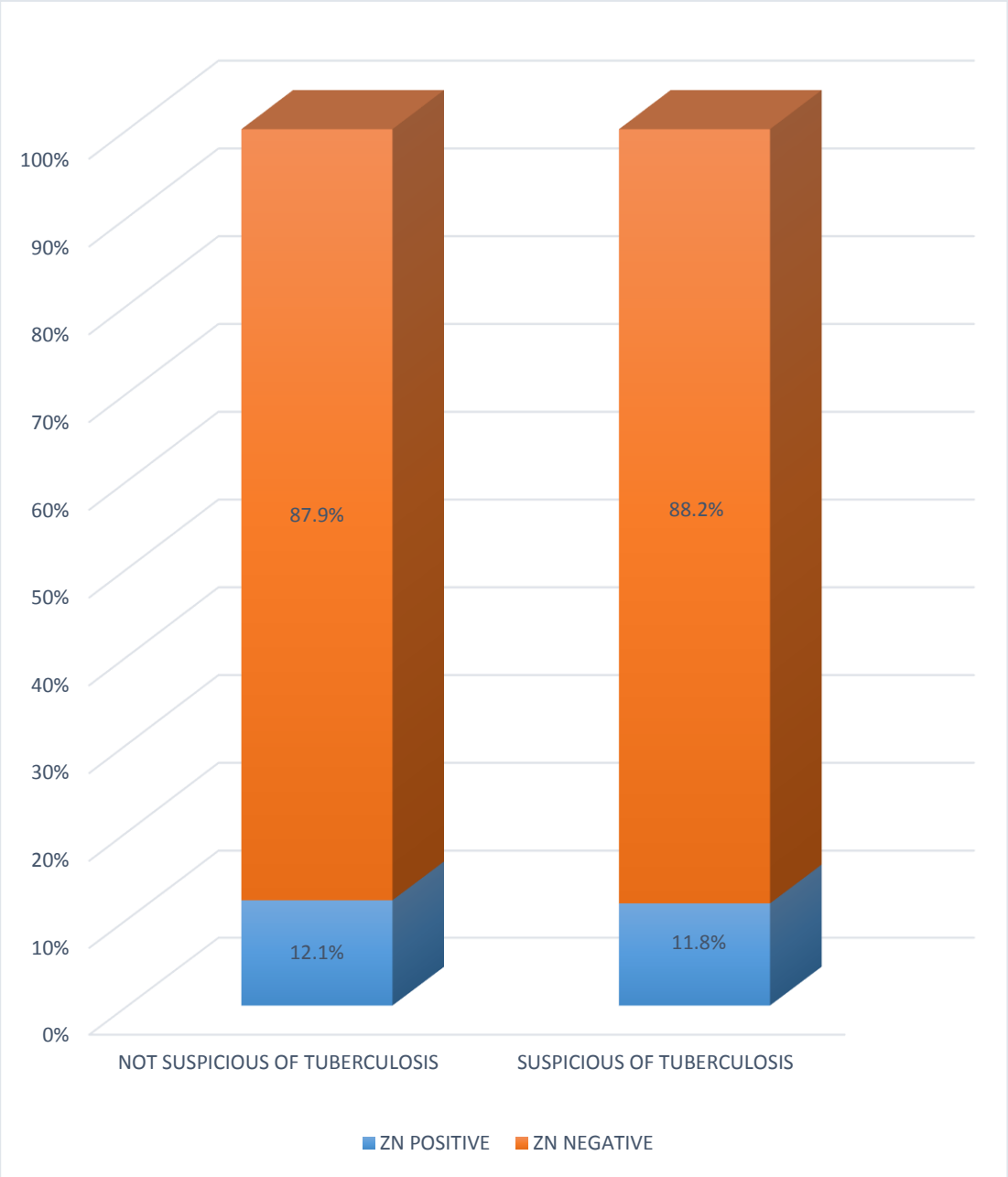


2 out of 17 cases which had a clinical suspicion of tuberculosis were positive by Ziehl-Neelsen staining, whereas 4 out of the remaining 33 cases were positive by Ziehl-Neelsen staining. (Table 12, Chart 10)

TABLE 12: COMPARISON OF CLINICAL SUSPICION OF TUBERCULOSIS WITH ZIEHL-NEELSEN STAINING

CLINICAL SUSPICION OF TUBERCULOSIS * ZIEHL-NEELSEN STAINING CROSSTABULATION					
			Ziehl-Neelsen staining		Total
			Positive	Negative	
Clinical Suspicion of Tuberculosis	No	Count	4	29	33
		% within Ziehl-Neelsen Staining	66.7%	65.9%	66.0%
	Yes	Count	2	15	17
		% within Ziehl-Neelsen staining	33.3%	34.1%	34.0%
Total		Count	6	44	50
		% within Ziehl-Neelsen Staining	100.0%	100.0%	100.0%

CHART 10: COMPARISON OF CLINICAL SUSPICION OF TUBERCULOSIS WITH ZIEHL-NEELSEN STAINING

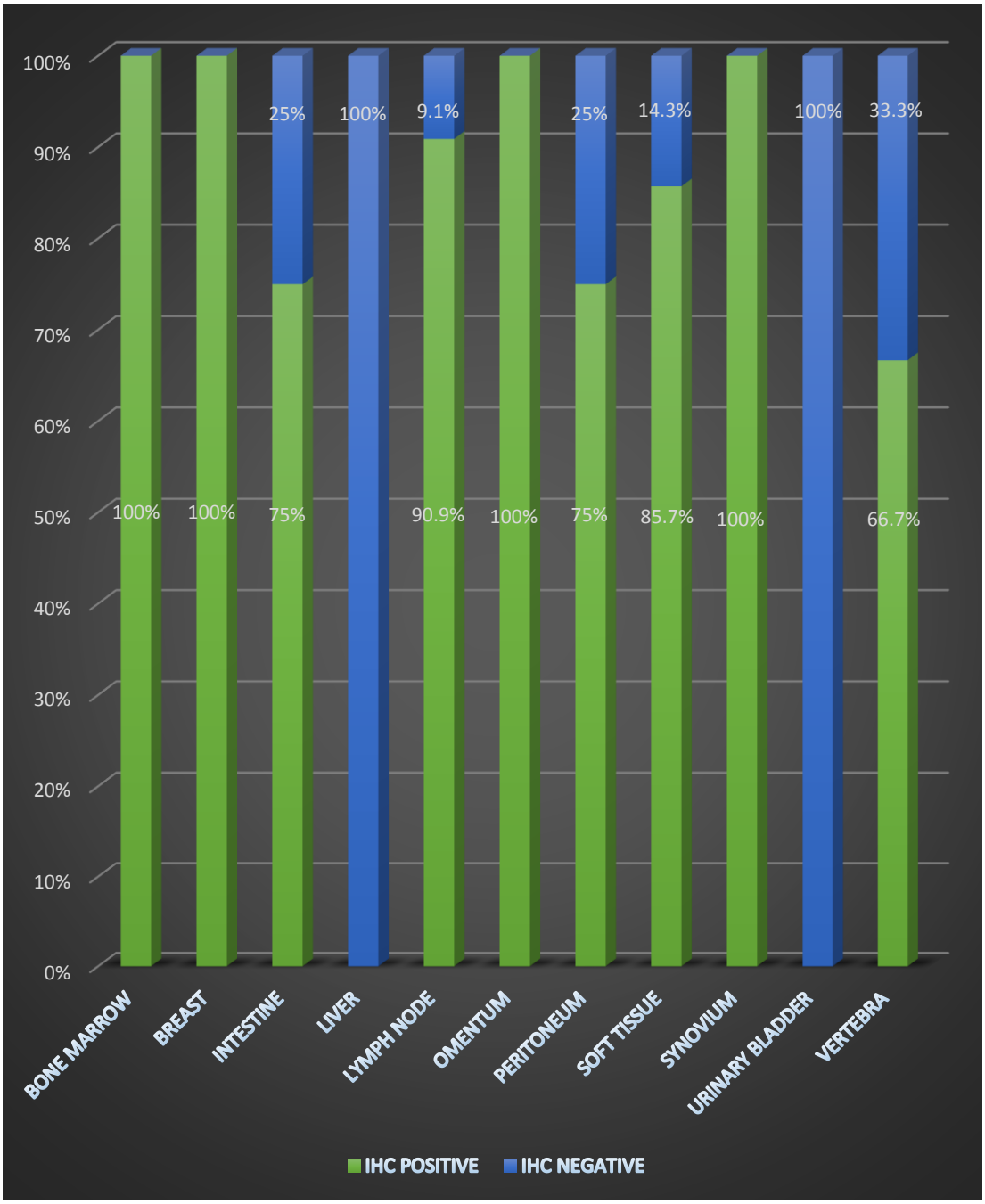


Out of the 9 cases which were negative for immunohistochemistry, the tissue samples were lymph node (2), intestine (2), soft tissue (1), vertebra (1), liver (1), peritoneum (1) and urinary bladder (1). (Table 13, Chart 11)

TABLE 13: COMPARISON OF ORGAN SYSTEMS WITH IMMUNOHISTOCHEMICAL EXPRESSION

			IMMUNOHISTOCHEMISTRY											Total
			B. MARROW	BREAST	INTESTINE	LIVER	LYMPHNODE	OMENTUM	PERITONEUM	SOFT TISSUE	SYNOVIUM	U. BLADDER	VERTEBRA	
IHC	POS	Count	1	1	6	0	20	1	3	6	1	0	2	41
		% within IHC	100%	100%	75%	0%	90.9%	100%	75%	85.7%	100%	0%	66.7%	82%
	NEG	Count	0	0	2	1	2	0	1	1	0	1	1	9
		% within IHC	0%	0%	25%	100%	9.1%	0%	25%	14.3%	0%	100%	33.3%	18%
Total		Count	1	1	8	1	22	1	4	7	1	1	3	50
		% within IHC	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%

CHART 11: COMPARISON OF ORGAN SYSTEMS WITH IMMUNOHISTOCHEMICAL EXPRESSION



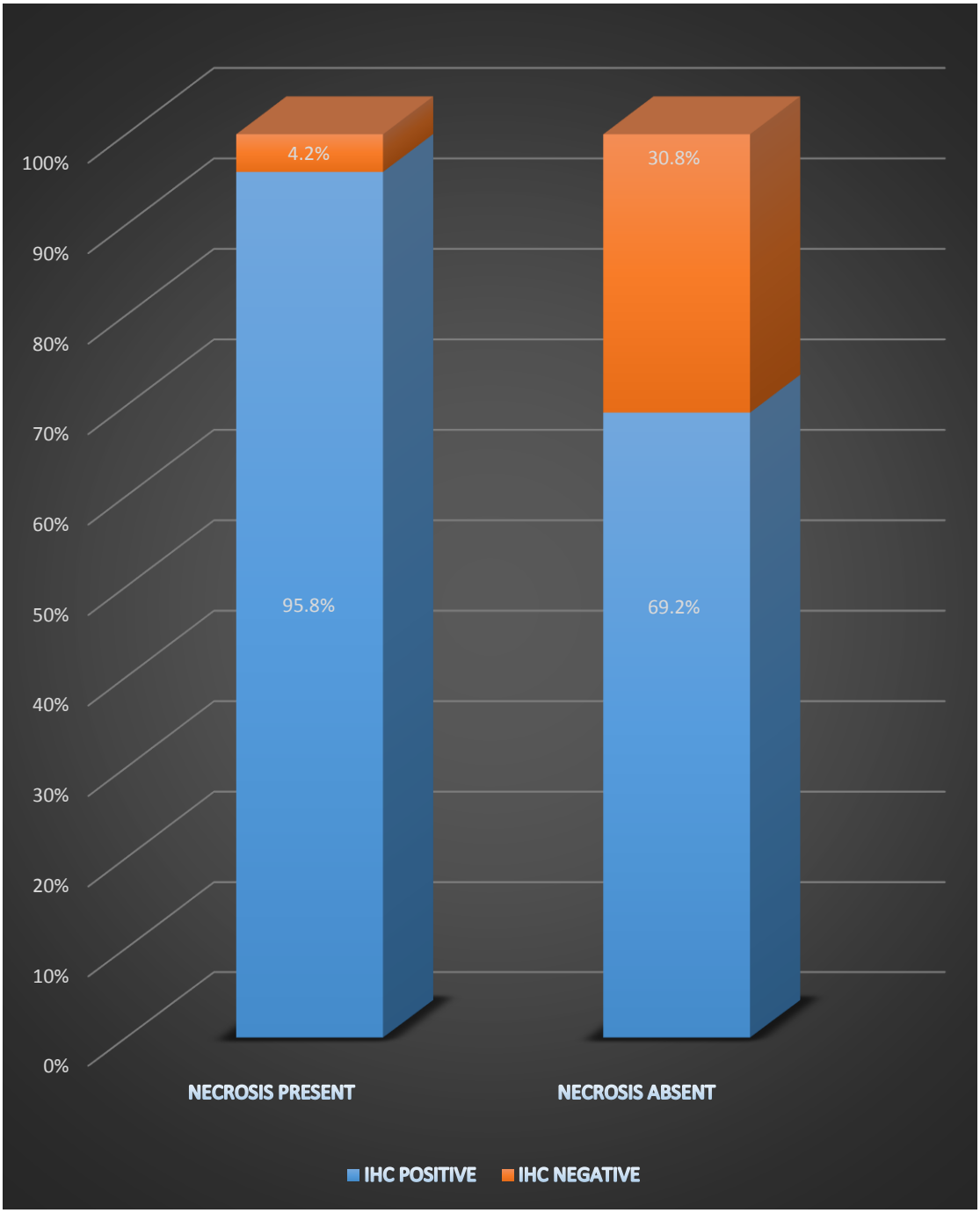
Presence of necrosis had no effect on the expression of immunohistochemistry, with P value more than 0.005 (Table 14, Chart 12)

TABLE 14: CORRELATION OF NECROSIS WITH IMMUNOHISTOCHEMICAL EXPRESSION

NECROSIS - IMMUNOHISTOCHEMISTRY CROSS TABULATION					
			IMMUNOHISTOCHEMISTRY		Total
			POSITIVE	NEGATIVE	
NECROSIS	PRESENT	Count	23	1	24
		% within Immuno histochemistry	56.1%	11.1%	48.0%
	ABSENT	Count	18	8	26
		% within Immuno histochemistry	43.9%	88.9%	52.0%
TOTAL		Count	41	9	50
		% within Immuno histochemistry	100.0%	100.0%	100.0%

Pearson Chi-Square=5.984* p=0.014

CHART 12: CORRELATION OF NECROSIS WITH IMMUNOHISTOCHEMICAL EXPRESSION



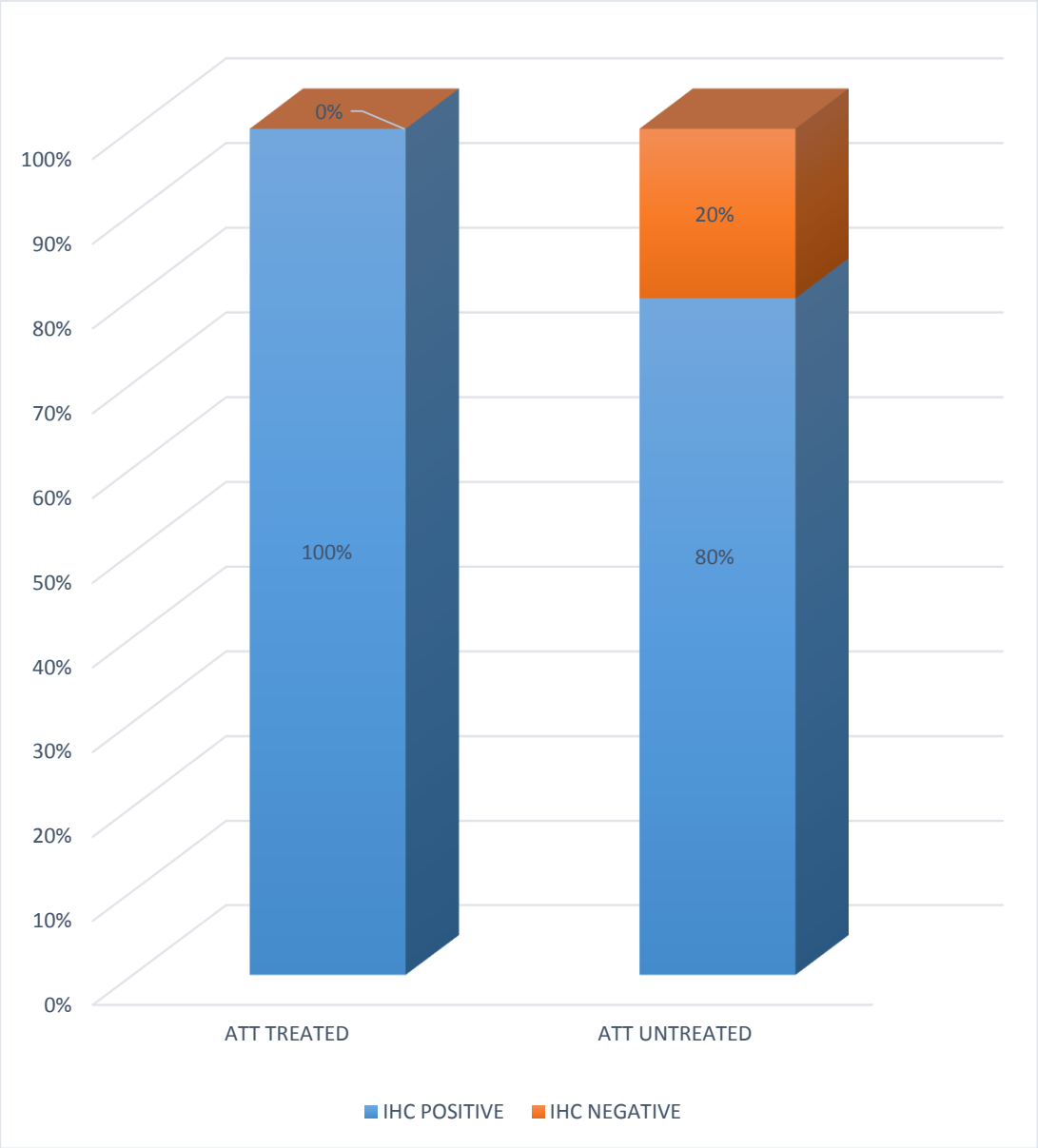
Prior treatment with anti-tuberculous drugs had no effect on the immunohistochemical expression with P value more than 0.005 (Table 15, Chart 13)

TABLE 15: CORRELATION OF ANTI-TUBERCULOUS THERAPY WITH IMMUNOHISTOCHEMICAL EXPRESSION

Anti-tuberculous Therapy * Immunohistochemistry Crosstabulation					
			Immunohistochemistry		Total
			Positive	Negative	
Anti-tuberculous therapy	Treated	Count	5	0	5
		% within IHC	12.2%	0.0%	10.0%
	Untreated	Count	36	9	45
		% within IHC	87.8%	100.0%	90.0%
Total		Count	41	9	50
		% within IHC	100.0%	100.0%	100.0%

Pearson Chi-Square=1.220 p=0.269

CHART 13: CORRELATION OF ANTI-TUBERCULOUS THERAPY WITH IMMUNOHISTOCHEMICAL EXPRESSION

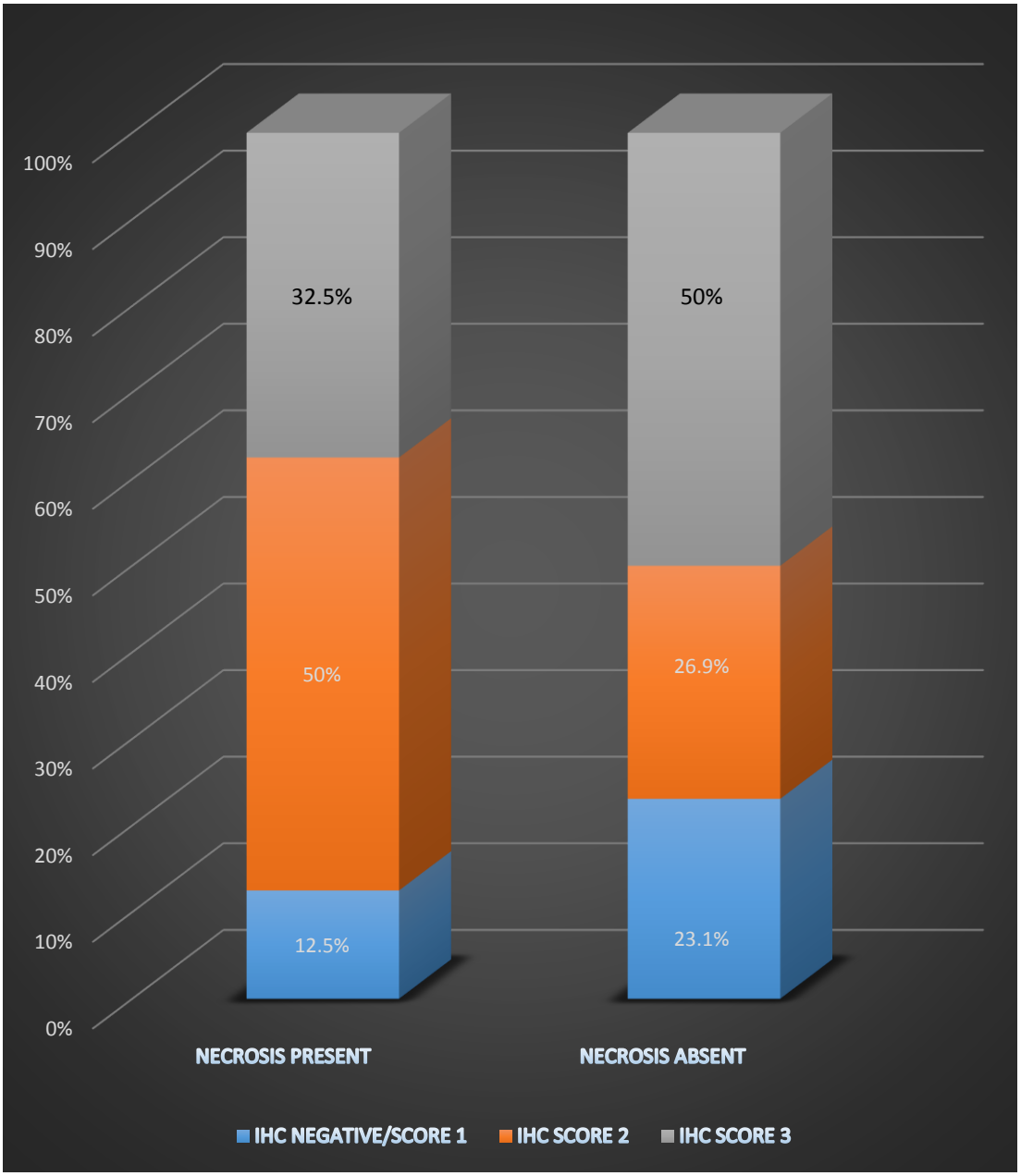


Out of the 41 cases which were positive by immunohistochemistry, 22 cases had strong intensity staining (score 3) and 19 cases had moderate staining intensity (score 2). 3 cases had mild intensity staining (score 1) and were considered as negative along with the remaining 6 cases. The presence of necrosis had no impact on the staining intensity. (Table 16, Chart 14)

TABLE 16: COMPARISON OF NECROSIS WITH INTENSITY OF IMMUNOHISTOCHEMICAL EXPRESSION

Necrosis * Immunohistochemistry Staining Intensity Crosstabulation						
			Immunohistochemistry			Total
			Negative/ Score 1	Score 2	Score 3	
Necrosis	Present	Count	3	12	9	24
		% within IHC	33.3%	63.2%	40.9%	48%
	Absent	Count	6	7	13	26
		% within IHC	66.7%	36.8%	59.1%	52%
Total		Count	9	19	22	50
		% within IHC	100.0%	100.0%	100.0%	100.0%

CHART 14: COMPARISON OF NECROSIS WITH INTENSITY OF IMMUNOHISTOCHEMICAL EXPRESSION



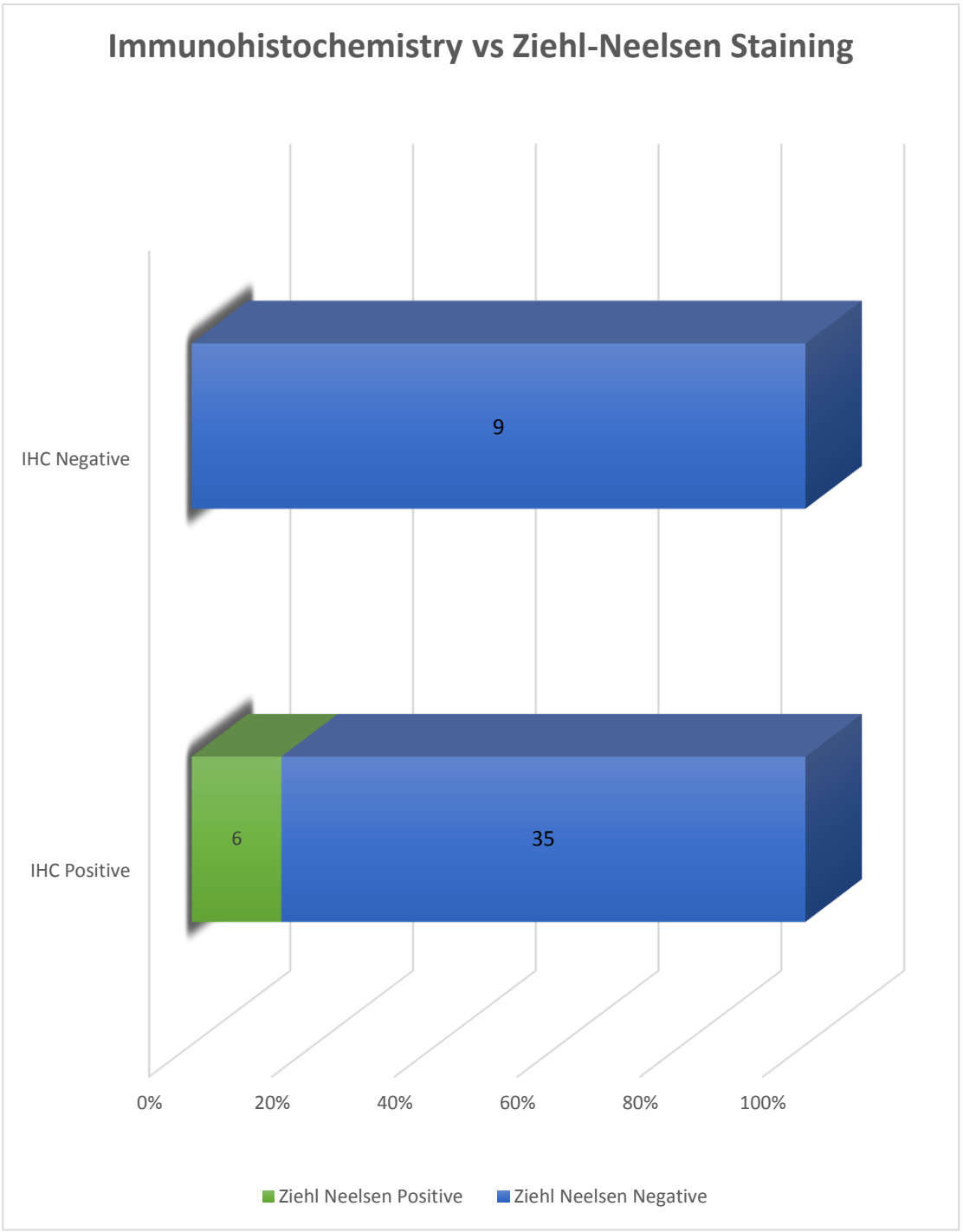
12% of cases were positive for acid fast bacilli by Ziehl-Neelsen staining, whereas 82% of cases were positive by immunohistochemical staining. The P value is less than 0.001 by McNemar test which is statistically significant which shows the sensitivity of immunohistochemistry is better than Ziehl-Neelsen staining. (Table 17, Chart 15)

TABLE 17: COMPARISON OF IMMUNOHISTOCHEMICAL STAINING WITH ZIEHL-NEELSEN STAINING

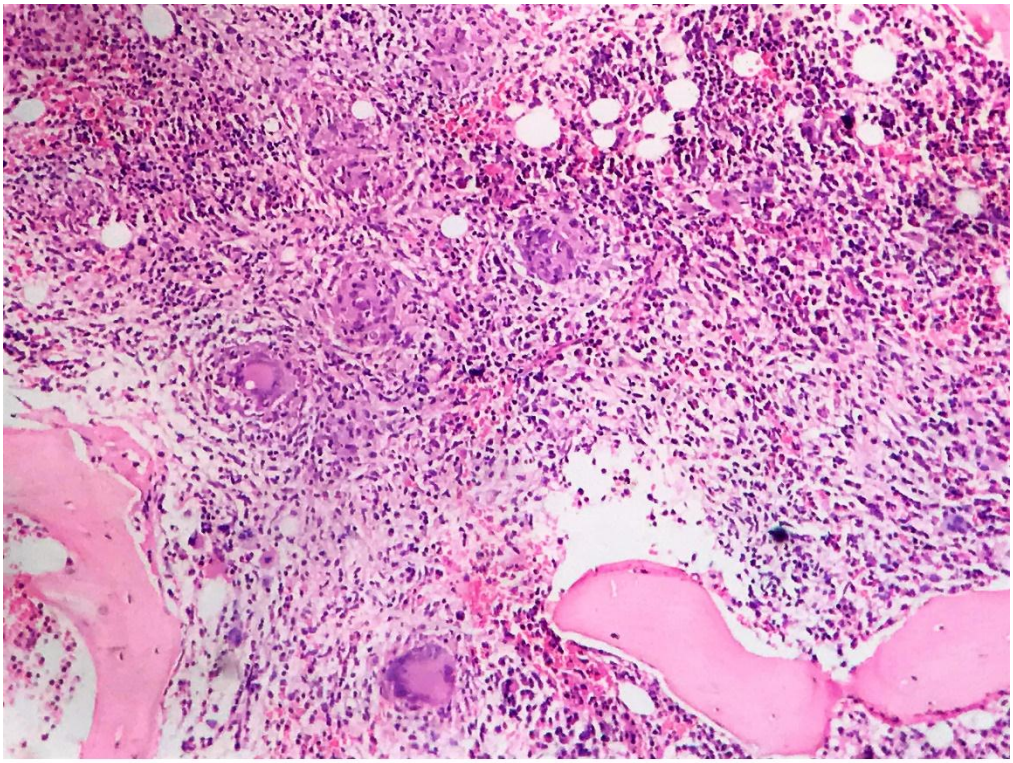
Immunohistochemistry * Ziehl-Neelsen Stain Cross tabulation					
			Ziehl-Neelsen Stain		Total
			POSITIVE	NEGATIVE	
Immunohistochemistry (IHC)	POSITIVE	Count	6	35	41
		% within IHC	14.6%	85.4%	100.0%
	NEGATIVE	Count	0	9	9
		% within IHC	0.0%	100.0%	100.0%
Total		Count	6	44	50
		% within IHC	12.0%	88.0%	100.0%

McNemar Test P<0.001

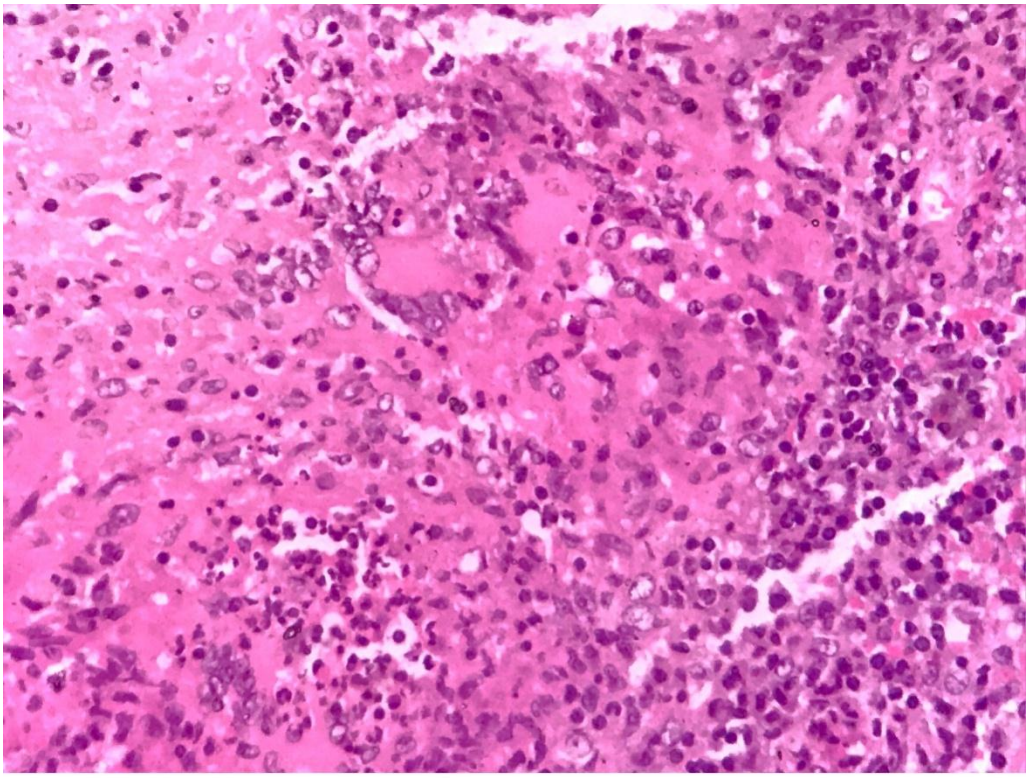
CHART 15: COMPARISON OF IMMUNOHISTOCHEMICAL STAINING WITH ZIEHL-NEELSEN STAINING



COLOUR PLATES

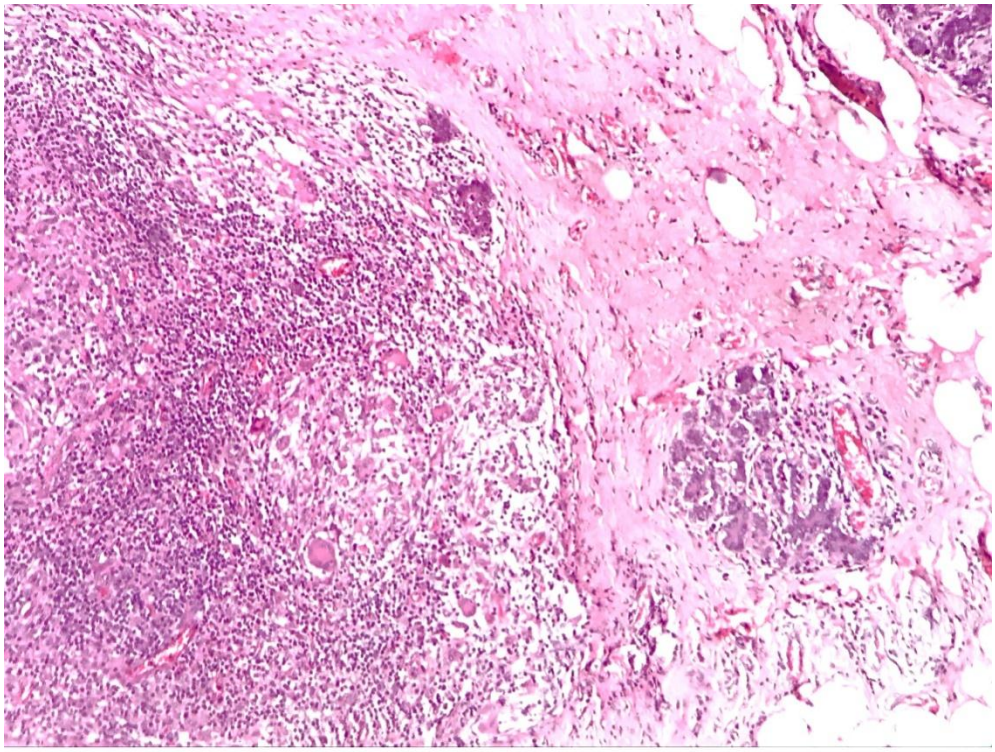


Bone marrow showing epithelioid granuloma (H&E, 100x)

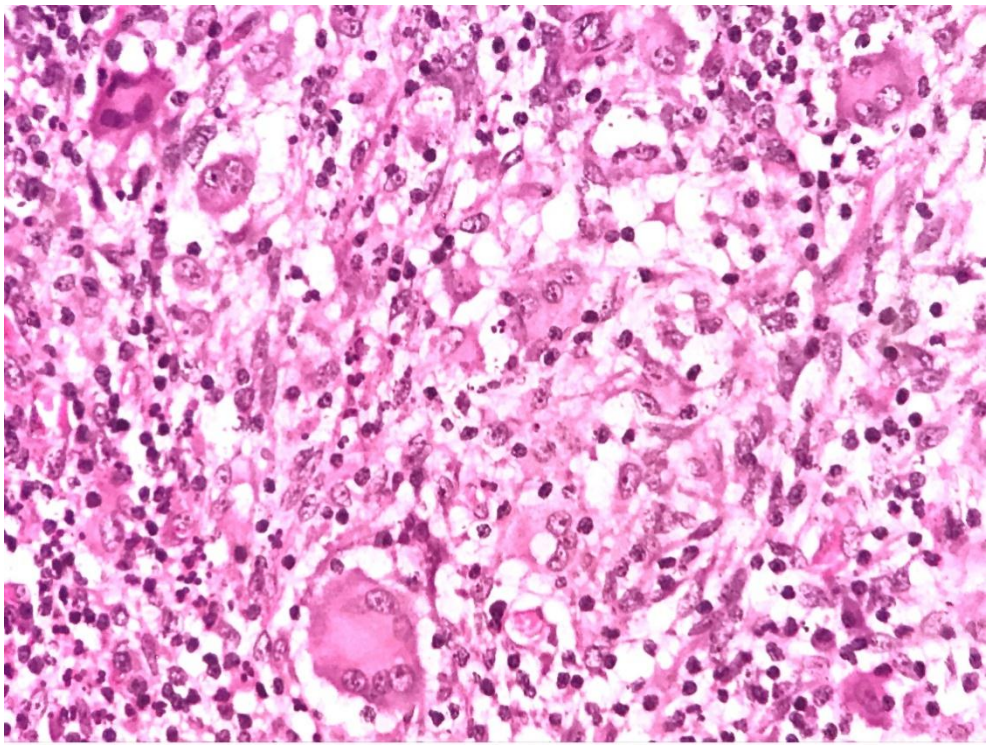


Epithelioid granuloma with Langhans giant cell (H&E, 400x)

(Lesion in Urinary bladder)

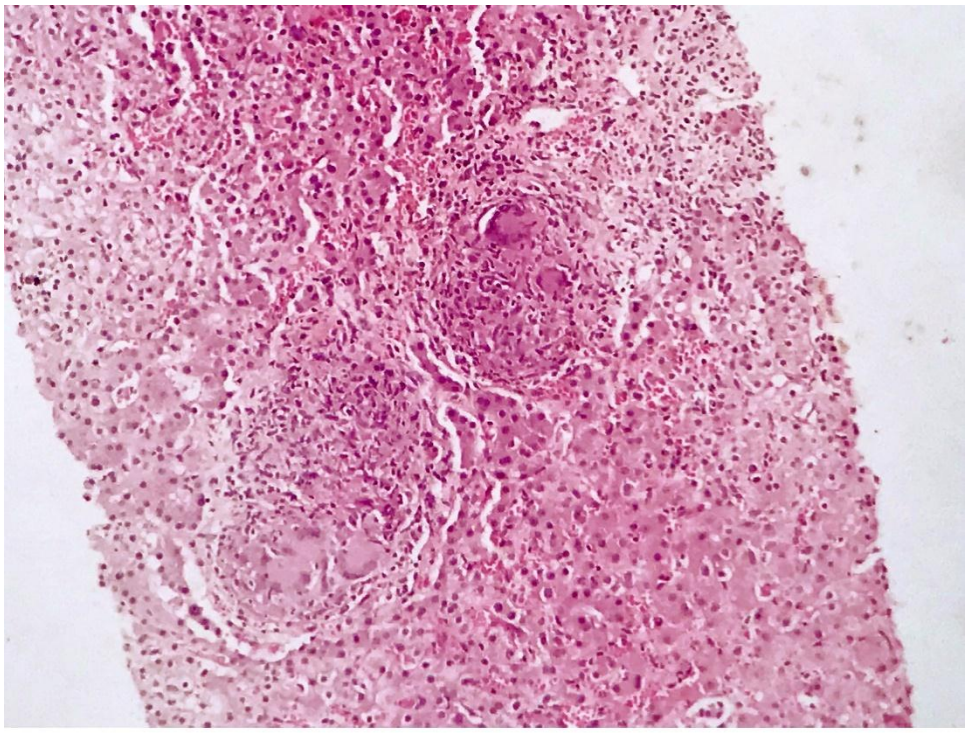


Breast parenchyma showing epithelioid granuloma (H&E, 100x)

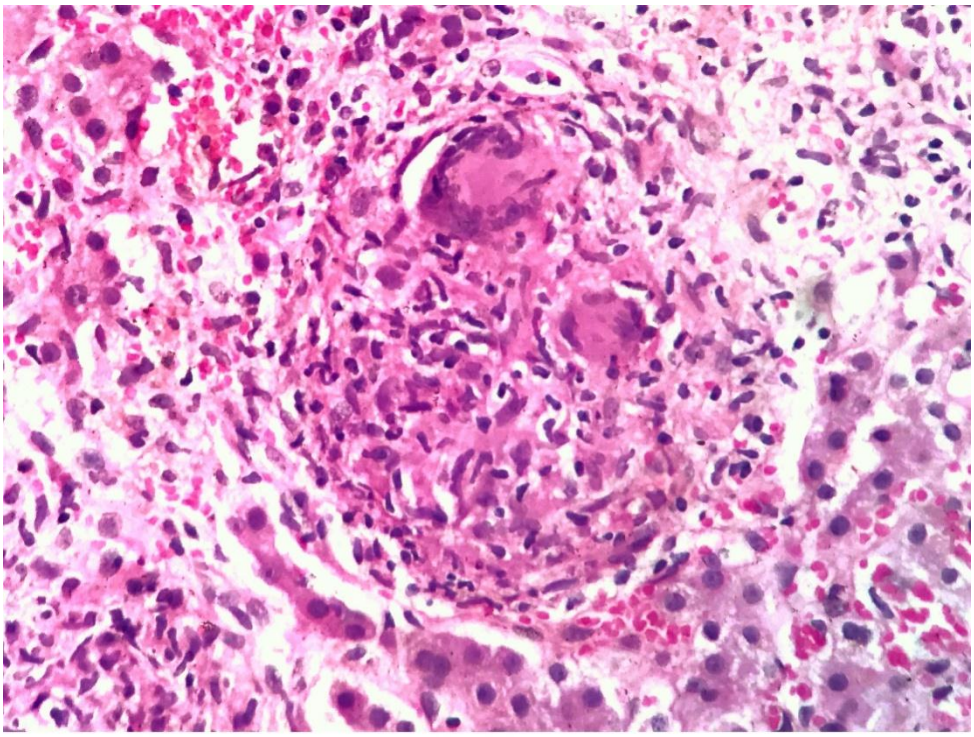


Epithelioid granuloma with Langhans giant cells in breast

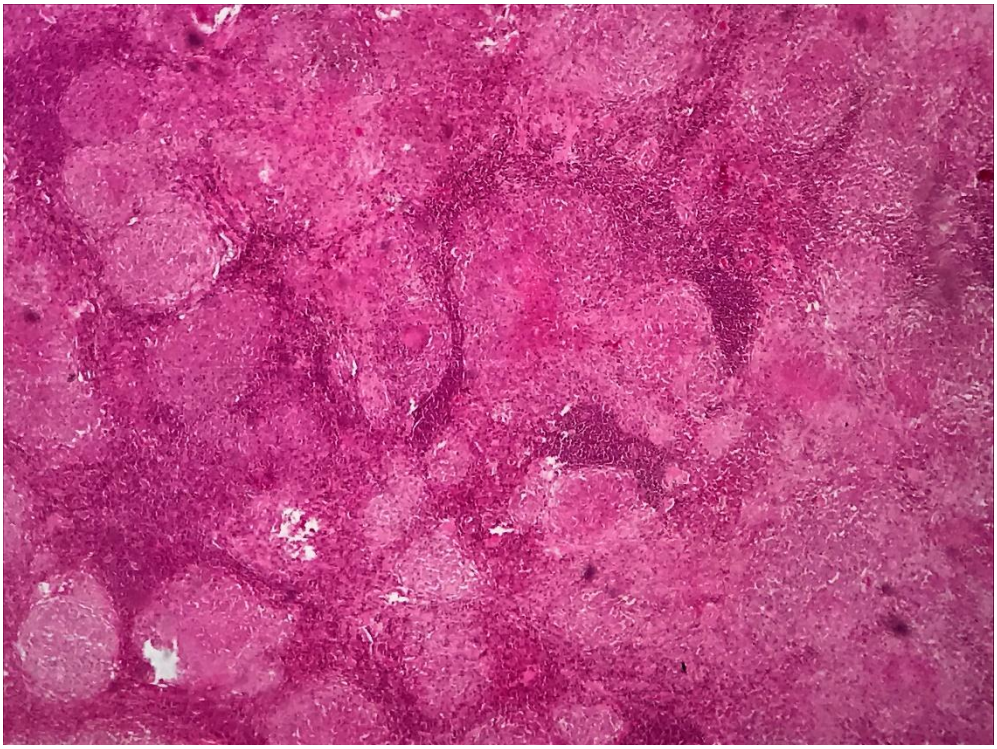
(H&E, 400x)



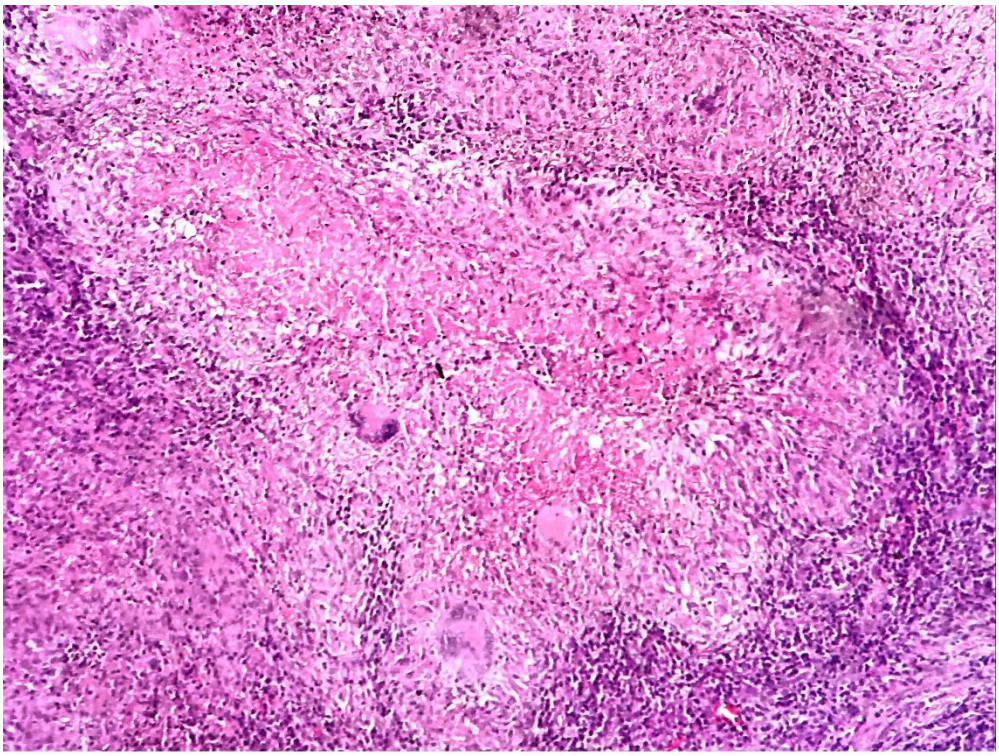
Liver parenchyma with epithelioid granuloma (H&E, 100x)



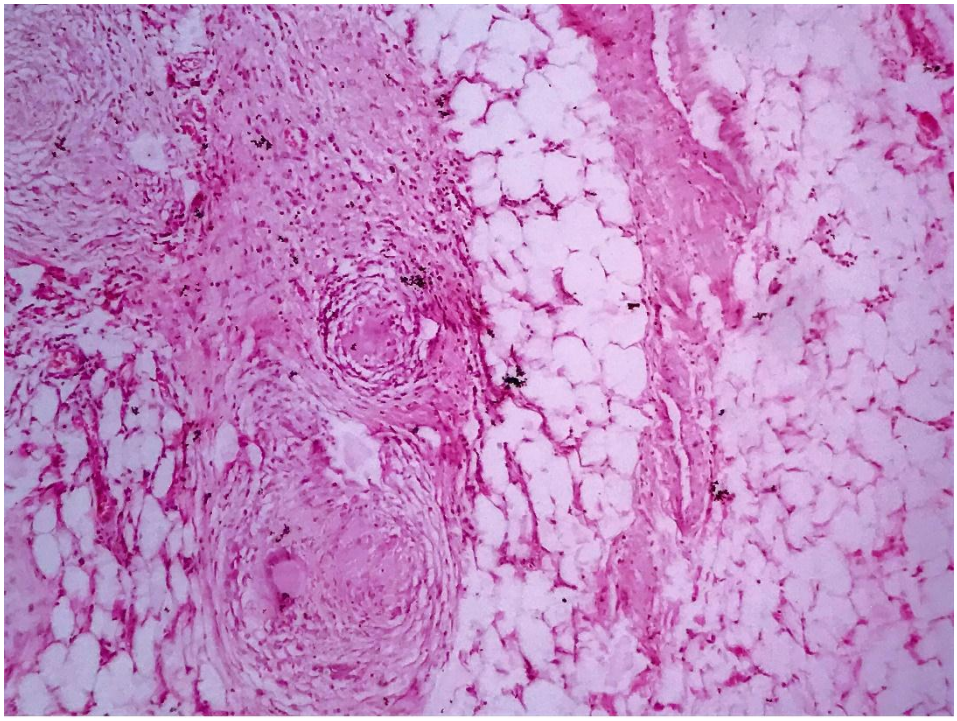
Liver parenchyma with epithelioid granuloma and Langhans giant cells (H&E, 400x)



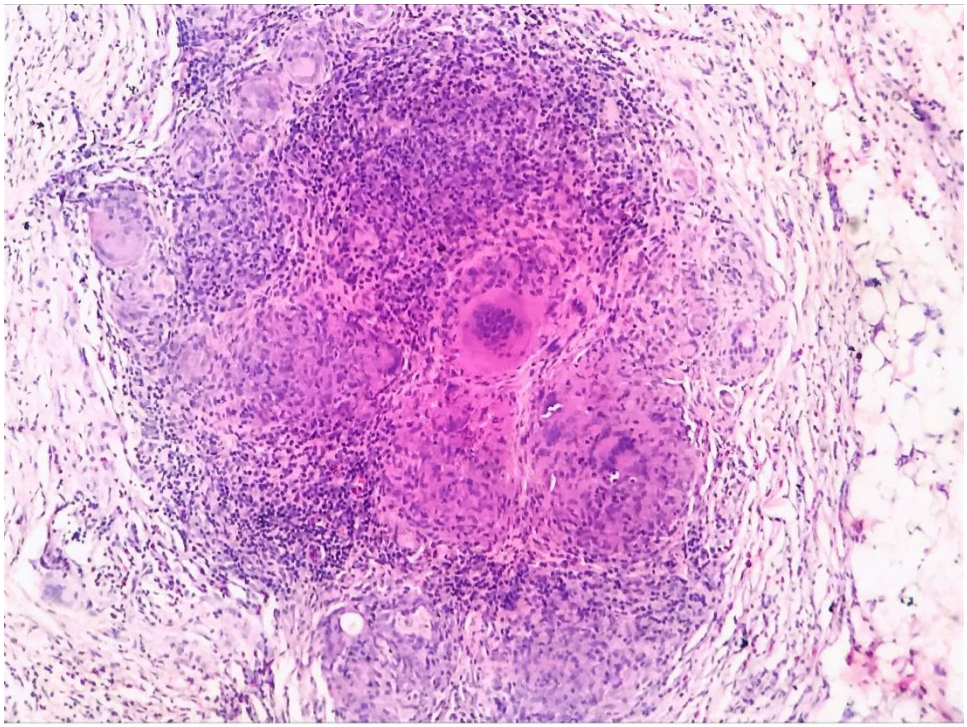
Lymph node parenchyma with multiple epithelioid granuloma and areas of necrosis (H&E, 40x)



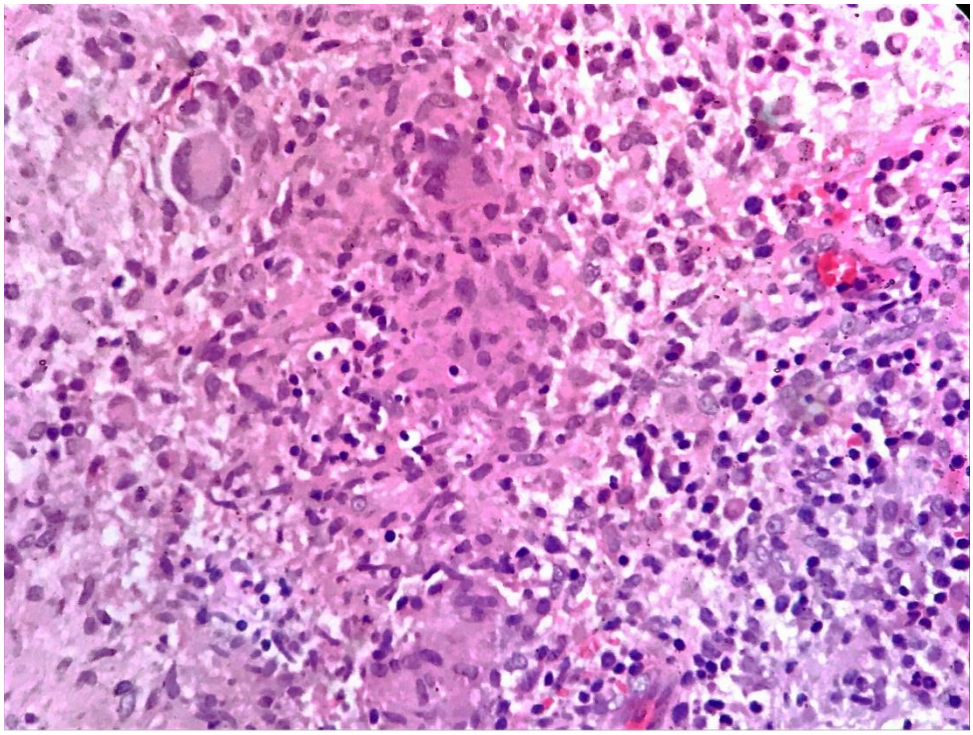
Lymph node parenchyma with epithelioid granuloma, areas of necrosis and Langhans giant cells (H&E, 100x)



Omentum showing epithelioid granuloma with Langhans giant cell (H&E, 100x)

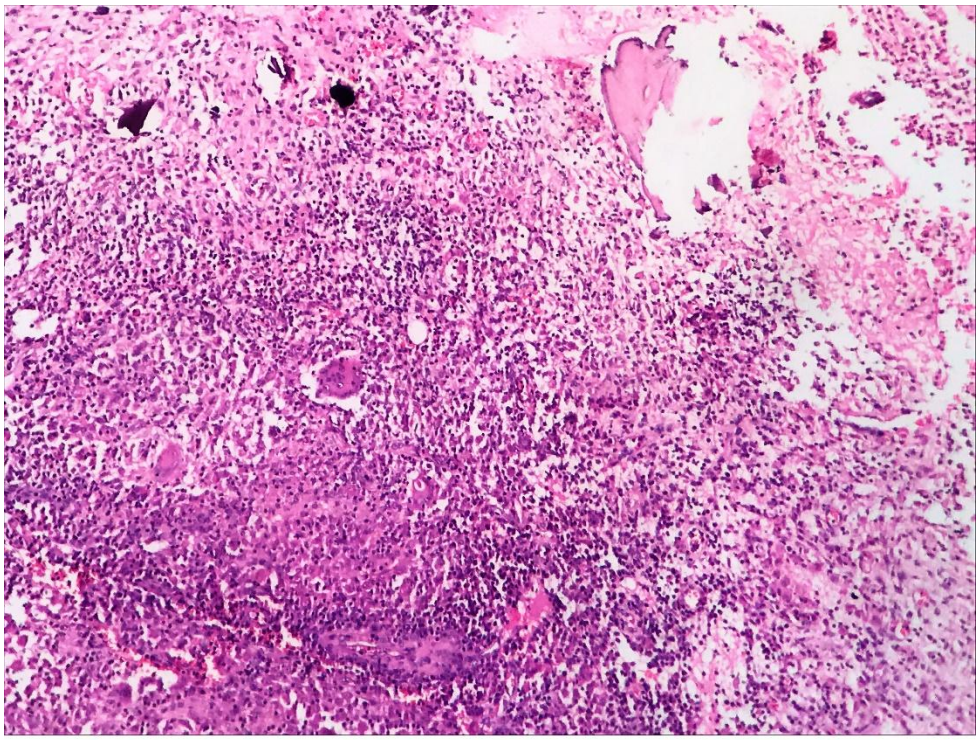


Peritoneum showing epithelioid granuloma with Langhans and foreign body type of giant cells (H&E, 100x)

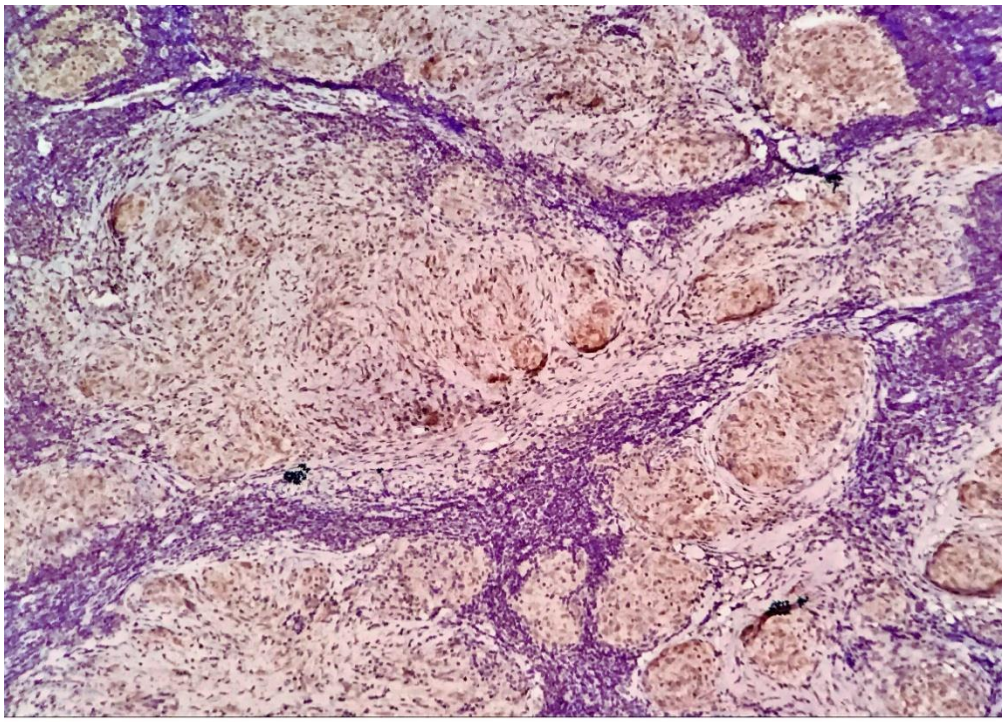


Epithelioid granuloma and Langhans giant cell (H&E, 400x)

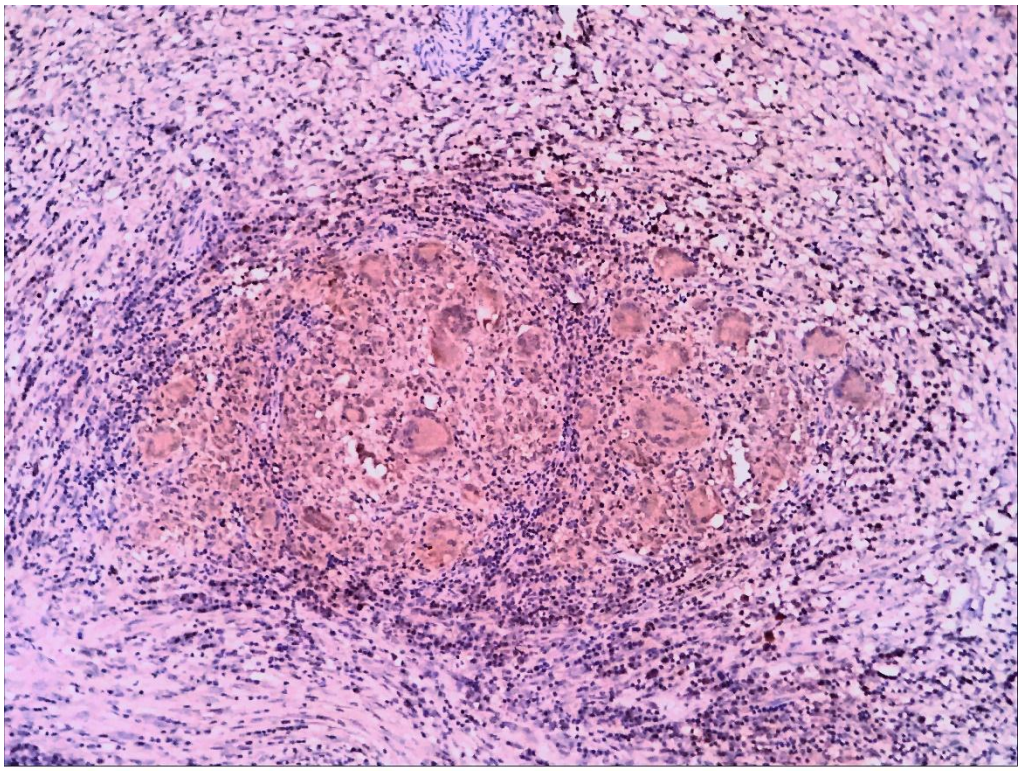
(Lesion in soft tissue)



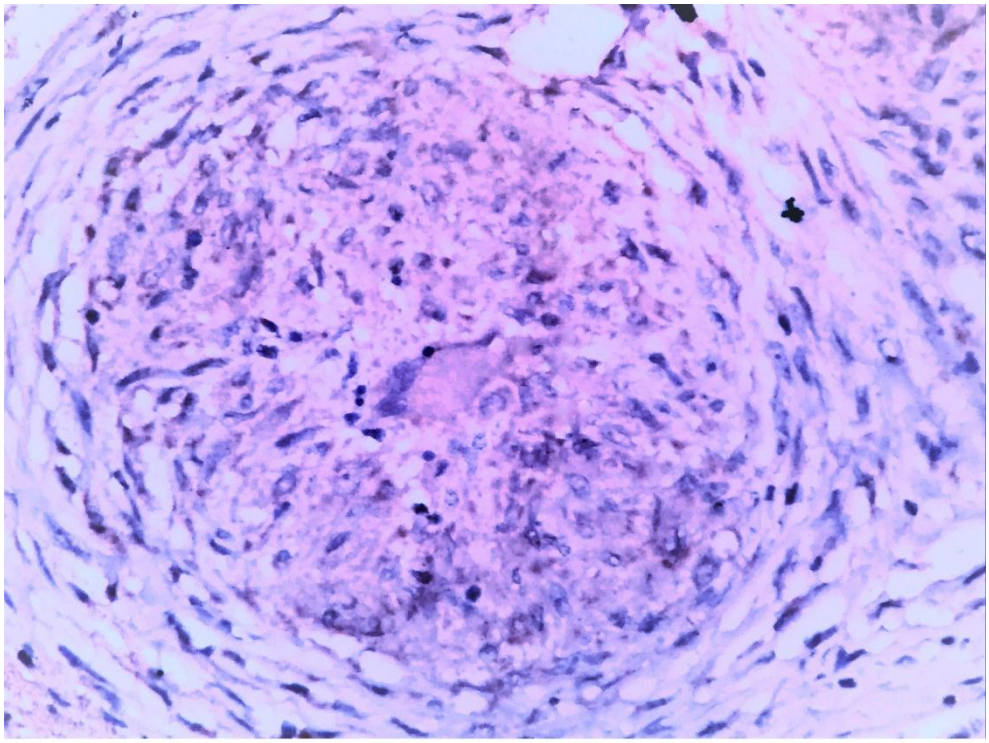
Bony spicules with epithelioid granuloma and Langhans giant cells (H&E, 100x) (Lesion in vertebra)



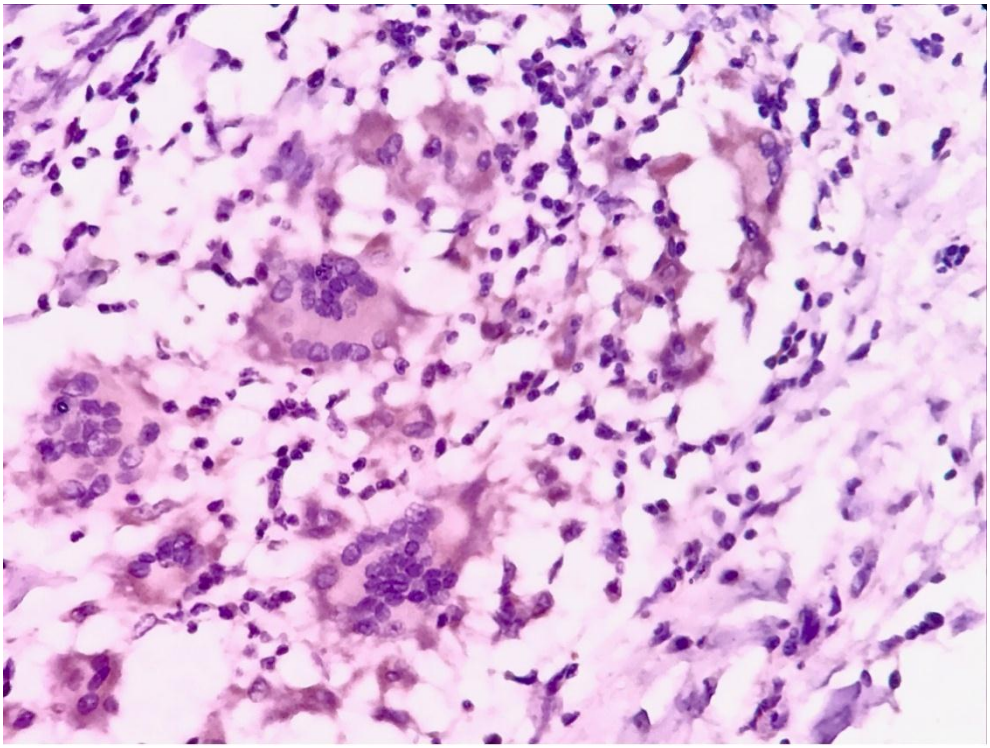
IHC : Positive staining within the granulomas and multi nucleated giant cells (100x)



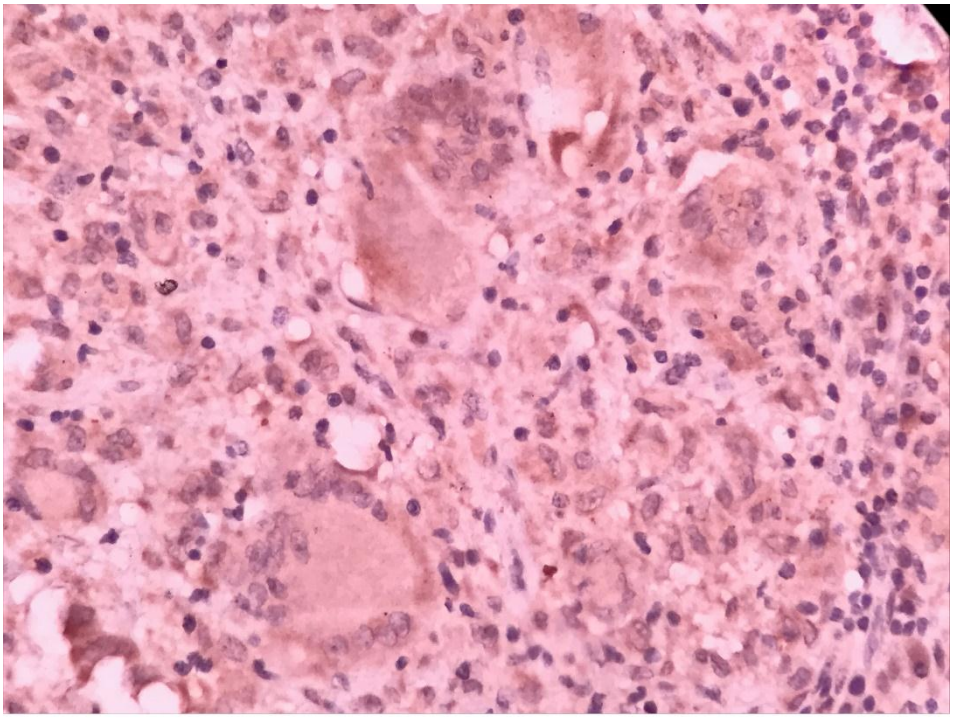
IHC : Positive staining within the granulomas and multi nucleated giant cells (100x)



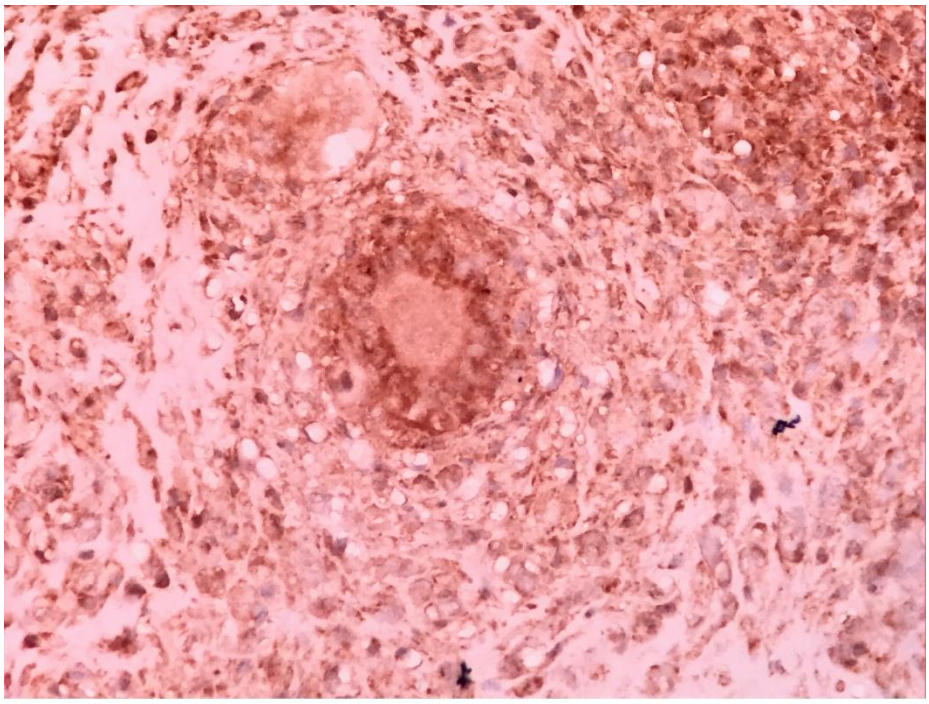
IHC : Absent / Negative staining in the granuloma (400x)



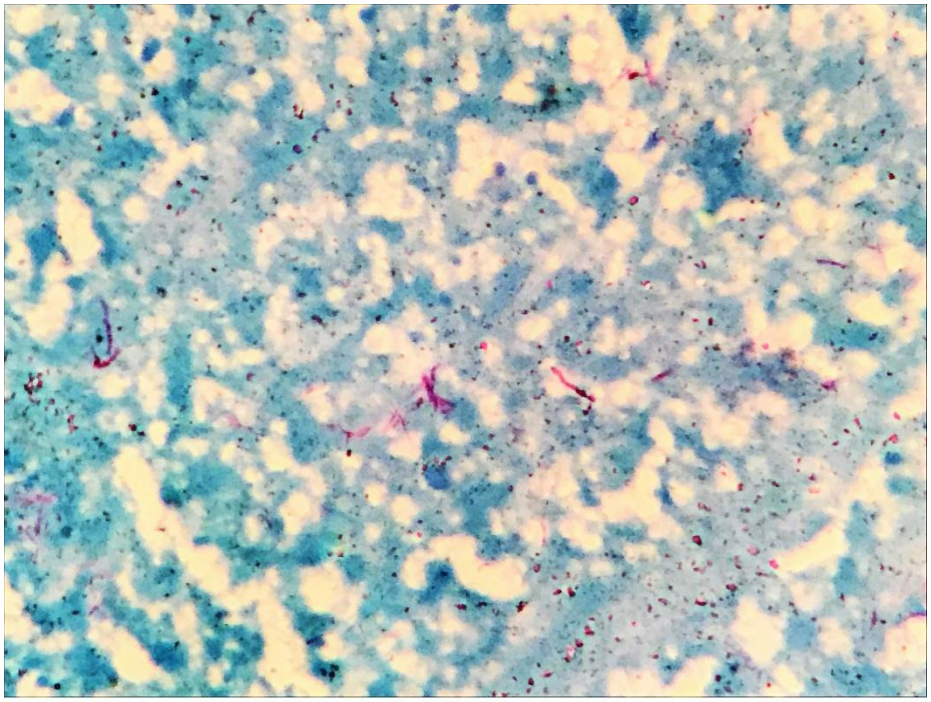
IHC : Mild intensity staining – Score 1 (400x)



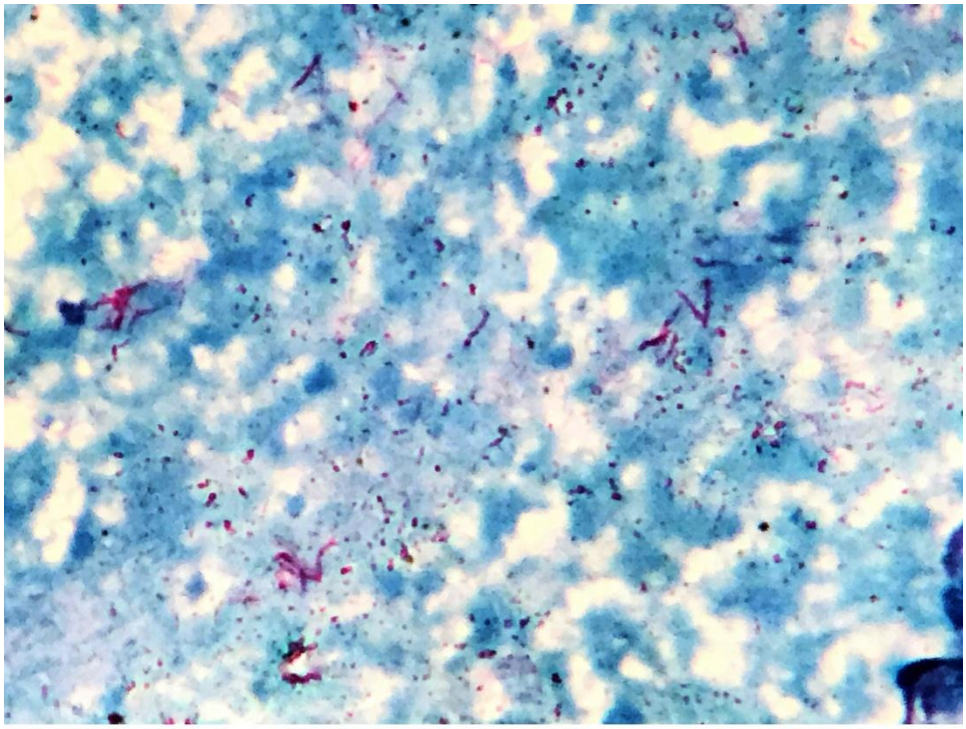
IHC : Moderate intensity – Score 2 – Cytoplasmic granular staining within the epithelioid histiocytes and multinucleated giant cells (400x)



IHC : Strong intensity staining – Score 3 – Cytoplasmic granular staining within the epithelioid histiocytes and multinucleated giant cells (400x)



Ziehl – Neelsen stain showing acid fast tubercle bacilli (Bright Red) in a blue background



Ziehl – Neelsen stain showing acid fast tubercle bacilli (Bright Red) in a blue background (Oil immersion)

DISCUSSION

DISCUSSION

Tuberculosis is the leading cause of death among infectious diseases in India, which incidentally has the highest tuberculosis burden in the world. Early diagnosis and treatment of tuberculosis, with the disease being curable makes it all the more pertinent. The diagnosis of extra pulmonary tuberculosis has always been a stumbling block.

Lymph node tuberculosis accounts for the majority of extra pulmonary tuberculosis. In the study by Khandkar et al, lymph node involvement was seen in 51% cases of extra pulmonary tuberculosis[190].

In the study by Sharma et al, lymph node involvement accounted for 35% of extra pulmonary tuberculosis[191].

In the study by Dandapat et al, 41.5% cases of extra pulmonary tuberculosis were lymph node tuberculosis[191].

In the study by Jha et al, lymph node tuberculosis accounted for 63% of extra pulmonary tuberculosis[191].

In the study by Castro et al, 46% of extra pulmonary tuberculosis cases were lymph node tuberculosis[191].

In the present study, 44% cases of extra pulmonary tuberculosis were lymph node tuberculosis.

TABLE 18: INCIDENCE OF LYMPH NODE TUBERCULOSIS AMONG EXTRAPULMONARY TUBERCULOSIS

STUDY	PERCENTAGE OF LYMPH NODE TUBERCULOSIS AMONG EPTB
Khandkar et al	51%
Sharma et al	35%
Dandapat et al	41.5%
Jha et al	63%
Castro et al	46%
Present Study	44%

There is not much of a difference in the sex distribution in extra pulmonary tuberculosis, with many studies showing a slight male preponderance.

In the study by Shrivatsava et al, the male: female ratio was 1.16:1 (Males: 53.8%, Females: 46.2%) [192].

In the study by Prakasha et al, the male: female ratio was 1.06:1 (Males: 51.5%, Females: 48.5%) [193].

In the study by Bag et al, the male:female ratio was 1.4:1 (Males: 58.4%, Females: 41.6%) [194].

In the present study, the male: female ratio is 1.08:1 (Males-52%, Females-48%)

TABLE 19: SEX DISTRIBTUION OF PATIENTS WITH EXTRA PULMONARY TUBERCULOSIS

STUDY	MALES	FEMALES	MALE: FEMALE RATIO	STUDY SAMPLE
Shrivatsava et al	**53.8%**	**46.2%**	**1.16: 1**	**130**
Prakasha et al	**51.5%**	**48.5%**	**1.06: 1**	**528**
Bag et al	**58.4%**	**41.6%**	**1.4: 1**	**2596**
Present study	**52%**	**48%**	**1.08: 1**	**50**

In the study by Shrivatsava et al, the age group of 21-40 years had the highest proportion of extra pulmonary tuberculosis accounting for 41.53% [192].

In the study by Jamil et al, 49.17% of cases were in the third and fourth decade group [195].

In the study by Gaur et al, 59.2% of cases of extra pulmonary tuberculosis were in the third and fourth decade[196].

In the present study, the major proportion (60%) of cases of extra pulmonary tuberculosis (presumptive diagnosis) were in the third and fourth decade of life.

TABLE 20: COMMON AGE DISTRIBUTION OF PATIENTS WITH EXTRA PULMONARY TUBERCULOSIS

STUDY	PERCENTAGE OF PATIENTS IN THIRD & FOURTH DECADE OF LIFE	STUDY SAMPLE
Shrivatsava et al	**41.53%**	**130**
Jamil et al	**49.17%**	**769**
Gaur et al	**59.2%**	**252**
Present study	**60%**	**50**

Presence of the mycobacterial antigens can be elicited by Immunohistochemistry as shown by several studies which are mentioned later and our present study. Positive cases show coarse or fine granular cytoplasmic staining corresponding to the fragments of TB bacilli or intact bacilli can be seen within the macrophages, fibroblasts, plasma cells, lymphocytes and even endothelial cells outside the granuloma[197].

One of the major limitations of immunohistochemistry technique is the background staining which may cause increased false positive rate, especially if the interpreter is unfamiliar with the technique. Proper antibody dilution, reducing endogenous enzyme interference with peroxide blocking reagents are necessary to reduce the background staining.

Another major limitation of polyclonal mycobacterium tuberculosis antibody is the cross reactivity with non-tuberculous mycobacteria like M. avium, M. phlei, and M. parafortuitum, which can be overcome by using species specific monoclonal antibody.

In the study by Humphrey et al, IHC positivity was 77.7% among a sample of 59 cases[198].

In the study by Barbolini et al, all 23 cases in the study were positive by IHC staining[198].

In the study by Padmavathy et al, immunohistochemistry was positive in 34 out of 50 cases[198].

In the study by Goel at al, the positivity rate for immunohistochemistry was 100%[198].

In the study by Purohit et al, two types of antibodies (anti-MPT and anti-BCG) were used for 120 cases, the positivity rate of each was 80% and 76.6% respectively[197].

In the study by Baba et al, two types of antibodies viz. anti-MPT and anti-BCG were used. The positivity of each antibody was 80% and 48% respectively[198].

In the present study, immunohistochemical analysis revealed positivity in 41 out of 50 cases, which translated to a positivity rate of 82%

TABLE 21: IMMUNOHISTOCHEMISTRY POSITIVITY IN EXTRA PULMONARY TUBERCULOSIS

STUDY	METHOD & ANTIBODY USED	SAMPLE SIZE	IHC POSITIVITY
Humphrey et al	Immunoperoxidase-anti peroxidase; polyclonal anti -BCG	59	77.7%
Barbolini et al	Indirect avidin-biotin complex; monoclonal antibody	23	100%
Padmavathy et al	Indirect immunoperoxidase; polyclonal anti-BCG	50	68%
Goel at al	Streptavidin-biotin; monoclonal antibody	36	100%
Purohit et al	Anti-MPT antibody Anti-BCG antibody	120 120	80% 76.6%
Baba et al	Anti-MPT antibody Anti-BCG antibody	25 25	80% 48%
Present study	Immunoperoxidase-anti peroxidase; polyclonal anti -MTB	50	82%

Ziehl-Neelsen staining which is a fast and cheap procedure, has a variable sensitivity which depends on the number of bacilli in the tissue. The search for the organism is in itself a laborious process, because they are small and few in number, besides the fact that the fragmented bacilli are not stained. Mycolic acid which is essential for the demonstration of Ziehl-Neelsen staining, is soluble in organic solvents which led to the hypothesis that the use of various solvents in the tissue processing during formalin fixation and paraffin embedding affects the integrity of mycolate which results in the frequent negative staining[30].

In a study by Luo D, Ziehl-Neelsen staining was positive in 47 out of 137 cases (34.3%) [199].

In a study by Nassaji et al, Ziehl-Neelsen staining was positive in 59 cases out of 226 cases (26.1%) [200].

In a study on 252 patients with extra pulmonary tuberculosis by Cagatay et al, staining was positive in 17.8% of patients[200].

In another study by Chakravorty et al, the overall positivity of conventional smear microscopy was only 3.9% (three of 76) for detecting extra pulmonary tuberculosis[200].

Salian et al have shown 25% (15/60) positivity by Ziehl-Neelsen staining on histopathological specimens[200].

In a study conducted by Ajantha et al on 182 tissue samples from suspected extra pulmonary tuberculosis cases for testing of Mycobacterium tuberculosis, Ziehl-Neelsen staining was positive only in 3.3% of samples[200].

In a study by Padmavathy et al, all the 50 tissue sections were negative for AFB by Ziehl-Neelsen staining[201].

In the study by Mukherjee et al., Ziehl-Neelsen staining demonstrated the tubercle bacilli in 22 out of 50 cases of lymph node tuberculosis, which translates to a positivity rate of 44% [202].

In the present study, Ziehl-Neelsen staining was positive in 6 out of 50 cases (12%).

TABLE 22: ZIEHL-NEELSEN POSITIVITY IN EXTRA PULMONARY TUBERCULOSIS

STUDY	SAMPLE SIZE	POSITIVITY RATE
Luo D	**137**	**34.3%**
Nassaji et al	**226**	**26.1%**
Cagatay et al	**252**	**17.8%**
Chakravorty et al	**76**	**3.9%**
Salian et al	**60**	**25%**
Ajantha et al	**182**	**3.3%**
Padmavathy et al	**50**	**0%**
Mukherjee et al	**50**	**44%**
Present Study	**50**	**12%**

Literature has shown that studies have been done to compare the results of Ziehl-Neelsen staining and immunohistochemistry.

In the study by Radhakrishnan et al, in a sample size of 10, the IHC positivity was 100% and Ziehl-Neelsen staining positivity was 0%[198].

In the study by Mukherjee et al, IHC positivity was 87% and Ziehl-Neelsen staining positivity was 44% in a sample of 50 cases[198].

In the study by Padmavathy et al, IHC positivity was 68% and Ziehl-Neelsen staining was 0% among 50 cases[198].

In the study by Mustafa et al, with a sample of 55 cases, IHC positivity was 64% and Ziehl-Neelsen staining positivity was 0%[198].

In the present study the IHC positivity was 82% and Ziehl-Neelsen positivity was 12% among 50 cases of extra pulmonary tuberculosis.

Thus, from the present study and various other studies, it has been shown that immunohistochemistry is a better test than Ziehl-Neelsen staining, with the positivity rate of immunohistochemistry ranging from 64% to 100% (82% in the present study) and the positivity rate of Ziehl-Neelsen staining ranging from 0% to 44% (12% in the present study)

As evidenced by our study, the presence of necrosis and prior treatment with anti-tuberculous drugs had no negative impact on the immunohistochemical staining characteristics.

TABLE 23: COMPARISON OF IHC ANALYSIS AND ZIEHL-NEELSEN STAINING

STUDY	METHOD & ANTIBODY USED	SAMPLE SIZE	IHC POSITIVITY	ZIEHL-NEELSEN STAINING POSITIVITY
Radhakrishnan et al	Peroxidase-anti-peroxidase; IgG anti mycobacterial antibody	10	100%	0%
Mukherjee et al	Avidin-biotin complex; polyclonal anti-BCG	50	87%	44%
Mustafa et al	Polyclonal anti-BCG antibody	55	64%	0%
Padmavathy et al	Indirect Immunoperoxidase; Polyclonal anti-BCG	50	68%	0%
Kohli et al	Streptavidin-biotin; polyclonal anti mycobacterial antibody	100	72%	23%
Present Study	Immunoperoxidase-anti peroxidase; polyclonal anti - MTB	50	82%	12%

SUMMARY

SUMMARY

- For the study period of two years from August 2015 to July 2017, a total of 24617 specimens were received at the Institute of Pathology, Madras Medical College & Rajiv Gandhi Government General Hospital for histopathological examination.
- Out of the 24617 cases of histopathological specimens received, 349 cases were granulomatous lesions in extra pulmonary sites.
- Out of the 349 cases, 198 cases were of suspected tuberculous etiology based on the clinical suspicion, presence of necrosis with or without caseation or Langhans giant cells.
- Lymph nodes accounted for the majority of extra pulmonary granulomatous lesions, constituting about 44% of all lesions, followed by Intestine (16%), soft tissues (14%), peritoneum (8%) and vertebra (6%).
- Other rare sites of occurrence included bone marrow, breast, liver, omentum, synovium and urinary bladder which all accounted for 2% each.
- Maximum incidence of the disease was found in the age group of 21-40 years which constituted about 60% followed by the age group of above 40 years which formed about 32%.
- Males accounted for 52% of the cases with females accounting for 48% of cases.

- Out of the 50 cases, only one patient had history of infection with Human Immunodeficiency Virus (HIV), who presented with cervical lymphadenopathy
- Among the 50 cases, 5 patients had previously undergone anti-tuberculous therapy.
- Among the 50 granulomatous lesions, necrosis was present in 24 cases on histopathological examination which constituted about 48% of cases as opposed to 52% of cases without necrosis.
- Among the 50 cases which were subjected to immunohistochemical analysis using polyclonal anti-mycobacterium tuberculosis antibody, 41 cases (82%) were positive for tubercle bacilli.
- Out of the 50 cases which were subjected to Ziehl-Neelsen staining, 6 cases were positive for the bacilli.
- 14 out of 17 cases in whom there was a clinical suspicion of tuberculosis, were positive by immunohistochemical staining, whereas 27 out of the remaining 33 cases were positive by immunohistochemical staining.
- 2 out of 17 cases which had a clinical suspicion of tuberculosis were positive by Ziehl-Neelsen staining, whereas 4 out of the remaining 33 cases were positive by Ziehl-Neelsen staining
- Presence of necrosis had no effect on the expression of immunohistochemistry, with P value more than 0.005

- Prior treatment with anti-tuberculous drugs had no effect on the immunohistochemical expression with P value more than 0.005
- Among the 41 cases which were positive by immunohistochemistry, 22 cases had strong intensity staining (score 3) and 19 cases had moderate staining intensity (score 2). 3 cases which had mild / weak intensity staining (score 1) along with the remaining 6 cases which had no staining were considered as negative.
- The presence of necrosis had no impact on the staining intensity of immunohistochemistry.
- 12% of cases were positive for acid fast bacilli by Ziehl-Neelsen staining, whereas 82% of cases were positive by immunohistochemical staining. The P value was found to be less than 0.001 by McNemar test which is statistically significant which shows that immunohistochemistry is better than Ziehl-Neelsen staining for localization of the tubercle bacilli.

CONCLUSION

CONCLUSION

To conclude, extra pulmonary tuberculosis the commonest presentation of which is lymph node tuberculosis poses a diagnostic challenge, which necessitates the need for a rapid, cost effective yet sensitive test. The presence of necrosis combined with the prior treatment by anti-tuberculous drugs compound the diagnostic difficulties.

With Ziehl-Neelsen staining, the sensitivity varies across a large spectrum from 0% to 45%, making the test less reliable. The sensitivity of the test largely depends on the load of bacilli with at least 10,000 bacilli per ml of specimen required for the stain to demonstrate the bacilli, which is difficult in extra pulmonary tuberculosis due to the less bacillary load. Furthermore, Ziehl-Neelsen stain can only highlight the intact bacilli, not the fragmented bacilli.

With Immunohistochemistry, as evidenced by studies mentioned earlier, the sensitivity has fared way better than Ziehl-Neelsen staining. In our study too, Immunohistochemistry has been shown to be a better test than Ziehl-Neelsen staining and can highlight the intact as well as the fragmented bacilli within the necrotic areas and remain unaffected by treatment.

Immunohistochemistry can be a reasonable if not better alternative to Ziehl-Neelsen staining or as an adjunct in the diagnosis of extra pulmonary tuberculosis. It is a simple, robust, cheap and sensitive test that can be employed in routine pathology laboratories where results can be available in a day, prompting early diagnosis and treatment, as opposed to the culture which

takes weeks to obtain a result or Polymerase Chain Reaction which is quite costly and not available in many of the places in developing countries. Besides aiding in the diagnosis, immunohistochemistry also helps in avoiding the misdiagnosis, preventing the unwarranted empirical use of anti-tuberculous drugs.

RECOMMENDATIONS

RECOMMENDATIONS

1. Further studies with large sample size and employing immunohistochemistry, with Polymerase chain reaction/culture as the gold standard may be necessary to validate the sensitivity of Immunohistochemistry.

2. The use of species specific monoclonal antibody as opposed to the polyclonal antibody helps in distinguishing positivity due to cross reaction with non-tuberculous mycobacteria.

3. Immunohistochemistry can be a useful and cheap alternative in the rapid diagnosis of extra pulmonary tuberculosis.

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ANNEXURES

ANNEXURE 1

Class	Type	Description
0	No TB exposure Not infected	No history of exposure Negative reaction to tuberculin skin test
1	TB exposure No evidence of infection	History of exposure Negative reaction to tuberculin skin test
2	TB infection No disease	Positive reaction to tuberculin skin test Negative bacteriologic studies (if done) No clinical, bacteriologic, or radiographic evidence of TB
3	TB, clinically active	*M. tuberculosis* cultured (if done) Clinical, bacteriologic, or radiographic evidence of current disease
4	TB Not clinically active	History of episode(s) of TB **(or)** Abnormal but stable radiographic findings Positive reaction to the tuberculin skin test Negative bacteriologic studies (if done) **(and)** No clinical or radiographic evidence of current disease
5	TB suspect	Diagnosis pending TB disease should be ruled in or out within 3 months

ANNEXURE 2

SCORING SYSTEM FOR IMMUNOHISTOCHEMICAL MARKER POLYCLONAL ANTI-MYCOBACTERIUM TUBERCULOSIS ANTIBODY (CYTOPLASMIC STAINING)

INTENSITY OF STAINING	SCORE
ABSENT	0
MILD	1
MODERATE	2
STRONG	3

ANNEXURE 3 - INFORMATION SHEET

- We are conducting a study on suspected tuberculous lesions among patients attending Government General Hospital, Chennai and for that your specimen may be valuable to us.
- The purpose of this study is to aid in the early and rapid diagnosis of extra pulmonary tuberculosis with the help of certain special tests and immunohistochemical markers.
- We are selecting certain cases and if your specimen is found eligible, we may be using your specimen to perform extra tests and special studies which in any way do not affect your final report or management.
- The privacy of the patients in the research will be maintained throughout the study. In the event of any publication or presentation resulting from the research, no personally identifiable information will be shared.
- Taking part in this study is voluntary. You are free to decide whether to participate in this study or to withdraw at any time; your decision will not result in any loss of benefits to which you are otherwise entitled.
- The results of the special study may be intimated to you at the end of the study period or during the study if anything is found abnormal which may aid in the management or treatment.

Signature of investigator **Signature of participant**

Date:

ANNEXURE 4 -ஆ

ஆராய்ச்சி தகவல் தாள்

ஆராய்ச்சி தலைப்பு: சந்தேகத்திற்குரிய காச நோய் திசுக்களில் ஜீல் நீல்சன் நிறமியையும் இம்முனோ ஹிஸ்டோகெமிஸ்ட்ரியையும் ஒப்பிட்டுபார்க்கும் ஆய்வு.

ஆய்வாளர் : மரு.கு.வீரராகவன்

நோய்குறியியல்துறை

சென்னை மருத்துவக்கல்லூரி,

சென்னை-600 003.

தங்களது திசு (அறுவை சிகிச்சை செய்யப்பட்டகட்டி) இங்கு பெற்றுக்கொள்ளப்பட்டது.

இராஜிவ்காந்தி அரசு பொது மருத்துவமனைக்கு வரும் நோயாளிகளிடம் இருந்து பெறப்பட்ட சந்தேகத்திற்குரிய காச நோய் திசுக்களை பற்றிய ஒரு ஆராய்ச்சி இங்கு நடைபெற்று வருகிறது.

இந்த திசுக்களில் Mycobaterium tuberculosis என்னும் பாக்டீரியா உள்ளதா என்பதை ஆராய்வதே இந்த ஆய்வின் நோக்கமாகும்.

நீங்களும் இந்த ஆராய்ச்சியில் பங்கேற்க நாங்கள் விரும்புகிறோம். இந்த ஆராய்ச்சியில் உங்களுடைய திசுக்களை வைத்து சில சிறப்புப் பரிசோதனைக்கு உட்படுத்தி தகவல்களை ஆராய்வோம். இதனால் தங்களது நோய்யின் ஆய்வரிக்கையும் அல்லது சிகிச்சையோ பாதிப்புக்குள்ளாகாது என்பதையும் தெரிவித்துக் கொள்கிறேன்.

முடிவுகளை அல்லது கருத்துக்களை வெளியிடும் போதும் அல்லது ஆராய்ச்சியின் போதும் தங்களது பெயரோ அல்லது அடையாளங்களையோ வெளியிட மாட்டோம் என்பதையும் தெரிவித்துக் கொள்கிறோம்.

இந்த ஆராய்ச்சியில் பங்கேற்பது தங்களுடைய விருப்பத்தில் பேரில் தான் இருக்கிறது. மேலும் நீங்கள் எந்நேரமும் இந்த ஆராய்ச்சியில் இருந்து பின்வாங்கலாம் என்பதையும் தெரிவித்துக் கொள்கிறோம்.

இந்த சிறப்புப் பரிசோதனையின் முடிவுகளை ஆராய்ச்சியின் போது அல்லது ஆராய்ச்சியின் முடிவின் போதும் தங்களுக்கு அறிவிப்போம் என்பதையும் தெரிவித்துக் கொள்கிறோம்.

இந்த ஆய்வைப்பற்றிய சந்தேகங்களுக்கு தொடர்பு கொள்ள வேண்டியவர் : மரு.கு.வீரராகவன், செல் : 7299114346

பங்கேற்பாளர் கையொப்பம்..... இடம் : ..... தேதி: .....

பங்கேற்பாளர் பெயர் மற்றும் விலாசம் :

ஆராய்ச்சியாளர் கையொப்பம் ..... இடம் : ..... தேதி:.....

ANNEXURE 5 - INFORMED CONSENT FORM

Title of the study: **A COMPARATIVE STUDY ON ZIEHL NEELSEN STAINING AND IMMUNOHISTOCHEMICAL ANALYSIS IN SUSPECTED TUBERCULOUS LESIONS**

Name of the Participant :

Name of the Principal (Co-Investigator) : Dr. G. Veera Raghavan

Name of the Institution :Madras Medical College

Name and address of the sponsor / agency (ies) (if any) :

Documentation of the informed consent

I _____ have read the information in this form (or it has been read tome). Iwas free to ask any questions and they have been answered. I am over 18 years of age and,exercising myfree power of choice, hereby give my consent to be included as a participant in "**A COMPARATIVE STUDY ON ZIEHL NEELSEN STAINING AND IMMUNOHISTOCHEMICAL ANALYSIS IN SUSPECTED TUBERCULOUS LESIONS".**

1. I have read and understood this consent form and the information provided to me.
2. I have had the consent document explained to me.
3. I have been explained about the nature of the study in which the resected soft tissue tumors will be subjected to immunohistochemistry and histopathological examination.
4. I have been explained about my rights and responsibilities by the investigator. I have the right to withdraw from the study at any time.
5. I have been informed the investigator of all the treatments I am taking or have taken in the past _____ months including any native (alternative) treatment.
6. I hereby give permission to the investigators to release the information obtained from me as result of participation in this study to the sponsors, regulatory authorities, Govt. agencies, and IEC. I understand that they are publicly presented.
7. I have understood that my identity will be kept confidential if my data are publicly presented
8. I have had my questions answered to my satisfaction.
9. I have decided to be in the research study.

I am aware that if I have any question during this study, I should contact the investigator. By signing this consent form I attest that the information given in this document has been clearly explained to me and understood by me, I will be given a copy of this consent document.

For adult participants:

Name and signature / thumb impression of the participant (or legal representative if participant incompetent)

Name _____ Signature_____ Date_____

Name and Signature of impartial witness (required for illiterate patients):

Name _____ Signature_____ Date_____

Address and contact number of the impartial witness:

Name and Signature of the investigator or his representative obtaining consent:

Name _____ Signature_____ Date_____

ANNEXURE 6

ஆராய்ச்சி ஒப்புதல் கடிதம்

ஆராய்ச்சி தலைப்பு: சந்தேகத்திற்குரிய காச நோய் திசுக்களில் ஜீல் நீல்சன் நிறமியையும் இம்முனோ ஹிஸ்டோகெமிஸ்ட்ரியையும் ஒப்பிட்டுபார்க்கும் ஆய்வு.

சென்னை மருத்துவக் கல்லூரி நோய் குறியியல் துறையில் பயிலும் முதுகலை மருத்துவர் கு.வீரராகவன் அவர்கள் மேற்கொள்ளும் இந்த ஆய்வில் பங்குகொள்ள ..... ஆகிய நான் முழுமனதுடன் சம்மதிக்கிறேன்.

எனக்கு விளக்கப்பட்ட விஷயங்களை நான் புரிந்துக் கொண்டு எனது சம்மதத்தை தெரிவிக்கின்றேன்.

இந்த ஆராய்ச்சியில் பிறரின் நிர்பந்தமின்றி எனது சொந்த விருப்பத்தின் பேரில் தான் பங்கு பெறுகிறேன் மற்றும் நான் இந்த ஆராய்ச்சியில் இருந்து எந்நேரமும் பின் வாங்கலாம் என்பதையும் இதனால் எந்த பாதிப்பும் ஏற்படாது என்பதையும் நான் புரிந்துக் கொண்டேன்.

நான் காசநோய் குறித்த இந்த ஆராய்ச்சி விவரங்களை கொண்ட தகவல் தாளை பெற்றுக் கொண்டேன்.

நான் என்னுடைய சுயநினைவுடன் மற்றும் முழு சுதந்திரத்துடன் இந்த மருத்துவ ஆராய்ச்சியில் என்னை சேர்த்துக் கொள்ள சம்மதிக்கிறேன்.

எனக்கு அறுவை சிகிச்சை செய்யப்பட்டு நோய் குறியியல் துறையில் சதை பரிசோதனை பயன்பட்ட மெழுகுக்கட்டிகளை வைத்து ஆராய்ச்சி மற்றும் சிறப்பு பரிசோதனை செய்து கொள்ள சம்மதம் தெரிவிக்கிறேன்.

பங்கேற்பாளர் கையொப்பம்..... இடம் : ..... தேதி: .....

பங்கேற்பாளர் பெயர் மற்றும் விலாசம் :

ஆராய்ச்சியாளர் கையொப்பம் ..... இடம் : ..... தேதி:.....

MASTER CHART

MASTER CHART

S. No	Biopsy No.	Age	Sex	HIV Status	Anti tuberculous therapy History	Clinical Suspicion of TB	Site	Necrosis	Immuno Histo chemistry	IHC Staining Intensity Score	Ziehl Neelsen Stain
1	7/16	44	M	Negative	Untreated	No	Ileum	Present	Positive	2	Negative
2	513/16	26	F	Negative	Untreated	No	Breast	Absent	Positive	3	Negative
3	540/16	24	F	Negative	Untreated	No	Lymph node	Present	Positive	2	Negative
4	1022/16	34	M	Negative	Untreated	Yes	Omentum	Absent	Positive	3	Positive
5	1434/16	36	M	Negative	Untreated	Yes	Lymph node	Present	Positive	3	Negative
6	1767/16	52	M	Negative	Untreated	No	Lymph node	Present	Positive	3	Negative
7	5814/16	23	M	Negative	Untreated	No	Bone marrow	Absent	Positive	2	Negative
8	5939/16	23	M	Negative	Untreated	No	Liver	Absent	Negative	0	Negative
9	6025/16	20	F	Negative	Untreated	Yes	Vertebra	Absent	Positive	3	Negative
10	7028/16	34	M	Negative	Untreated	No	Ileocecal Junction	Absent	Positive	3	Negative
11	7033/16	55	M	Negative	Untreated	No	Ileocecal Junction	Absent	Positive	3	Positive
12	7581/16	50	M	Negative	Untreated	Yes	Peritoneum	Absent	Positive	3	Negative
13	8015/16	19	M	Negative	Untreated	No	Soft Tissue	Absent	Positive	2	Negative
14	8109/16	55	M	Negative	Untreated	No	Lymph node	Present	Positive	3	Negative
15	8761/16	26	F	Negative	Treated	Yes	Vertebra	Present	Positive	3	Negative
16	8857/16	46	M	Negative	Untreated	No	Ileocecal Junction	Absent	Positive	3	Negative
17	9116/16	53	M	Negative	Untreated	No	Soft Tissue	Present	Positive	2	Negative
18	9235/16	60	M	Negative	Untreated	No	Peritoneum	Present	Positive	3	Negative
19	11537/16	25	M	Negative	Untreated	No	Lymph node	Present	Positive	3	Positive
20	11950/16	62	M	Negative	Untreated	No	Jejunum	Absent	Positive	2	Negative
21	12106/16	23	F	Negative	Untreated	No	Urinary Bladder	Absent	Negative	0	Negative
22	8/17	52	F	Negative	Untreated	No	Soft Tissue	Absent	Negative	0	Negative
23	257/17	28	F	Negative	Untreated	Yes	Lymph node	Present	Positive	3	Negative
24	295/17	20	M	Negative	Untreated	Yes	Lymph node	Present	Negative	1	Negative
25	327/17	26	M	Negative	Untreated	No	Lymph node	Present	Positive	3	Positive
26	358/17	34	M	Negative	Treated	Yes	Lymph node	Absent	Positive	3	Positive
27	452/17	22	F	Negative	Treated	Yes	Lymph node	Present	Positive	2	Negative
28	772/17	27	F	Negative	Untreated	No	Abscess Wall	Present	Positive	2	Negative
29	843/17	30	F	Negative	Untreated	Yes	Lymph node	Present	Positive	2	Negative
30	1162/17	23	M	Negative	Untreated	No	Lymph node	Present	Positive	3	Negative
31	1297/17	42	F	Negative	Treated	Yes	Soft Tissue	Absent	Positive	2	Negative
32	1507/17	36	F	Negative	Untreated	Yes	Lymph node	Present	Positive	3	Negative
33	1655/17	24	M	Negative	Untreated	No	Synovium	Present	Positive	3	Negative
34	1725/17	50	M	Positive	Untreated	No	Lymph node	Present	Positive	3	Negative
35	1870/17	50	M	Negative	Untreated	No	Ileum	Absent	Negative	1	Negative
36	1925/17	32	F	Negative	Untreated	Yes	Lymph node	Present	Positive	2	Negative
37	2159/17	29	M	Negative	Untreated	No	Lymph node	Present	Positive	2	Negative
38	2290/17	59	M	Negative	Untreated	No	Ileum	Absent	Positive	2	Negative
39	2414/17	30	F	Negative	Treated	Yes	Lymph node	Absent	Positive	2	Negative
40	2634/17	54	F	Negative	Untreated	Yes	Lymph node	Absent	Negative	1	Negative
41	2644/17	23	F	Negative	Untreated	No	Soft Tissue	Absent	Positive	2	Negative
42	2945/17	26	F	Negative	Untreated	No	Soft Tissue	Absent	Positive	3	Positive
43	3120/17	45	F	Negative	Untreated	No	Vertebra	Absent	Negative	0	Negative
44	3421/17	23	M	Negative	Untreated	No	Ileum	Absent	Negative	0	Negative
45	3660/17	16	F	Negative	Untreated	No	Lymph node	Absent	Positive	2	Negative
46	3678/17	25	M	Negative	Untreated	No	Lymph node	Present	Positive	2	Negative
47	3953/17	23	F	Negative	Untreated	Yes	Peritoneum	Absent	Negative	0	Negative
48	4083/17	36	F	Negative	Untreated	No	Lymph node	Present	Positive	3	Negative
49	4148/17	30	F	Negative	Untreated	Yes	Peritoneum	Absent	Positive	2	Negative
50	4222/17	23	F	Negative	Untreated	No	Lymph node	Present	Positive	2	Negative