1. Introduction

Tuberculosis (TB) is a disease caused by *Mycobacterium Tuberculosis* (MTB). TB remains a major global health problem; it ranks as the second leading cause of death from an infectious disease worldwide (Rodriguez and Castaneda 2012). In 2013, an estimate of 9.0 million people developed TB and 1.5 million died from the disease (1.1 million among HIV-negative people and 0.4 million among HIV-positive people). Though most TB cases and deaths occur among men, the burden of disease among women is also high. In 2013, there were an estimate of 3.3 million cases and 510 000 TB deaths among women, as well as an estimate of 550 000 cases and 80 000 deaths among children (WHO, Global tuberculosis report, 2014).

The largest number of new TB cases occurred in the South-East Asia and Western Pacific Regions, accounting for 56% of new cases globally. However, Africa carried the greatest proportion of new cases per population with 280 cases per 100 000 population (WHO, Tuberculosis factsheet, 2014). The situation in Sudan is even grimmer. In 2013 the country has incidence, prevalence and Mortality of 192, 115 and 28 cases (per 100 000 population) respectively (WHO, Tuberculosis country profiles, 2014).

The common primary route of TB infection is lung and called pulmonary tuberculosis (PTB), from which the organism can spread to many secondary sites including lymph node (LN), bone, joint, and cause extra pulmonary tuberculosis (EPTB) (Jensen, *et al*. 2005).However, in Sudan, approximately 74% of TB is pulmonary and the rest is extra pulmonary, but this data can vary from country to another (Tajeldin and Abdel Aziem, 2012).

The primary symptoms of TB infection is chronic productive cough with fever, loss of weight, loss of energy, poor appetite, and night sweats (Robert and Serafino, 2013).

The diagnosis of PTB relies on clinical examination, radiology (commonly chest X-rays), tuberculin skin test, blood tests, as well as microscopic examination and microbiological culture of sputum and body fluids (Hopewell, *et al.* 2006).For EPTB, the most suitable method is the use of biopsy, which should be subjected to Ziehl-Neelsen stain (ZN) and histopathology. Unfortunately, both methods have several limitations (Bayazıt, *et al.* 2004). However, availability of a rapid, sensitive, specific and reliable diagnosis is an essential element in the management of EPTB.

Recently, many complementary diagnostic tools have increasingly been developed for diagnosis of EPTB (Park, *et al.* 2003). MTB IS6110 oligonucleotides is an attractive target for polymerase chain reaction (PCR) amplification, and has been tested in intestinal TB and Crohn's disease (Jin, *et al.* 2010). The 38- kilo Dalton (KD) protein is one of the most important antigens of MTB, which is responsible for binding of carbohydrate to the protein (Morten and Harald, 1992). This protein recently used to produce Monoclonal anti 38-KD for immunohistochemical (IHC) assay.

1.1. Rationale

The most frequent method used for diagnosis of EPTB is the conventional histopathology applying hematoxylin and eosin H and E, in which the consequent diagnosis is based on histopathological evidences. However, these evidences may be missed for other conditions. Therefore, they usually confirmed by ZN stain which has a very low sensitivity which necessiate the need for alternative more specific methods. Therefore, the present study try

to evaluate the diagnostic utility of PCR, IHC and ZN stain in detection of MTB in histological sections.

1.2. Research objectives

General objective:

To evaluate the diagnostic utility of PCR, IHC and ZN stain in detection of MTB in histological sections.

Specific objectives:

To evaluate the diagnostic utility of IHC in detection of MTB in histological sections using anti 38-KD antibody with application of PCR as gold standard.

To assess the diagnostic utility of ZN stain in histopathological sections using PCR as gold standard.

2. Review of Literature

2.1. Tuberculosis

Tuberculosis is a disease caused by *Mycobacterium Tuberculosis* Species, which is belong to the Kingdom *Bacteria*, Phylum *Actinobacteria*, Class *Actinobacteria*, Subclass *Actinobacteridae*, Order *Actinomycetales*, Suborder *Corynebacterineae* Family *Mycobacteriaceae*, Genus *Mycobacterium* (Daniel, 2006).

The genus *Mycobacterium* is non-motile and non-sporulated rods. They are grouped in the supra-generic rank of *actinomycetes* that, unusually, have a high content (61-71 %) of guanine plus cytosine in the genomic deoxyribonucleic acid (DNA), and high lipid content in the wall, probably the highest among all bacteria. These quirky lipids may act as carbon and energy reserves, it also involved in the structure and function of membranes and membranous organelles within the cell (Niederweis, 2003).The waxy coat confers the distinctive characteristics of the genus MTB acid fastness, hydrophobicity, resistance to injury. It probably also contribute to the slow growth rate of MTB by restricting the uptake of nutrients (Mostowy, *et al.* 2005).Most of the genus *Mycobacterium*live and replicate freely innatural environmental system. Only a few of thembecame successful pathogens of higher vertebrates. The MTB species that cannot replicate in the environment are *Mycobacterium leprae, Mycobacteriumlepraemurium, Mycobacterium avium* subspecies*, Paratuberculosis,* and themembers of the *Mycobacterium tuberculosis* complex (Niemann, *et al.* 2004).

M. tuberculosis complex are generically called the tuberclebacillus, *M. tuberculosis,* and the regional variants or subtypes*M. africanum* and *M. canettii* are primarily pathogenic in humans, while *M. bovis* and *M. microti*

are the causative agents of TB in animals, and can be transmitted to humans. Some particular strains like *M. caprae* and *M.pinnipedi* isolated from goats and seals are identified as *M. bovis* subspecies (Sola, *et al.* 2003; Mostowy, *et al.* 2005). However, the close affiliation among the members of the complex is endorsed by high genomic DNA similarity (Niemann, *et al.* 2004).

In smears stained with carbol fuchsine or auramine and examined under light microscope, the tubercle bacilli typically appear as straight or slightly curved rods. The size and shape of bacilli may vary from short cocco bacilli to long rods according to growth conditions and age of the culture (Soolingen, *et al.* 1997). The dimensions of the bacilli vary from 1-10 μm in length (usually 3-5 μm), and 0.2-0.6 μm in width. However, MTB is rarely pleomorphic, it does not elongate into filaments, and does not branch in chains (Chauhan, *et al.* 2006).

The intracellular bacilli described as being significantly elongated compared to broth-grown bacilli and remarkably, to display bud-like structures (Chauhan, *et al.* 2006). In tissue, the bacilli are more numerous within the phagocytic cells, and become sparser and unevenly colored, when the disease was controlled, this due to partial loss of the internal contents (Garton, *et al.* 2002, Cimino, *et al.* 2006). With electron microscopy, some inner dense granules of bacilli can be identified. These granules act as energy store in the cell and site of oxidation-reduction reactions (Brennan and Draper 1994).

2.1.1. MTB characteristic and environmental properties

*Cell structure:*The most important cell structure of the bacilli is the cell envelope (Draper and Daffe, 2005; Kremer and Besra, 2005). The envelope is composed of the cytoplasmic membrane, a cell wall, and an outer capsule.

The cytoplasmic membrane is reach with lipopolysaccharides, and it contains proteins act as sensors that measuring the concentration of molecules in the environment, as well as carriers of nutrients and ions (Mahapatra, *et al*. 2005). The cell wall is constituted by an inner peptidoglycan layer, and outer layer which consist of free lipids. The cell wall is responsible for the shape-forming property and the structural integrity of the bacterium (Soolingen, *et al*. 1997).There are also certain proteins called porins forming hydrophilic channels that permit the passive passage of aqueous solutes through the mycolic acid layer (Smith, 2003; Riley, 2006).

2.1.1.1. Acid fastness

The expression "acid-fastness" describes the resistance of certain microorganisms to decolorization with acid-alcohol solutions after staining with arylmethane dyes such as carbol fuchsin. The property depends on the role of cell wall lipid in trapping the dyes. This feature is of greatest practical importance in identifying the MTB, particularly in pathological specimens (Indrigo, *et al.* 2002). Indeed, there is a parallelism between the increasing degree of acid fastness displayed by MTB, and the increasing length of mycolic acid chains in their walls. This correspondence suggests that the chemical binding of the dye to these molecules might be a determinant for acid fastness, this acid fastness in aqueous solution retain for a long time, even after heating. However, the acid fastness property of the MTB is obliterated by cell trauma or autolysis and infection by specific mycobacteriophages or treatment with antibiotics targeting cell wall synthesis, such as isoniazid (Mohamad, *et al*. 2004).

2.1.1.2. Cord formation

The cord formation characteristics of the MTB have been attributed to the trehalose 6, 6'-dimycolate. This compound, also known as cord factor, it generate hydrophobic interaction and aggregate in elongated structures similar to cords, and it responsible from all biological activities related to pathogenicity, toxicity, and protection against the host response ((Behling, *et al*. 1993; Indrigo , *et al.* 2002).

In general, fresh virulent MTB produce rough textured colonies on solid media, expanded gummy veils on the liquid media and serpentines on microscopic smears. In contrast, non-virulent *Mycobacteria* usually develop smooth colonies on solid media, form discrete mats in liquid media and distribute randomly in loose aggregates when smeared. The recognition of these colony formation provides a reliable distinction of MTBfrom other mycobacteria in cultured specimens and even in sputum smears (Palomino, *et al.* 2007).

2.1.1.3. Permeability barriers

The tightly packed mycolic acids provide the bacillus with an efficient protection and an exceptional impermeability. In addition to the capsule, an even thicker layer of carbohydrate and protein outside the lipid layer which obstructs the diffusion of large molecules, such as enzymes, and protects the lipid layer itself. (Niederweis, 2003; Mailaender, *et al*. 2004).

2.1.1.4. Nutritional and environmental requirements for growth

MTB is prototrophic and heterotrophic (Neyrolles, *et al*. 2006)**,** can grow in salt solutions using glycerol as a carbon source, ammonium ions and asparagine as nitrogen sources. MTB also is able to metabolize glycerol into pyruvate (Wayne and Sramed 1994). However, trace elements found by the microorganism in the water, inorganic ions, small molecules,

macromolecules, and iron in form of insoluble ferric salts have either a structural or a functional role in the cell. Also exochelins and mycobactins are the major siderophores hydrophobic compounds located within the cell wall to introduce the iron into the cytoplasm. A deficiency in these elements frequently reduces the virulence of bacterial pathogens (Voss, *et al*. 2000).

In nature, the MTB grows most successfully in tissues with high oxygen tension, such as the lungs. Carbon dioxide is essential for MTB growth, and may be taken from the atmosphere. The temperature and hydrogen ion concentration ranges, in which the bacillus is able to multiply, are relatively narrow (Voss, *et al.* 2000).

2.1.1.5. Resistance to physical and chemical challenges

MTB survives to some extent in the acid or alkaline microenvironment because of its interaction with the defensive mechanism of the host, as well as the acid contents of the stomach (Palomino, *et al.* 2007).The microorganism also withstands at very low temperatures. Its viability may be increasingly preserved for a long term between 2-4°C to -70°C. When ultra-frozen, the viability of the bacilli remains almost intact as well as the taxonomic, serologic, immunologic, and pathogenic properties. After thawing, they may require re-adaptation to recover full metabolic activity (Kim, 1979). On the other hand, the bacilli are very sensitive to heat, sunlight and ultraviolet (UV) irradiation (Huber, *et al.* 1970; Collins, 1971). Moreover, the bacilli can tolerates low oxygen tension as demonstrated in undisturbed liquid culture media where the self-generated microaerophilic sediment contains non-dividing, yet viable, bacilli. The bacilli may survive for many years in this condition but need a minimal concentration of oxygen to induce the switch into a fermentative metabolism (Wayne and Sramed, 1994). However, the tight structure of the cell wall of the tubercle bacillus is

undoubtedly the shield that preserves the position and function of the metabolic and replicating machinery of MTB (Palomino, *et al.* 2007).

2.1.2. Epidemiology of Tuberculosis

More than two billion people (about one-third of the world population) are estimated to be infected with *M. tuberculosis*(Lonnroth and Raviglione, 2008). The global incidence of tuberculosis peaked around 2003 and appears to be declining slowly (WHO, Global Tuberculosis Report, 2013).

In 2013, the global burden of TB in the World is estimated in 9.0 million incident cases of TB (range, 8.3 million–9.0 million), for rates ranging 123.7-133.9 cases per 100,000 population. However, the incidence can be very different among the WHO Regions in the World, being higher in Africa where those estimates can reach 274.8 (250.9 - 298.7) cases per 100,000 populations, **Table 2.1.** The absolute number of incident cases has been decreasing slowly, since the early 2000s. In 2013 most of the estimated cases occurred in the WHO regions of Southeast Asia and the western Pacific collectively accounted for (58%) of world TB cases, followed by the African region (27%), smaller proportions of cases occurred in the eastern Mediterranean region (8%), the European region and the region of the Americas accounted (4%), (3%) of world TB cases respectively. In 2013 the largest number of incident cases per country was in India (uncertainty range 2.0 million– 2.4 million) corresponding to 26% of global cases, China (0.9 million–1.1 million), South Africa (0.4 million–0.6 million), Nigeria (0.34 million-0.88 million) and Pakistan (0.37 million-0.65 million). Of the 9.0 million incident cases, an estimated 0.55 million were children, 3.3 million (2.7–3.6 million) occurred among women, 1.0 million (0.9 million-1.3 million) occurred among HIV infected people (WHO, Global Tuberculosis Report 2014).

The incidence rate varies widely among countries. The lowest rates are found predominantly in high-income countries including most countries in Western Europe, Canada, the United States of America, Australia, and New Zealand. In these countries, the incidence rate is less than 10 cases per 100,000 populations (Philippe, *et al*. 2015). The highest rates are found in high burden counties which have incidence rate of 150 -300 cases per 100,000 population. However, the high burden counties with markedly lower rate in 2013 were Brazil, China and Russian Federation, while rates were above than 500 per cases 100,000 population in Mozambique, South Africa, and Zimbabwe (WHO, Global *Tuberculosis* Report, 2014).

In Sudan, the absolute incidence rates have been decreasing since 2010. The country in 2010, 2011, 2012, and 2013 has incidence rates 110, 131,119, and 108 cases per 100,000 populations respectively (WHO, *Tuberculosis* country profile, 2014).

Regard the prevalence of TB, which for some countries is still difficult to estimate and in many cases even are not reported and/or analyzed. According the WHO, for 2013 the global prevalence was estimated in 11 million (range, 11.0–14.0 million) prevalent cases of TB, for rates ranging 160.1 - 203.8cases per 100,000 population globally. However, the prevalence can be very different among the WHO Regions in the World, being higher in Africa where those estimates can reach 334.5 (274.8 - 394.3) cases per 100,000 populations **Table 2.1.**The Region of the Americas halved the 1990 level of TB prevalence by around 2005, well in advance of the target year of 2015, and the best estimate suggests that the Western Pacific Region achieved the 50% reduction target in 2012. Reaching the 50% reduction target by 2015 appears feasible in the South-East Asia Region (WHO, Global *Tuberculosis* Report, 2014). By 2013, the prevalence rate in

Sudan had fallen since 2012. In 2012 and 2013, the country has prevalence rate 207(per 100 000 population) and 192 (per 100 000 population) respectively (WHO, *Tuberculosis* country profile, 2013; WHO, *Tuberculosis* country profile, 2014).

Regard the deaths, WHO estimates indicate that around 1.5 million (1.1-1.6 million) TB deaths occur in 2013. Of 1.5 million about 940,000 among HIV-negative people and the remaining among HIV-positive people. These deaths included 410,000 among women and 74,000 among HIV-negative children. Approximately 75% of total TB deaths occurred in the African and South-East Asia Regions (both including and excluding TB deaths among HIV-positive people). India and South Africa accounted for about one-third of global TB deaths (Philippe, *et al*. 2015).

The global number of TB deaths per 100,000 populations ranging 13.4 - 17.5 per 100.1000 populations **Table 2.1.** In 2013 and 17.6 when TB deaths among HIV-positive people are included. There is considerable variation among countries, ranging from less than two TB death per 100,000 population in most countries in westernEurope, Canada, the United States of America, Australia and New Zealand to more than 40 deaths per 100,000 population in much of the African region as well as three high burden countries in Asia (Bangladesh, Cambodia, and Myanmar) (WHO, global tuberculosis report, 2013).

Table 2.1. Estimated epidemiological burden of TB incidence, prevalence and mortality according to different regions distributed by the WHO in 2013

WHO regions	Incidence rates (cases/ 100,000pop)	Prevalence rates (cases/ 100,000pop)	Mortality rates (deaths/ 100,000pop)
WHO African Region	250.9 - 298.7	274.8 - 394.3	26.3- 33.5
WHO Region of the Americas	26.8 - 30.0	27.9 - 43.9	1.8 - 2.5
WHO Eastern Mediterranean Region	97.2 - 122.3	112.3 - 251.4	12.4 - 20.1
WHO European Region	43.5 - 50.2	245.4 - 312.3	5.4 - 8.4
WHO South-East Asia Region	177.0 - 204.7	23.8 - 39.8	20.5 - 35.4
WHO Western Pacific Region	83.4 - 100.1	205.7 - 361.4	6.7 - 8.3
Global burden	**123.7 - 133.9**	**160.1 - 203.8**	**13.4 - 17.5**

(Adapted from WHO Global Tuberculosis report 2014)

2.1.3. **Transmission of Mycobacterium Tuberculosis**

Tuberculosis is spread from person to person through the air by droplet nuclei, particles that contain *M. tuberculosis*complex. Droplet nuclei are produced when persons with pulmonary or laryngeal tuberculosis cough, sneeze, speak, or sing (Riley, 1993). These droplet nuclei are small enough to reach the alveoli within the lungs, where the organisms replicate (Horsburgh, 1996). However, four factors determine the likelihood of

transmission of MTBwhich include, the number of organisms being expelled intothe air, the concentration of organisms in the air which determinedby the volume of the space and its ventilation, thelength of time an exposed person breathes the contaminatedair, and presumably the immune status of the exposed individual (Riley, 1993; Wright, *et al.* 2009). Furthermore, the procedures that can result in the dissemination of droplet nuclei include endotracheal intubation, bronchoscopy, sputum induction, aerosol treatments, irrigation of a tuberculous abscess, and autopsy (Jong, *et al.* 2010). On the other hand, isolated extrapulmonary tuberculosis is not contagious, although such patients require careful evaluation for pulmonary or laryngeal TB (Lonnroth, and Raviglione, 2008).

2.1.4. Conditions enhancing TB infection

In general, a relatively small proportion of people infected with *M. tuberculosis* will go on to develop TB disease, (WHO, Global tuberculosis control, 2011). However, there are many of risk factors linked to poverty enhance the probability of TB infection. These factors include, crowded living conditions, malnutrition, and exposure to indoor air pollution from cooking fires. Also the susceptibility to TB may be affected by factors, such as tobacco, smoking, and silicosis, exposure to smoke from cooking fires and excessive alcohol as well as HIV infection (Selwyn, *et al.* 1989; Vynnyckyand Fine, 2000). Also both age and sex have biological and social effects, which are difficult to distinguish. The risk of developing primary TB is lower in children than in adults (Vynnycky and Fine, 1997), the children are more likely to develop severe forms of disease in organs other than the lungs (e.g. tuberculous meningitis). Young women (15–44 years) may be more likely than men to develop active TB following infection (Radhakrishna, *et al.* 2003).

HIV pandemic has had a devastating impact on TB control, and TB has emerged as one of the principal causes of HIV associated morbidity and mortality worldwide (Parrish, *et al*. 1998; Condos, *et al*. 1998; Frieden, *et al*. 2003). The risk of TB increases after HIV as a result of impairment in innate immune function. This impairment might potentially increase host susceptibility to TB infection following exposure (Corbett and Raviglione, 2005). However, themajority of HIV-uninfected individuals with latent MTB infection do not develop TB disease as their cellular immune function is adequate to maintain (Migliori, *et al.* 2007). In contrast, HIV-infected patients with latent MTB infection have a high risk of reactivation, with approximately 10% of such individuals developing active TB (Corbett and Raviglione, 2005).

2.1.5. Tuberculosis: Immune Response and Pathogenesis

The infection starts with penetration of a few mycobacteria deep into lung alveoli. The MTB are absorbed by alveolar macrophages in alveoli, and begin to live as an intracellular parasite (Rohde, *et al.* 2007). The pathogenesis processes start after the formation of a phagosome, and enveloped cytoplasmic vacuole that encapsulating the phagocytized bacterium. MTB survive and even reproduce within the phagosome by blockage of phagosome maturation. This process prevents acidification of its own internal environment below pH 6.4, as well as fusion with acidic lysosomes at pH 4.8 (Deretic, *et al.* 2006). This process is mediated by MTB cell wall lipid**,** andsome secreted glycoproteins (Russell, 2001; Makinoshima and Glickman, 2005). Macrophage becomes activated via immune processes induced by interferon γ (IFN-γ) that produced by T cells. Also macrophages become able to reduce the effect of MTB on phagosome

maturation and adjust the internal pH to the bactericidal value 5.2 (Fratti, *et al.* 1998).

Mycobacteria produce diverse lipids, lipoproteins, and glycolipids involved in these early recognition and activation reactions (Stewart, *et al.* 2003). These induce the production of interlukin (IL) IL-10, IL-12 and IFN-γ activator. The interaction of these immune system elements starts the attraction of other immune system cells to the infection site and the formation of tuberculous granulomas, which is the key element of TB pathogenesis (Geijtenbeek, *et al.* 2003).

2.1.5.1. Granuloma Formation

The innate immunity pathways starting in infected macrophages immediately after phagosome formation (Russell, 2007).One of the substances that are produced early in this process is tumor necrosis factor α (TNFα), which is the main factor activating macrophage during inflammation. Other early signaling molecules are chemokines CCL2 and CXCL10 and cytokines IL-1- α, IL-1, and IL-18.Also this stage is marked by the start of the inflammatory process, neutrophils are the first cells involved in this process (Segal,2005; Eruslanov, *et al.* 2005; Nathan, 2006). In response to cytokine and chemokine production by infected cells, mononuclear phagocytes start their migration to the primary infection. These phagocytes differentiate to tissue macrophages, and then capture the reproduced mycobacteria released after degradation of initially infected alveolar macrophages, to form the core of the developing tuberculous granuloma. In the center of a tuberculous granuloma, common mononuclear phagocytes are accompanied by numerous, foamy macrophages, giant multinucleate cells. The respiratory function is become progressively lost as

porous alveolar tissue is replaced by infiltrates and granulomatous fibrous tissue (Hunter, *et al*. 2006; Lay, *et al*. 2007).

2.1.5.2. Granuloma maturation and immune response

The cells that form the initial infection still produce TNF-α and enhance the expression of the genes for the chemokine ligands of the CXCR3 receptors (CXCL9, CXCL10, and CXCL11) (Fuller, *et al.* 2003). These factors induce lymphocyte mobilization from the bloodstream to the focus. Natural killer T cells are mobilized first and are followed by all other populations of CD4^{+}, CD8^{+}, and B cells. These cells located outside the macrophage core of the granuloma, T cells interact with living macrophages and perform the adaptive immune response to mycobacterial antigens, while the central zone of the focus is gradually necrotized. The formation of the central necrotic zone and exterior immunologically active lymphocyte layers crown granuloma maturation (North and Jung, 2004; Fortin, *et al*. 2007).

2.1.6. Tuberculosis clinical manifestation

The clinical manifestations of tuberculosis are dependent on a number of factors: age, immune status, co-existing diseases, immunization status virulence of the infecting organism, host-microbe interaction and organ involved by TB (Robert and Serafino, 2013).

2.1.6.1. Clinical manifestations PTB

Cough is the commonest presentation, which initially it may be nonproductive, but as inflammation and tissue necrosis ensue, sputum is produced. Hemoptysis is occasionally a presenting symptom, but usually results from previous disease and may not indicate active TB. Inflammation of the lung parenchyma adjacent to a pleural surface may cause pleuritic

pain. Also dyspnoea will occur in case of extensive disease and may result in respiratory failure (Grzybowski, *et al*. 1971).

2.1.6.2. Clinical manifestations EPTB

The presenting features of EPTB are generally nonspecific, and characterized by the following systemic effects which include, fever, weight loss, night sweats, anorexia, and weakness. The Physical findings include hepatomegaly, pulmonary findings, lymphadenopathy, and splenomegaly (Robert and Serafino, 2013).

2.1.6.2.1. Pleural tuberculosis

There are two mechanisms by which the pleural space becomes involved in TB. Early on a few organisms may gain access to the pleural space and, in the presence of cell-mediated immunity, cause a hypersensitivity response. The second involvement results from a large number of organisms spilling into the pleural space, usually from rupture of a cavity or an adjacent parenchymal focus via a bronchopleural fistula. The clinical manifestation of these involvements is an acute illness with fever and pleuritic pain. If the effusion is large, dyspnoea may occur (Robert and Serafino, 2013).

2.1.6.2.2. Lymph node tuberculosis

Tuberculous lymphadenitis usually presents as painless swelling of one or more lymph nodes. The nodes most commonly involved are those of the posterior or anterior cervical chain or those in the supraclavicular fossa. With continuing disease the nodes may become matted and the overlying skin inflamed. Rupture of the node may result in formation of a sinus tract, which is slow to heal. Intrathoracic adenopathy may compress bronchi, causing atelectasis leading to lung infection, and perhaps bronchiectasis being particularly common in children (Kent, 1967).

2.1.6.2.3. Genitourinary tuberculosis

This type of TB tends to present with local symptoms while the systemic symptoms being less common. Dysuria, hematuria and frequency of micturition are common. However, often there is advanced renal destruction by the time of diagnosis. In women genital involvement is more common without renal tuberculosis and may present with pelvic pain, menstrual irregularities and infertility (Christensen, 1974). In men a painless or only slightly painful scrotal mass is probably the most common presenting symptom of genital involvement. Symptoms of prostatitis, orchitis on epididymitis may also occur (Simon, *et al.* 1977).

2.1.6.2.4. Skeletal tuberculosis

The usual presenting symptom is pain. Swelling of the involved joint may be noted with limitation of motion. Also the systemic symptoms of infection are not common (Robert and Serafino, 2013).The epiphyseal region of bones is highly vascularized in infants and young children, therefore, bone involvement is much more common in these groups. Delay in diagnosis can be catastrophic in vertebral tuberculosis, where compression of the spinal cord may cause severe and irreversible neurologic sequelae (Gutman, 1993).

2.1.6.2.5. Central nervous system tuberculosis

Meningitis can result from direct meningeal seeding and proliferation during TB infection. The consequences of subarachnoid space contamination are diffuse meningitis or localized arteritis. In tuberculous meningitis the process is located primarily at the base of the brain (Gutman, 1993). Symptoms include those related to cranial nerve involvement as well as headache, decreased level of consciousness and neck stiffness. In most series more than 50% of patients with meningitis have abnormalities on

chest film, consistent with an old or current tuberculous process and often miliary TB (Robert and Serafino, 2013).

2.1.6.2.6. Abdominal tuberculosis

TB can involve any intra-abdominal organ and the peritoneum as well as it will occur in any location from the mouth to the anus. Therefore, clinical manifestations depend on the areas of involvement. The most common sites of involvement are the terminal ileum and caecum. In the terminal ileum or caecum the most common manifestations are pain, which may be misdiagnosed as appendicitis or intestinal obstruction. Rectal lesions usually present as anal fissures, fistulae or perirectal abscesses. TB peritonitis frequently presents with pain often accompanied by abdominal swelling (Bhansali, 1977). Nonspecific systemic symptoms like fever, weight loss, and anorexia are also common. The combination of fever and abdominal tenderness in a person with ascites should always prompt an evaluation for intra-abdominal TB infection (Robert and Serafino, 2013).

2.1.6.2.7. Pericardial tuberculosis

The symptoms, physical findings, and laboratory abnormalities may be the result of either the infectious process itself or the pericardial inflammation causing pain, effusion and eventually hemodynamic effects. The systemic symptoms produced by the infection are nonspecific. Fever, weight loss and night sweats are common (Schepers, 1962). Cardiopulmonary symptoms tend to occur later and include cough, dyspnea, orthopnea, ankle swelling and chest pain (Robert and Serafino, 2013).

2.2. Tuberculous lymphadenitis

Tuberculous lymphadenitis is the most common manifestations of all EPTB (Prasanta and Ashok, 2009). The most commonly involved lymph

nodes were cervical, followed by axilliary, inguinal, abdominal and supra clavicular sites (Majeed and Bukhari, 2011; Hussain, *et al*. 2011).

The incidence of TBL has increased in parallel with the increase in the incidence of MTB infection worldwide (WHO, Global tuberculosis report, 2013). TBL is seen in nearly (35%) of EPTB, which constituted about (15 to 20%) of all cases of TB (Mohapatra and Janmeja, 2009; Cortez, *et al*. 2011). The epidemiology of TBL varies between developed and developing countries. In developed countries, most cases of TBL occur among adult immigrants from TB endemic countries (Geldmacher, *et al*. 2002). This was illustrated by case series of TBL in France and Germany in which about 70% of cases occurred in immigrants. In the German study, two-thirds of patients had immigrated >3 years prior to diagnosis (Geldmacher, *et al*. 2002). In the United States, the rate of TBL is higher among Asian Pacific Islanders and in females. Rarely, TBL can also occur in travelers to endemic areas (Fontanilla, *et al*. 2011).

In developing countries where TB is endemic, TBL occurs in up to 60 % of HIV-infected patients with TB, and is frequently accompanied by signs of pulmonary involvement (Lee, *et al.* 2000; Atomiya, *et al.* 2002).In most series, TBL is more common among women than among men (composite ratio, 1.4:1) (Chen, *et al*. 1992; Fontanilla, *et al*. 2011).

2.2.1. Tuberculous lymphadenitis pathogenesis

Tuberculous lymphadenitis is a local manifestation of the systemic disease (Kent, 1967). It may occur during primary tuberculous infection or as a result of reactivation of latent infection. Primary infection occurs on initial exposure to tubercle bacilli. Inhaled droplet nuclei are small enough to pass mucociliary defenses of bronchi and lodge in terminal alveoli. The bacilli multiply in the lung, and called Ghon focus. The infection may

spread from primary focus to regional lymph nodes and the organism may continue to spread via the lymphatic system to other nodes or may pass through the nodes to reach blood stream, from where it can spread to all organ of the body (Dandapat, *et al.* 1990). Hilar, mediastinal and paratracheal lymph nodes are the first site of spread of infection from the lung parenchyma (Shriner, *et al.* 1992; Jha, *et al.* 2001).

After lymph node involvement progressive multiplication of the MTB occurs, this is accompanied by marked hyperemia, swelling, necrosis and caseation of the center of the nodes, and followed by inflammation, progressive swelling and matting with other nodes. The center of the enlarging gland becomes soft and caseous material may rupture into surrounding tissue or through skin with sinus formation (Chao, *et al.* 2002). The common clinical manifestation of TBL calledScrofuloderma which is MTB infection of the skin caused by direct extension of MTB into the skin from underlying structures or by contact exposure to TB. The uncommon manifestations observed in patients with mediastinal lymph node involvement include dysphagia (Singh, *et al.* 1996) oesophago mediastinal fistula (Ohtake, *et al.* 1996) and tracheo-oesophageal fistula (Im, *et al.* 1990). Upper abdominal and mediastinal lymph nodes involvement may cause thoracic duct obstruction and chylothorax, chylous ascites. Rarely, biliary obstruction due to enlarged lymph nodes can result in obstructive jaundice (Paredes, *et al.* 1990).

Histologically, tuberculous granuloma consists of a necrotic center surrounded by epithelioid histiocytes, multinucleated (Langhans) giant cells, and lymphocytes. The necrotic center consists of amorphous eosinophilic material with scattered nuclear fragments. Epithelioid histiocytes are highly activated macrophages that secrete a variety of cytokines. However, this

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morphological feature of tuberculous granuloma can be seen many different pathological conditions. The most common stains identification of *M. tuberculosis* in tissue sections are Ziehl-Neelsen, Kinyoun, and Fite-Faraco, all of which has very low sensitivity and lack of standardization (Wu, *et al*. 2012). The mycobacteria may also be detected by IHC with a higher reported degree of sensitivity and specificity (Ulrichs, *et al*. 2005; Mustafa, *et al*. 2006). Also PCR-based methods are highly specific (Wilson, 2011; Linasmita, *et al*. 2012).

However, the differential diagnosis of granulomatous lymphadenitis is wide and includes a diverse list of etiologies such as non-tuberculous mycobacteria, fungal organisms, autoimmune diseases, sarcoidosis, regional malignancies (eg, classical Hodgkin lymphoma, seminoma/dysgerminoma), and drug-mediated immune reactions (Roberto, *et al*. 2013).

2.3. Diagnosis of Tuberculosis

Sputum microscopy and culture with subsequent drug-susceptibility testing are currently recommended as standard methods for diagnosing active tuberculosis. The use of solid culture medium is more cost-effective in resource poor countries. Moreover tuberculin skin tests and Interferon-gamma release assays have no role in the diagnosis of active TB. However the use of imaging techniques nucleic acid amplification tests, and histopathologic examination of biopsy samples supplement these evaluations (Hopewell, *et al*. 2006).

2.3.1. Chest radiography

Chest radiography is indicated for all persons being evaluated active TB. Cavitation, fibrosis, and/or enlargement of the hilar and mediastinal lymph nodes may be present radiographic film of patient infected with TB. In some

cases, radiographic film may present as lobar or segmental infiltrates, lung mass, scattered fibronodular lesions ("miliary"). However, Radiographic features depend on the type of infection. In primary TB, the process is generally seen as middle or lower lung zone infiltrate, associated with hilar adenopathy (Yeon, *et al*. 2008).

In post-primary PTB, also known as reactivation TB or secondary TB which occur years later, cavitation may take place, as a result of specific cell-mediated immunity (so-called progressive primary" tuberculosis) (Collins and Stern, 2007). The most frequent sites of secondary TB are the apical and posterior segments of the right upper lobe and the apical-posterior segment of the left upper lobe. Healing of the tuberculous lesions usually results in development of a scar with loss of lung parenchymal volume and, often, calcification which can be appear in radiologic film (Naidich, *et al*. 2007). Old, healed TB presents a different radiologic appearance from active TB. Dense pulmonary nodules, with or without visible calcification, may be seen in the hilar area. Smaller nodules, with or without fibrotic scars, are often seen in the upper lobes (Collins and Stern, 2007).

In early HIV infection, the nature of the radiographic findings tends to have the typical radiographic findings described above. With more advanced HIV disease, the radiographic findings become more "atypical": cavitation is uncommon and lower lung zone or diffuse infiltrates and intra-thoracic adenopathy is frequent (Yeon, *et al*. 2008).

2.3.2. Tuberculin skin test (TST)

This test measures a patient's immune response to MTB antigens. TST is based on the fact that, infection with TBproduces a delayed-type hypersensitivity reactionto certain antigenic components of the organism called tuberculin(Edwards, 1960).

The recommended method of TST is the Mentel Mantoux test, also known as the Mantoux screening test, tuberculin sensitivity test, Pirquet test, or purified protein derivative (PPD) test, TST is done by injecting 5 tuberculin units of tuberculin PPD into the skin in the arm. The test becomes positive 2–10 weeks after the MTB infection. Positive reactions (>10-mm induration) can occur in MTB infection. Negative reactions (< 4-mm induration) represent a lack of tuberculin sensitization. However, false-negative reactions can occur in about 20% of all persons with active tuberculosis (Mori, *et al*. 2004). TST may be positive in different conditions, like, metabolic disease, malnutrition, live virus vaccination, malignancy, immunosuppressive drugs, newborns, elderly people, and inadequate test application.The sensitivity of the TST for active TB varies considerably, from 65% to 94% (Fine,*et al*. 1999).This sensitivity is decreased in certain populations with disseminated tuberculosis (Huebner, *et al*. 1993).

2.3.3. Interferon-gamma release assays (IGRAs)

Serum Interferon-gamma release assays (IGRAs) are in vitro tests of whole blood or mononuclear cells, IGRAs based on IFN-γ that release after T-cell stimulation by MTBspecific proteins like early-secreted antigenic target-6 (ESAT-6).The test is measures and quantifies IFN-γ (IU/mL) released from blood collected in special tubes that are coated with ESAT-6 antigen. The result of IGRA is reported both qualitatively (positive, negative, indeterminate, or borderline) and quantitatively (IU/mL). Reversion of IGRA test results from positive to negative has been observed, particularly in those with negative results on the initial TST (Fietta, *et al*. 2003;Menzies, *et al*. 2007). However, although IGRS has the advantage of being able to differentiate *MTB* infection from non MTB infections, but it cannot distinguish active TB from latent infection. Because ESAT-6

proteins are also present in other *Mycobacterium species*(Menzies, 2007; Mack,Mazurek, *et al.* 2010).

2.3.4. Sputum-based diagnosis

To establish the final diagnosis of PTB, respiratory samples should submit to the laboratory for microscopy acid fast bacilli (AFB) smear, culture and nucleic acid amplification (NAA) assays. The technique used to obtain the respiratory sample strongly influences the ability to detect pulmonary tuberculosis. Expectorated sputum is the starting point. Three sputum samples should be collected on three separate days and stained for AFB (American Thoracic Society, 1997; Havlir and Barnes, 1999).The sensitivity of expectorated sputum ranges from 34% to 80%, which tends to be highest in patients who have cavitary disease and lowest in patients who have weak cough (Braun, 1993; Dunlap, *et al.* 1995).

However; if a patient is suspected of having PTB, but is smear negative on expectorated sputum or is unable to produce sputum for testing, further diagnostic testing may be warranted (Valway, *et al.* 1998). The options include sputum induction (SI) or Flexible fiberoptic bronchoscopy (FOB). SI can carry out by inhalation of an aerosol of sterile hypertonic saline using nebulizer. This can be used to stimulate the production of sputum. The FOB techniqueincludes Bronchial Aspiration Lavage (BAL), bronchial washings (BW), bronchial brushings (BB), transbronchial biopsy (TBB), and post bronchoscopy sputum collection (PBS). The technique provides maximal visualization of the tracheobronchial tree, results in an exceedingly low complication rate, and does not require general anesthesia (Shinnick and Good, 1995).

2.3.4.1. Stains for mycobacteria

MTB can be rapidly and inexpensively detected directly from pretreated and concentrated respiratory specimens, body fluids, and tissue using acid-fast stains. A Gram stain is not reliable to detect MTB at which MTB appear as non-stained "ghosts" or as beaded Gram-positive bacilli. Therefore, acid-fast stains, such as the Ziehl-Neelsen stain or the fluorescent auramine-rhodamine stain are recommended for MTB staining (Chegou and Hoek, 2011).

The acid-fast stain forms a complex between the unique mycolic acids of the MTB cell wall and the dye. This complex formation makes the mycobacteria resistant to destaining by acid-alcohols, providing the basis for the "acid-fast" terminology. Non-acid fast bacteria do not retain the acid-fast dye in the presence of the acid-alcohol decolorizer, and are often stained in a subsequent step by counterstain. Commonly utilized acid-fast stains is the Ziehl-Neelsen stain, which is lack for sensitivity and a large number of bacilli (104-106/mL) are required for a positive stain (Chegou and Hoek, 2011). Therefore concentration step provides increase sensitivity over direct smear microscopy (Steingart and Henry, 2006). Moreover, the stain is also non-specific and the reader cannot determine the species of MTB present in a positive smear (Attorri and Dunbar, 2000; Julian and Roldan, 2010).

Fluorescent stains such as auramine O are also used alone or in combination with rhodamine B. The fluorescent stains exhibit high binding property to DNA and RNA providing enhanced sensitivity for examining concentrated specimens. The fluorescent stains, stain the bacilli, to be appear as long slender rods (1-10µm long & 0.2-0.6µm wide) and are often slightly curved or bent (Pfyffer and Palicova, 2011).

2.3.4.2. Culture

The growth of MTB in culture is considered as gold standard for identification of a case of tuberculosis. The sensitivity of culture is excellent, ranging from 80% to 93% with high specificity of 98%, which is much better than an acid-fast smear. Therefore, only 10- 100 viable organisms/mL of specimen is required for a positive culture. Media for the growth of MTB complex is the same as that used for other MTB species and generally includes both a solid and a liquid-based medium. Solid media utilized is either egg-based such as the Lowenstein-Jensen (L-J) medium or agar-based such as Middle brooks 7H10 medium. Antimicrobial agents can be added to help with elimination of contaminating organismswhich may have a more rapid growth rate than MTB. In general, MTB colonies are seen more rapidly on agar-basedmedium (10-12 days) as opposed to egg-based medium (18-24 days) (Liu and Gregor, 1973). Uses of Middle brook 7H11 medium containing casein is reported toimprove the recovery of drug-resistant isolates of MTB (Cruciani and Scarparo, 2004; Pfyffer and Palicova, 2011).

However, cultures for MTB complex should be incubated at 35-37ºC in an atmosphere of 5-10% CO_2 for primary cultures on solid medium. Since MTB complex grows slowly in culture, the culture plates should be examined for growth twice per week (Levidiotou and Vrioni, 2003).

2.3.5. Diagnosis of tuberculous lymphadenitis

The diagnosis of smear-positive PTB has been considerably established, but the diagnosis of smear-negative PTB, TB–HIV co-infection and EPTB poses serious challenges (Golden and Vikram, 2005). Diagnosis of EPTB, in particular, is difficult. This may due to nature of the specimens, lack of adequate clinical sample volumes and non-uniform distribution of bacteria

in those specimens as well as the disease localized in sites that are difficult to access (Cherian, 2004; Cheng, *et al.* 2005; Galimi, 2011). On other hand, the diagnosis of TBL is challenging as it mimics many pathologic processes (sarcoidosis, leprosy, fungal and NTM infections) and yields inconsistent histopathologic findings in the absence of AFB (Osores, *et al.* 2006; Derese. *et al.* 2012). However, various methods are employed for the diagnosis of TBL as well as EPTB such as smear microscopy, culture identification, histopathology, radiologic examination, and nucleic acid amplification tests (Katoch, 2004; Lange and Mori, 2010).

2.3.5.1. Radiology and imaging

Chest radiograph, ultrasound, computerized tomography (CT) and Magnetic resonance imaging (MRI) of the neck can be performed for all patients with TBL. Ultrasound of the neck can demonstrate multiloculated cystic lesions that are surrounded with thick capsule (Prasanta and Ashok, 2009).

On CT scan, the presence of collection of nodal masses with central luscency, a thick irregular rim of contrast enhancement and inner nodularity, a varying degree of homogeneous augmentation in smaller nodes as well as subcutaneous manifestations of inflammation, such as thickening of the overlying skin, congestion of the lymphatics and thickening of the adjacent muscles may suggest mycobacterial cervical lymphadenitis. However, these findings may also be seen in other diseases like lymphoma and metastatic lymphadenopathy (Kim, *et al.* 1993; Nadel, *et al.* 1996).

MRI may reveal discrete, matted and confluent masses. Necrotic foci, when present, which are more frequently peripheral rather than central, and this together with the soft tissue edema may be of value in differentiating

mycobacterial cervical lymphadenitis from metastatic nodes (Hirunwiwatkul, *et al.* 2002).

2.3.5.2. Fine-needle aspiration cytology (FNAC)

Fine-needle aspiration cytology a less invasive and cost-effective procedure than excision biopsy, that has assumed an important role in the diagnosis of TBL (Chakravorty and Tyagi, 2005; Derese, *et al.* 2012). The amount of material obtained in the FNA is usually so small, that it is often inadequate to perform AFB smear and culture examination (Kidane, *et al.* 2002; Prasanta and Ashok, 2009). FNA cytology also has difficulty in differentiating TB from other granulomatous diseases (Baek, *et al.* 2000). It has sensitivity and specificity of 88% and 96%, respectively in the diagnosis of TBL (Chao, *et al.* 2002), but the combination of FNA with culture or a Mantoux test further increases the diagnostic yield in MTB cervical lymphadenitis (Ellison, *et al.* 1999). Several researchers have performed PCR from the remainders of FNA after cytological examination, but this clinical application of PCR along with FNA cytology could reduce the necessity for open biopsy as the process of biopsy is invasive and leaves unwanted scar tissues in the neck causing aesthetic problems (Baek, *et al.* 2000; Supiyaphun, *et al.* 2010).

Smear microscopy is widely used in the diagnosis of TBL, it can be obtained either from a draining sinus or by FNA. However, in pulmonary specimen, ZN smear has very low and variable sensitivity values ranging (0–40%) and could not differentiate between MTB and non-MTB (Haldar, *et al.* 2011; Derese *et al.* 2012). Moreover, the ZN stain have several limitations and are often unhelpful in establishing the diagnosis of TBL in FNA sample. Because the Acid-fast bacilli AFB positivity in FNA sample depends on the bacillary load of the specimen and the type of the material

(Hussain, *et al.* 2011). Furthermore, different studies have reported a wide range of AFB positivity in FNA sample and histological sample ranging from as low as 0% to as high as 75% (Hussain, *et al.* 2011).

Culture of MTB is diagnostic for TBL, and it has variable sensitivities ranging (0-80%) in different extrapulmonary tuberculosis specimens (Abbara and Davidson, 2011). However, a negative culture result should not exclude the diagnosis of TBL, because the adequacy of sample play an important role of the result. The presence of 10–100 bacilli per cubic millimeter of the specimen is enough for a positive culture result. Different media can be used to culture the fresh aspiration TBL such as L-J, Middle brook. But unfortunately several weeks are needed to obtain this culture to obtain result, which may prolong the initiation of treatment (Padmavathy, *et al.* 2003; Sharma and Mohan, 2004; Takahashi, *et al.* 2008; Abbara and Davidson, 2011).

2.3.5.3. Histopathology

Diagnosis of TBL from tissue samples is usually made by histopathologic examination, that largely based on histopathology morphological evidence (presence of granuloma with or without caseation and calcification) which is not good, followed by acid fast stain to confirm the presence of bacilli, but ZN staining has very low sensitivity and often is less specific (Liu, *et al.* 2007; Almadi, *et al,* 2009). However, histology does not distinguish between the granuloma of TBL other granulomatous diseases such as non-MTB, sarcoidosis, leprosy and systemic lupus (Bravo and Gotuzzo, 2007; Chawla, *et al.* 2009).

2.3.5.4. Immunohistochemistry

Immunohistochemistry refers to the process of detecting antigens (e.g., proteins) in cells of a tissue section. This process accomplished with antibodies that recognize the target protein of interest in the tissue section. The antibody-antigen complex can be visualized using either chromogenic detection system, or fluorescent detection system. Since inception in the 1940s, IHC has gradually developed into major diagnostic tool in diagnostic cellular pathology. The identification of specific or highly selective cellular epitopes, in routinely processed paraffin wax embedded tissue with an antibody and appropriate labeling system, has made a significant impact on histological diagnosis (Ramos, 2005).

IHC involves a series of uniform steps, typically beginning with antigen retrieval. The Methods is varying in terms of reagents and methods. The process involves using of proteolytic enzymes or heating of histologic sections using pressure cooking, microwave, in baths of appropriate buffers. The standard goal of this process is unmasking antigens hidden by formalin cross-links (Shi, *et al.* 2000). The first definitive step of IHC following antigen retrieval is the application of a specific primary antibody, followed by extensive washing to remove excess amounts of primary antibody. A species-specific secondary antibody is then applied, which binds to the primary antibody. The secondary antibody is typically conjugated to biotin, horseradish peroxidase, or some other tag. Finally, a detection reagent is applied that includes a chromagen or a fluorescently tagged molecule to visualize the localization of the primary antibody (Ramos, 2005).

Since 1990, Few IHC studies have suggested the probable role of immunohistochemical staining in establishing mycobacterial etiology of caseating granulomas of lymph nodes and tissue specimens with TB (Orelle,

et al. 1991; Kutzner, *et al.* 1998). Most of these studies have used polyclonal antibodies, which resulting in false positive reactions due to antigenic cross reactivity with other bacteria and fungi (Ulrichs, *et al*. 2005). Recently, IHC assumed greater significance in the diagnosis of several infectious diseases including TB. The role of IHC in the diagnosis of TB in formalin-fixed paraffin embedded lymph node biopsies have been reported in several published studies (Mukherjee, *et al.* 2002; Manju, *et al*. 2007; Ihama, *et al*. 2012; Goel, *et al.* 2012).

However, different mycobacterial antigens have been detected using IHC method in pleural tuberculosis (Baba, *et al*. 2008), intracranial tuberculoma (Sumi, *et al.* 2001), abdominal and lymph node tuberculosis (Manju, *et al.* 2007). The sensitivity and specificity of this technique have been reported by many worker using different antibodies such as, Anti-Bacilli Calmette–Guérin (BCG), Anti MPT-64, anti-ESAT-6, anti-HspX, anti-Tb8.4 anti-PlcA, anti-35 KD and anti 38-KD (Goel, *et al*. 2008).

2.3.5.4.1. Anti-BCG antibody

Sensitivity and specificity of 74% and 95% respectively were reported by Ashoke, *et al*. (2002), they used a commercial anti-BCG antibody to detect MTB in 50 lymph node tissue. Moreover the positivity of this antibody was evaluated by Higuchi, *et al.* l981; Ulrichs, *et al.* (2005), both worker reported 100% positivity for this antibody. Indirect immunoperoxidase; polyclonal anti- BCG was positive in 68% of 50 case suspected to have lymph node TB in study carried by Padma, *et al.* (2005).

2.3.5.4.2. Anti MPT64 antibody

The MTB protein 64 (MPT-64) antigen is an MTB complex specific antigen secreted during bacterial growth. It is also termed as a 24 KD a secretary protein. This protein was used as diagnostic marker for diagnosis

of TBL in many different studies (Roche, *et al.* 1996; Goel and Budhwar, 2008; Madhu, *et al.* 2012; Yasushi, *et al.* 2012). Fifty-five cases of granulomatous lymphadenitis withhistologically suspected tuberculosis obtained from Norway and Tanzania were evaluated in comparative study carried by Tehmina, *et al.* (2006). They used ZN stain, MPT64 IHC, and IS11610 PCR as gold standard and found that, ZN satin had very low sensitivity (12%), while the classical tuberculosis histology had sensitivity, specificity, positive and negative predictive values of 92, 37, 60, and 81%, respectively, and IHC had sensitivity, specificity, positive and negative predictive values of 90, 83, 86, and 88%, respectively. The observed agreement between PCR and IHC was 87% (P. value less than 0.05). Moreover, formalin-fixed histologically diagnosed abdominal tuberculosis (n = 33) andcervical TBL (n = 120) biopsies were used by Manju, *et al.* (2007), to evaluate the diagnostic potential of IHC using an MPT64, in detection of abdominal and lymph node TB (LNTB). They reported that, the overall sensitivity, specificity, positive and negative predictive values of IHC with anti-MPT64 was 92%, 97%, 98%, and 85%, respectively.

In study conducted by Tadele, *et al.* (2014), at Tikur Anbessa Specialized Hospital and the United Vision Medical Services from December 2011 to June 2012. 51 Lymph node aspirates samples were collected and subjected to ZN staining and IHC with polyclonal anti-MPT64 antibody. PCR was used as gold standard in this study. Accordingly, the overall sensitivity and specificity, positive and negative predictive value of IHC was 88.1%, 89.5%, 82.2% and 93.2%, respectively.

Furthermore, human pulmonary (n = 3) and lymph node (n = 17) TB biopsies, and non-TB controls (n = 12) were studied by Tehmina, *et al.* (2014), to detect mycobacterial antigens (MPT32, MPT44, MPT46, MPT51,

MPT53, MPT59, MPT63, and MPT64) by using rabbit polyclonal antibodies. ZN stain, PCR 1S6110 and immunohistochemistry were performed. These workers reported that, plenty of bacilli were detectable with ZN stain in the lung biopsies with sensitivity of (65%), while (0%) sensitivity of ZN stain was detected in the lymph node biopsies. All the cases were shown to be positive by PCR. Of the secreted antigens, only MPT64 was consistently detected in both cases with sensitivity and specificity of (100%).

2.3.5.4.3. Anti ESAT-6 antibody

ESAT-6, is the prototype of a novel family of small proteins 6 kDa of unknown function produced by Actinobacteria. Commercial polyclonal anti-ESAT-6 antibody recently being used as diagnostic tool of TBL (Brodin, *et al*. 1996).

Many different studies wereevaluating the role of this antibody in diagnosis of TBL. Study carried out by Sumi and Radhakrishnan, (2009) was precisely designed to evaluate the potentialdiagnostic application of IHC using a panel of antibodies against MTBantigens for the diagnosis of TBL. IHC was performed on theformalin-fixed paraffin sections of lymph node biopsies using rabbit polyclonal antibodies against fourrecombinant mycobacterial proteins, that is, ESAT-6, HspX, Tb8.4 and PlcA. The results ofIHC were correlated with ZN staining method. IHC using ESAT-6 antibody, was found to be highly sensitive (88.6%) and highly specific (93.4%).

2.3.5.4.4. Monoclonal anti 38-KD antibody

The 38-KD protein is one of the most important antigens of MTB, it actively secreted and attached to the surface of the mycobacterial cell by a lipid tail that may also be responsible for binding of carbohydrate to the

protein, (Morten and Harald, 1992), this protein recently used to produce monoclonal anti 38KD for IHC assay. Review of published English literature shows reports of occasional application of IHC using polyclonal and monoclonal antibodies raised against 35 kDa, 65 kDa, 24KDa proteins (antigens) for detection of mycobacterial antigens in various clinical and experimental specimens and that too mainly in tissue sections (Choudhary, *et al*. 1994). Barbolini, *et al* (1989) experimented with four types of monoclonal antibodies raised in mice against different proteins of Mycobacterium tuberculosis and observed that antibody 61.3 to 35 KD protein of MTB was species specific for *Mycobacterium tuberculosis* complex and was not reactive to *Mycobacterium kansasii*.

Direct visualization of MTB or their products in clinical EPTB specimens, was done by Madhu, *et al.* (2012) using IHC. Immunostaining with monoclonal antibody to 38-kDa antigen of MTB was done in fresh and archival fine needle aspirates and tissue granulomata of 302 cases of EPTB and was compared with the PCR, conventional Z.N staining and culture. Diagnostic indices for all types of archival and fresh material varied from 64 to 76% in nucleic acid amplification and 96 to 98% in ICC. There was no significant difference in the diagnostic indices of ZN staining and/ or ICC in fresh or archival material whereas the sensitivity of PCR differed significantly in fresh versus archival material both in cytology (71.4% vs 52.1%) and histology (51.1% vs 38.8%).

The utility of immunohistochemical staining for the diagnosis of TB in intestinal tissue biopsy was investigated by Yasushi, *et al.* (2012). They used 10 patients (4 males and 6 females) with intestinal TB. Laboratory tests include, hsitopatholoy, Z.N staining, PCR using IS1160 and IHC stain using a commercially available species-specific monoclonal antibody to the 38-

kDa antigen of the MTB were used. The histopathological data revealed that tuberculous granulomas were present in 4 cases (40%). IHC staining with anti-MTB monoclonal antibody revealed positive findings in 4 patients (40%); the same patients in which granulomas were detected by hematoxylin and eosin staining.

The advantage of immunostaining over conventional Ziehl Neelsen staining was reported by Goel and Budhwar, (2007). They used IHC staining using species-specific monoclonal anti-body to 38 kDa protein of Mycobacterium tuberculosis complex and ZN staining for acid fast bacilli, the tests were done on 69cases, 36 cases of confirmed EPTB and 33 non-tuberculous cases, in archival formalin fixed paraffin embedded tissue sections. AFB positivity was observed in only 36.1% of tuberculous granulomas while IHC staining was positive in 100% of tuberculous granulomata with zero false positivity and negativity.

Species-specific IHC localization of MTB complex in fine needle aspirates of tuberculous lymphadenitis using antibody to 38 kDa immunodominant protein antigen was examined by Goel and Budhwar, (2008). The worker assessed the 38-kDa protein antigen as a diagnostic adjunct to conventional cytomorphology and its advantage over Ziehl-Neelsen microscopy. IHC staining was positive in 59 of 61 (96.7%) archival and 110 of 113 (97.3%) fresh FNA smears. ZN positivity for AFB was observed in 27 of 61 (44.2%) archival and 48 of 113 (42.4%) fresh FNA smears of TBL.

Sensitivity of 74% and specificity of 100% for IHC stain in detection of MTB complex were obtained by Ashok, *et al.* (2002). They used 50 cases of *tuberculous lymphadenitis* and 10 to assess the advantage of IHC over Z.N stain.Pooja, *et al.* (2014) assessed the role of IHC in detection of

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mycobacterial Antigen in 65 cases of extrapulmonary TB. They reported Sensitivity and specificity of immunostaining of 96.92% and 95%, respectively, with very low sensitivity of Z.N (21%).

2.3.5.5. Molecular Testing

In recent years, novel diagnostic modalities of nucleic acid amplification to detect EPTB have increasingly developed (Abbara and Davidson, 2011). These diagnostic techniques such as PCR which offering better accuracy than AFB smear microscopy and greater speed than culture (Katoch, 2004; Jacob, *et al*. 2008; Haldar, *et al*. 2011), and fluorescent in situ hybridization (FISH), which has greater sensitivity and specificity than PCR (2005; Fenhalls, 2002; Lehtola, *et al*. 2006).

2.3.5.5.1. Polymerase chain reaction

PCR is a fast and useful technique for the demonstration of MTB DNA fragments in patients with clinically suspected with TBL. Various gene targets such as IS6110, 16S rRNA gene, 65 kDa protein gene, MPT-64 protein gene, 38 kDa protein gene, and MTP-40 protein gene have been employed to evaluate the diagnostic utility of PCR in several clinical types of EPTB samples (Martins, *et al*. 2000; Bandyopadhyay, *et al*. 2008; Garcia, *et al*. 2009; Haldar, *et al*. 2011). IS6110 oligonucleotide is most commonly used gene in detection of MTB. The reason for widely used IS6110 in PCR tests is the presence of its multiple copies in MTB complex genome, which is believed to confer higher sensitivity (Lima, *et al*. 2003; Rafi, *et al*. 2007; Jin, *et al*. 2010).

As indicated in **Table 2.2** Sensitivity and specificity ranged from (50%-100%) and (80%-100%) were reported by many worker for IS6110 gene in different types of extrapulmonary specimens rather than TBL. In contrast,

IS6110 PCR gene, have been employed by many researcher to diagnose tuberculous lymphadenitis in formalin-fixed paraffin-embedded tissues to report sensitivity ranged from (42%-100%) and specificity ranged from (87%-100) for this gene. However, several inhibitors such as host proteins, blood and even eukaryotic DNA in extra-pulmonary specimens are from this variation in sensitivity of PCR results (Gan, *et al*. 2002; Haldar, *et al*. 2011; Sun, *et al.* 2011).

Table 2.2. Sensitivity and specificity of IS6110 PCR tests using different gene targets for the diagnosis of different types of EPTB

Reference	Type of EPTB	NAA test	Gene target	Sensitivity (100%)	Specificity (100%)
Totsch et al. (1996)	TB lymphadenitis	Nested PCR	IS6110	89	100
Moussa *et al.* (2000)	Genitourinary TB	Real-time PCR	IS6110	96	98
Gan *et al.* (2002)	Abdominal TB	PCR	IS6110	64	100
Ogusku *et al.* (2003)	Cutaneous TB	Nested PCR	IS6110	100	100
Verettas *et al.* (2003)	Osteoarticular TB	Nested PCR	IS6110	100	100
Pahwa *et al.* (2005)	TB lymphadenitis	PCR	IS6110	90	86
Jambhekar *et al.* (2006)	Osteoarticular TB	PCR	IS6110	85	80
Rebollo *et al.* (2006)	Disseminated TB	PCR	IS6110	50	100
Zamirian *et*	Pericardial TB	PCR	IS6110	80	80

al. (2007)					
Pandey *et al.* (2009)	Osteoarticular TB	Nested PCR	IS6110	73	94
Nopvichai *et al.* (2009)	TB lymphadenitis	PCR	IS6110	67	100
Jin *et al.* (2010)	Abdominal TB	PCR	IS6110	58	100
Sharma *et al.* (2010)	TB lymphadenitis	PCR	IS6110	100	92
Sharma *et al.* (2011)	Osteoarticular TB	PCR	IS6110 + MPB-64	82-100	100
Cortez *et al.* (2011)	TB lymphadenitis	PCR	IS6110	63	87
Derese *et al.* (2012)	TB lymphadenitis	PCR	IS1081	42	89

2.3.5.5.2. Fluorescence in situ hybridization

In situ hybridization (ISH) was independently developed by two research groups (Pardue and Gall, 1969; John, *et al.* 1969). Radiolabeled DNA or 28S RNA was hybridized to cytological preparations from Xenopus oocytes and detected by mi-croautoradiography. This technique allowed nucleic acid sequences to be examined inside cells without altering the cell's morphology or the integrity of its various compartments. Since then, ISH has been modified for studies of chromosomal development, chromosome analysis of tumors and leukemia, and cytogenetic studies of a wide range of species. It was finally introduced into bacteriology by Giovannoni, *et al.* (1988), who was the first to use radioactively labeled rRNA-directed oligonucleotide probes for the microscopic detection of bacteria. With the development of fluorescent labels (Pinkel, *et al.* 1986; Pinkel, *et al.* 1988),

radioactive labels were steadily supplanted by non-isotopic dyes. In 1989, DeLong for the first time used fluorescently labeled oligonucleotides for the detection of single microbial cells. Compared to the radioactive probes, fluorescent probes are safer, they offer better resolution and do not need additional detection steps. Moreover, fluorescent probes can be labeled with dyes of different emission wavelength thus enabling detection of several target sequences within a single hybridization step. Over the last decade, higher sensitivity and speed have made FISH a powerful tool for phylogenetic, ecologic, diagnostic, and environmental studies in microbiology (Amann *et al.* 1990).

Fluorescence in situ hybridization detects nucleic acid sequences by a fluorescently labeled probe that hybridizes specifically to its complementary target sequence within the intact cell. The procedure includes the following steps: (i) fixation of the specimen; (ii) preparation of the sample, possibly including specific pretreatment steps; (iii) hybridization with the respective probes for detecting the respective target sequences; (iv) washing steps to remove unbound probes; (v) mounting, visualization and documentation of results. There are different ways of labeling. Direct fluorescent labeling is most commonly used and is also the fastest, cheapest and easiest way because it does not require any further detection steps after hybridization (Spear, *et al.* 1999). Prior to hybridization bacteria have to be fixed and permeabilized for penetration of the fluorescent probes into the cell and to protect the RNA from degradation by endogenous RNAses. Fixation can employ precipitating agents like ethanol or methanol, cross-linking agents like aldehydes. Optimal fixation should result in good probe penetration, retention of the maximal level of target RNA (Jurtshuk, *et al.* 1992; Brown-Howland, *et al.* 1992; Roller, *et al.* 1994). For better attachment of

specimens to glass slides, it is recommended to treat the surfaces first with a coating agent. Chemicals that have been successfully used include gelatin (Amann, *et al*. 1990), poly-L-lysine (Lee, *et al*. 1999) or silanating agents (Moter, *et al*. 1998). However; Hybridization process must be carried out under stringent conditions for proper annealing of the probe to the target sequence. Stringency can be adjusted by varying either the formamide concentration or the hybridization temperature. Formamide decreases the melting temperature by weakening the hydrogen bonds, thus enabling lower temperatures to be used with high stringency. The hybridization takes place in a dark humid chamber, usually at temperatures between 378C and 508C. Hybridization time varies between 30 min and several hours. Afterwards slides are briefly rinsed with distilled water to remove unbound probe. Post-hybridization stringency washes are performed as required. To reduce the amount of toxic waste, stringency of the washing buffer can be regulated by varying the salt concentration instead of by using formamide. Finally, slides are rinsed with water again, dried and mounted. For microscopy, a conventional epifluorescence microscope equipped with narrow-band-pass filter sets for multicolour FISH can be used. The most striking problem and limitation of FISH is auto-fluorescence of certain bacteria like members of the genus Pseudomonas. (Margo and Bombardier, 1985; Graham, 1983), Legionella (Wilkinson, *et al*. 1990), cyanobacteria (Schonnhuber. *et al.* 1999) and archaeal species like methanogenes (Sorensen, *et al*. 1997) various tissues that contain elastin and collagen or blood cells like erythrocytes and eosinophile granulocytes usually exhibit bright autofluorescence (Van de Lest, *et al*. 1995; Monici, *et al*. 1995).

3. Materials and Methods

3.1. Study design

This is descriptive analytical cross sectional study aimed to evaluate the utility of PCR, IHC and ZN stain in detection of MTB in histological sections. The study was conducted during the period from July 2012 to July 2015. All samples were collected from different histopathology Laboratories in both public and private sectors (Total Lab care, Laboratories Administration).

3.2. Materials

Formalin- fixed, paraffin- Embedded (FFPE) blocks, were used in this study. Demographic and clinical data were retrieved from each target laboratory records. All specimens without histopathological evidences of tuberculosis were excluded.

3.3. Sample size

One hundred sixty one tissue blocks were included in this study. The age were ranging from 4 to 85 years old. The sample size was calculated using software available at http://www.surveysystem.com/sample-size-formula.htm

$$\text{Sample size} = \frac{t^2 \times p(1-p)}{m^2}$$

Description:

m = margin of error at 5% (standard value of 0.05).

t = confidence level at 95% (standard value of 1.96)

p = estimated prevalence of MTB in the study area

3.4. Sample processing

Three tissue sections (5 micron) were sectioned from each specimen in rotary microtome. These sections were used for performance H&E, ZN stain and IHC. Tissue sections of 30 micron were sectioned from each specimen for PCR assay. Procedures for performance of these techniques are described in the hard copy of this proposal.

3.4.1. Hematoxylin and Eosin

The first section of (5 μm) was stained using conventional H and E stain procedure for histopathology diagnosis (Bancroft and Marilyn, 2006). Then the slides were evaluated for the presence of MTB evidences.

3.4.2. Ziehl-Neelsen stain

All sections for ZN satin were dewaxed in xylene 5 for minutes, three times, and rehydrated through descending grades of ethyl alcohol beginning with 100% ethyl alcohol, then 90% ethanol, 70% ethanol and finally to distilled water, 4 minutes for each change, then the sections were placed in pre-heated working solution of carbol fuchsine at 58 -60oC water bath for 10 minutes, The were washed in running water for 2 minutes. Differentiation with 3% hydrochloric acid in 95% ethyl alcohol was done to all sections until no more color runs from the slide, then the sections were washed briefly in water to stop the reaction of acid alcohol. Counterstain with 0.25% methylene blue in 1% acetic acid 15 to 30 sec was done to all section. After that, the sections were dehydrated through ascending grades of alcohol and immersed in xylene for 5 minutes, then mounted with Distrene, Plasticiser, and Xylene (DPX) mounting media.

3.4.3. Anti 38KD Immunohistochemistry.

For immunohistochemical satin, 5 µm thick sections were cut on poly-L-lysine-coated slides from each paraffin wax blocks. The sections were de-paraffinized in 2 changes of xylene for 7 minutes each, and rehydrated using descending graded ethanol concentrations (100, 100, 100, 90, 70%), and three changes of distilled water 2 minutes each, followed by three changes of phosphate buffer Saline (PBS) pH 7.4 which was provided as stock solution and prepared as one bag in one liter of deionized water as adjusted by the manufacturers. The sections were retrieved in retrieval solution which was ready to use citrate buffer pH 6.0 as adjusted by the manufacturers, and the duration was adjusted after different time intervals to one hour. Slides left for natural cooling for 20 min, washed with PBS three changes 3 minutes each. The endogenous peroxidase activity was suppressed by Appling 3% hydrogen peroxide to the all sections for 15 minutes, followed by PBS three changes 3 minutes each. Also protein block was applied to all sections for 15 min, followed by PBS three change 3 minutes. The slides were placed in dark humid chamber of Ventana bench mark immunostainer machine for the upcoming steps. The excess water was drained and sealing with IHC sealing pen (Pap pen) was done around the tissue area, to prevent the solution wasting. Mouse monoclonal anti 38-KD antibody which was provided as stock solution and prepared as 1:50 using PBS pH 7.4 as adjusted by the manufacturers, was applied to all sections for 50 minutes, followed by PBS three change 3 minutes.

The immunoreactivity of antibody with target antigen was detected by using exposed detection system (universal immunohistochemical detection kit from Abcam, UK) in two steps. Initially anti mouse complementary antibody was added to all section for 15 min, followed by PBS wash three

changes 3 minutes, and subsequently followed by rabbit anti mouse conjugated Horse reddish peroxidase (HRP) anti body for 15 min, followed by PBS wash three changes 3 minutes, then (Diaminobenzidine)DAB stock solution and the DAB diluent were immediately mixed and applied to the sections for 10 minutes, controlled microscopically, followed by distilled water wash so as to stop the reaction. Nuclear stain was obtained by 3 minutes application of Mayer's Hematoxylin, followed by bluing for 10 minute in running tab water, to change the pH of the tissue sections and changing the color of Mayer's Hematoxylin from red to blue color, after that the sections were dehydrated through ascending grades of alcohol and immersed in xylene for 5 minutes, then mounted with DPX mounting media. During each IHC batch assay, negative and positive controls provided by the manufacturer were used, to detect the reactivity of antibody and to exclude the false positive result.

3.4.4. Polymerase chain reaction for detection of MTB

3.4.4.1. DNA Extraction

Ten μm tissue sections were cut and placed in ebendorf tubes. DNA extraction was done for all tissue samples by kit for rapid extraction of FFPE, of (Adilab biotechnologies co) Manufacturer Company for in vitro use.

Dewaxation was obtained by adding 1 ml of Xylene to the tissues in the ebendorf tube and vortex for 10 seconds, tissues were centrifuged for 3 minutes 13000/round per munite (RPM), incubated in the water path for 3 minutes at 50 degree Celsius. After that the tissues were centrifuged in the highest speed for 2 minutes, the supernatant xylene was disposed. 1 ml of methanol was added, vortex and centrifuged for 2 minutes in high speed and

the supernatant was discarded, this step was repeated 6 timed until no xylene remains in the tissues. Then the tissues allowed to dry for 10 minutes.

To the dry tissues 200 µl of Lysate together with 20µl of Proteinase K enzyme were added, mixed and incubated overnight at 37 degree celsius. 10µl of Protease K was added mixed well and incubated at 55 degree centigrade for 1 hour.

200µl of liquid CB was added and vortex for 30 seconds and then incubated at 70 degree celsiusfor 10 minutes.100µl of isopropanol was added with 30 minutes vortex. All content transferred to adsorption column (AC), centrifuged for 1 minute at 13000 RPM. The waste was discarded and 500µl of IR was added to the adsorption column, centrifuged for 30 seconds at 12000 RPM. The waste was discarded and 700µl of washing buffer was added with 12000 RPM centrifugation for 30 seconds. After removal of waste other 500 µl of washing buffer was added with 12000 RPM centrifugation for 30 seconds, then the ACs were transferred to clean tubes and centrifuged for 2 minutes at 13000 RPM. The AC were transferred to a new clean tube for the second time and elution buffer (EB) was added as 100µl , incubated at 65 degree centigrade in water bath for 5 minutes, then they were centrifuged for 1 minute at 12000 RPM. The extracted DNA is ready to use for PCR.

3.4.4.2. Amplification of IS1160

All the tubes required for the test and control were prepared and labeled. The master reaction mix was prepared by adding 5µl *n of PCR-mix-1, 10µl *n of 2.5x buffer and 0.5 µl *n of Taq F Polymerase, 15 µl of the reaction mix was added to each tube, and 25 µl of mineral oil was added to each tube. 10 µl of DNA was added to the test tubes and 10 µl of DNA buffed was added to the tube of Negative control, 10 µl was added from the internal

control to the internal control tube, and 10 µl of the positive control was added to the appropriate tube. The tubes mixed and transferred to thermocycler with the program obtained by the manufacturers described in **Table 3.2.**

Table 3.1. PCR Program used for amplification of IS16110 oligonucleotide.

Step	Temperature C^O	Time	Cycles
Thermocyclers with block temperature adjustment: ''MiniCycler'' ''PTC-100''(MJ research)			
1	95 C^O	Pause	
2	95 C^O	15min	1
3	95 C^O	30sec	42
	70 C^O	40sec	
4	72 C^O	2min	1
5	10 C^O	Storage	

3.4.4.3. Gel electrophoresis

The PCR products were visualized in 2% Agarose gel with 0.5 µg/ml ethidium bromide staining. The gel was prepared by dissolving 0.7 gm of agarose powder in 35 ml of 1X Tris/Borate/EDTA(TBE) buffer and heated at 65°c until the agarose completely dissolved, then left to cool at room temperature and 2 µl ethidium bromides was added. The comb was then placed appropriately in the electrophoresis tray and then gel was slowly poured and left to set for 30 min for solidification. In a clean Eppendorf tube 10 µl of 1000bp DNA ladder and PCR product was loaded on the gel. Gel-electrophoresis was performed at 120V and 36 Am for 60 minutes. Pictures

were taken by Gel documentation system (Gel mega, digital camera and software in a computer).

3.4.4.4. Interpretation of PCR result

According to manufacture MTB PCR kit (from Sacace technologies-Casera –Italy) manual, the PCR product length for MTB should be 390 bp.

3.5. Statistical analysis

Analysis of data was performed using Statistical Package for Social Sciences (SPSS) software version 20.The calculation of the expression of 38-KD among cases of lymphadenopathy was determined by obtaining Odd ratios. Variations were determined by using Qui-square and fisher's expect tests. Level of significance was set at P Value less than 0.05.

3.6. Ethical consideration

All specimens were collected after permission from the assigned Laboratories.

4. Results

In this study, 161 specimens were diagnosed as having tuberculosis witn H and E. The minimum age of was 4 years and the maximum was 80 years with a mean age of 51 years.

The study population was divided in to two group pediatric 42 (26%) and adults 119 (74%) as described in **Figure 4.1**. The distribution of study population by gender was 85(53%) were females, 76(47%) were males. Of 76(47%) males, 21 were pediatric and 55 were adults, and of 85(53%) were females of which 21 were pediatric and the remaining 64 were adults.

The study population was subsequently subdivided in to other groups according to age range started from <8 years up to 51+. The frequencies of patient's with TB infection were increased in age group (19-30), which represented 63 (39%) of study population, followed by age group (8-18) and (31-40) which represented 29 (18%) and 25 (16%) respectively. Age group 51+, <8 years and (40-50) represent 20 (12%), 13 (8%) and 11 (7%) respectively as indicated in **Table 4.1.**

Figure 4.2.Describes the distribution of the study population by lymph node site. The great majority of the specimens were obtained from cervical lymph node which representing 100 (62%), followed by axillary lymph node representing 17 (11%).The other sites included mediastinal, mesenteric, inguinal, and submandibular, constituting 10 (6%), 7 (4%), 7 (4%), and 4 (3%) respectively, the remaining was unknown representing 16 (10%).

4.1. Hematoxylin and eosin

By using H and E stain, both necrotic and non-necrotic granulomas with epithelioid cells and multinucleated giant cells characteristic of tuberculosis were observed in in our study population. Those showing histopathological

pattern containing (giant cells + granuloma + caseation) were considered as strong evidence as illustrated in **Photomicrograph 4.1**, and the other showing less evidences (e.g., ill-defined aggregates of epithelioid histiocytes only, granulomas without necrosis and giants cells, etc.) were considered as weaker evidence as illustrated in **Photomicrograph 4.2.**

Accordingly, of the 161 cases, 118 (73.3%) were categorized as having strong evidences (positive) and the remaining 43(26.7) were detected with weaker evidences (positive), cases as shown in **Figure 4.3.** Out of entire 118 (73.3%) strong evidences cases, 75(46.6%) were cervical lymph node, 7(4.3%), 12(7.5%), 5(3.1%), 5(3.1%), 3(1.9%), and 11(6.8%) were mediastinal, axillary, mesenteric, inguinal, sub-mandibular, and unknown respectively. On other hand of 43 (26.7%) cases were at negative level, 25(15.5%) were cervical, 3(1.9%), 5(3.1%), 2(1.2%), 2(1.2%), 1(0.6%), and 5(3.1%) were mediastinal, axillary, mesenteric, inguinal, sub-mandibular, and unknown respectively. Statistically, no significant association between TB evidences and lymph node site, *P-value*=0.998.

4.2. Ziehl-Neelsen stain

Of (n=161) studied lymph nodes, only 4 (2.5%) were demonstrated as positive in ZN (in all cases more than 5 bacilli were seen), and 157(97.5%) were negative with ZN stain. Out of entire 4 (2.5%) ZN positive cases, 3(1.9%) were cervical lymph node, and one case were inguinal. Of 157 (97.5%) ZN negative cases, 97(97.5%) were cervical, 10(6.2%), 17(10.6%), 7(4.3%), 6(3.7%), 4(2.5%), and 16(9.9%) were mediastinal, axillary, mesenteric, inguinal, sub-mandibular, and unknown respectively. Statistically, no significant association between ZN stain and lymph node site, *P-value*=0.998**.** Also out 4(2.5%) ZN positive cases were previously found as strong evidence positive, while the remaining 114(70.8%) strong

positive evidences were negative with ZN stain. Statistically, no significant association between TB evidences and ZN stain, *P-value*=0.221 as indicated in **Table 4.2**.

4.3. Anti 38-KD immunohistochemistry

Immunoexpression of monoclonal anti 38-KD was showed in area of giant cells and granuloma as shown in **Photomicrograph 4.3.** In this study out of 161 studied cases, 129 (80.1%) of cases were showed positive expression for 38-KD, the remaining 32 (19.9%) were negative. The correlation between lymph node site and IHC results were found as follow, out of entire 129 (80.1 %) IHC positive cases cases, 79(49.1%) were cervical lymph node, 7(4.3%), 13(8.1%), 6(3.7%), 6(3.7%), 3(1.9%), and 15(9.3%) were mediastinal, axillary, mesenteric, inguinal, sub-mandibular, and unknown respectively. On other hand of 32 (19.9%) IHC negative cases, 21(13%) were cervical, 3(1.9%), 4(2.5%), 1(0.6%), 1(0.6%), 1(0.6%), and 1(0.6%) were mediastinal, axillary, mesenteric, inguinal, sub-mandibular, and unknown respectively. Statistically, no significant association between IHC stain and lymph node site, *P-value* = 0.998. On other hand out of the entire 129(80.1%) IHC positive cases, 100 (62.2%) were identified as strong positive evidences and the remaining 29 (19.9%) were at negative level. Statistically, IHC stain is significantly associated with TB evidences *P-value* = 0.015 as shown in **Table 4.2.**

4.4. Polymerase chain reaction for IS6110

The IS6110 PCR was positive in test of Mycobacterium strains used as positive controls and negative for test that used as negative control **Photomicrograph 4.4.** Out of 161 studied lymph nodes, 135 (83.8%) were positive for IS1160 PCR and the remaining were negative, from the entire

135 (83.8%) PCR positive, 106(65.8%) cases were previously found as strong evidence positive and 29(18%) was found weak evidence. Of the remaining 26 (16.2%) PCR negative specimens, only 12(7.5%) specimens were showed strong evidence for TB, and 14(8.7%) were weak evidence, statistically, PCR is significantly associated with TB evidences, *P-value*=0.001 as showed in **Table 4.2.** Out of entire 135 (83.8%) PCR positive cases, 83 (51.6%) were cervical lymph node, 7(4.3%), 14(8.6%), 6(3.7%), 6(3.7%), 4 (2.5%), and 15 (9.3%) were mediastinal, axillary, mesenteric, inguinal, sub-mandibular, and unknown respectively. On the other hand, of 26 (16.2%) PCR negative cases, 17(10.5%) were cervical, 3(1.9%), 1(0.6%), 1(0.6%), 1(0.6%), 0(0.0%), and 1(0.6%) were mediastinal, axillary, mesenteric, inguinal, sub-mandibular, and unknown respectively. Statistically, no significant association between PCR result and lymph node site, *P-value*=0.749.

In this study PCR result also was correlated with other diagnostic methods. **Table 4.3** shows the correlation of PCR results with ZN stain, of entire 135 (83.8%) PCR positive cases, only 4(2.5%) cases were positive with ZN satin, the remaining 131(81.4%) were negative with ZN stain, statistically, no significant association between ZN results and PCR results, *P-value*=0.374. The correlation between PCR result and IHC results was described in **Table 4.4**, out of 135 (83.8%) PCR positive cases, 129(80.1%) were positive with IHC, while the remaining 6(3.7%) cases were negative with IHC. Statistically, IHC results is significantly associated with PCR result, *P-value*=0.000.

Table 4.5. Exploring the diagnostic validation of different tests using PCR as gold standard. Accordingly the sensitivity and specificity of histopathology diagnosis & ZN stain were 78.5%, 46.1% ($\chi 2$ = 11.6,

p<0.05) and 3.0%, 100% ($\chi 2 = 0.790$, p> 0.05) respectively. In contrast the sensitivity & specificity of anti 38KD IHC was 95.5%, 100% ($\chi 2 = 124.9$, p< 0.05) respectively.

Table 4.1. Distribution of the study population by age.

Age\ Year	Frequency	Percent	Cumulative Percent
<8 years	13	8.1	8.1
8-18	29	18.0	26.1
19-30	63	39.1	65.2
31-40	25	15.5	80.7
41-50	11	6.8	87.6
51+	20	12.4	**100.0**
Total	**161**	**100.0**	

Table 4.2. Compression of different techniques in identification of MTB

Results		ZN		Anti 38-KDa IHC		IS6110 PCR	
		+Ve	-Ve	+Ve	-Ve	+Ve	-Ve
H.E	Strong MTB Evidence	4 (2.4%)	114 (70.8%)	100 (62.1%)	18 (11.2%)	106 (65.8%)	12 (7.5%)
	Weak MTB Evidence	0 (0%)	43 (26.7%)	29 (18%)	14 (8.7%)	29 (18%)	14 (8.7%)
Total		**135 (2.5%)**	**157 (97.5%)**	**129 (80.1%)**	**32 (19.9%)**	**135 (83.9%)**	**26 (16.2%)**
P. Value		*0.221*		*0.015*		*0.001*	

Table 4.3. The correlation between ZN stain and PCR results.

Results		IS6110 PCR		Total
		Positive	*Negative*	
ZN Stain	*Positive*	4(2.5%)	0(0%)	4(2.5%)
	Negative	131(81.4%)	26(16.1%)	157(97.5%)
Total		**135(83.8%)**	**26(16.2%)**	**161(100%)**
P. Value = **0.374**				

Table 4.5. The correlation between IHC and PCR results.

Results		IS6110 PCR		Total
		Positive	*Negative*	
Anti 38-KDa IHC	*Positive*	129(80.1%)	0(0%)	129(80.1%)
	Negative	6(3.7%)	26(16.2%)	32(19.9%)
Total		**135(83.8%)**	**26(16.2%)**	**161(100%)**
P. Value = 0.000				

Table 4.6. Diagnostic validation of different test using PCR as gold standard.

Diagnostic method	Sensitivity %	Specificity%	PPV%	NPV%
H&E MTB evidence	87.5%	46.1%	100%	32.5%
ZN Stain	3%	100%	100%	16.7%
Anti 38-KD IHC	95.5%	100%	100%	81%
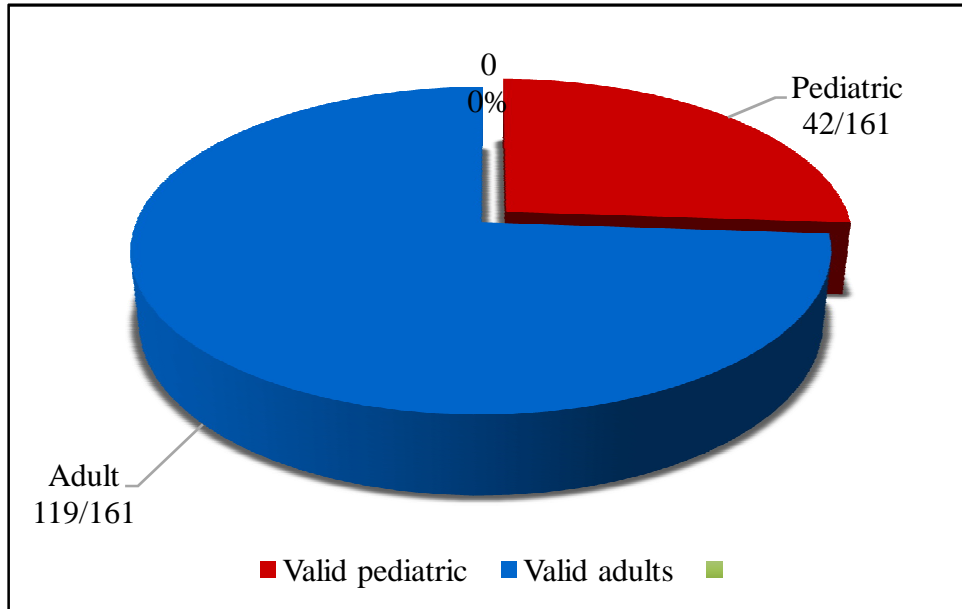
Figure 4.1. Description of the study population by age division.
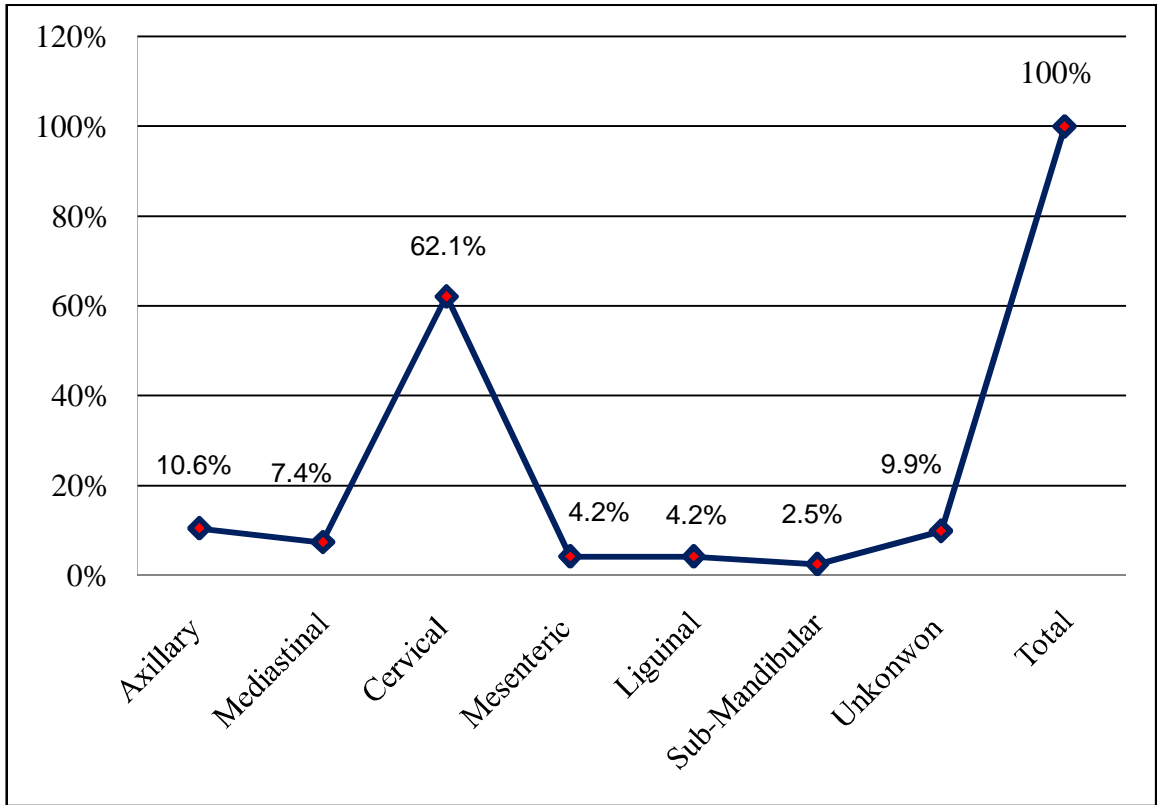
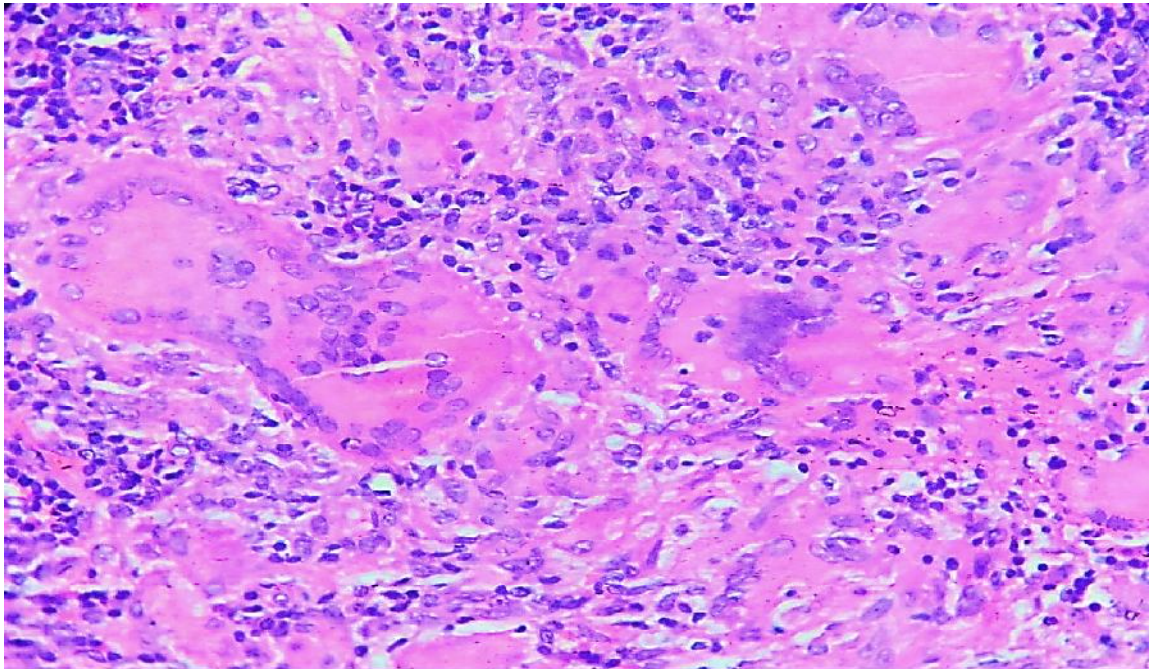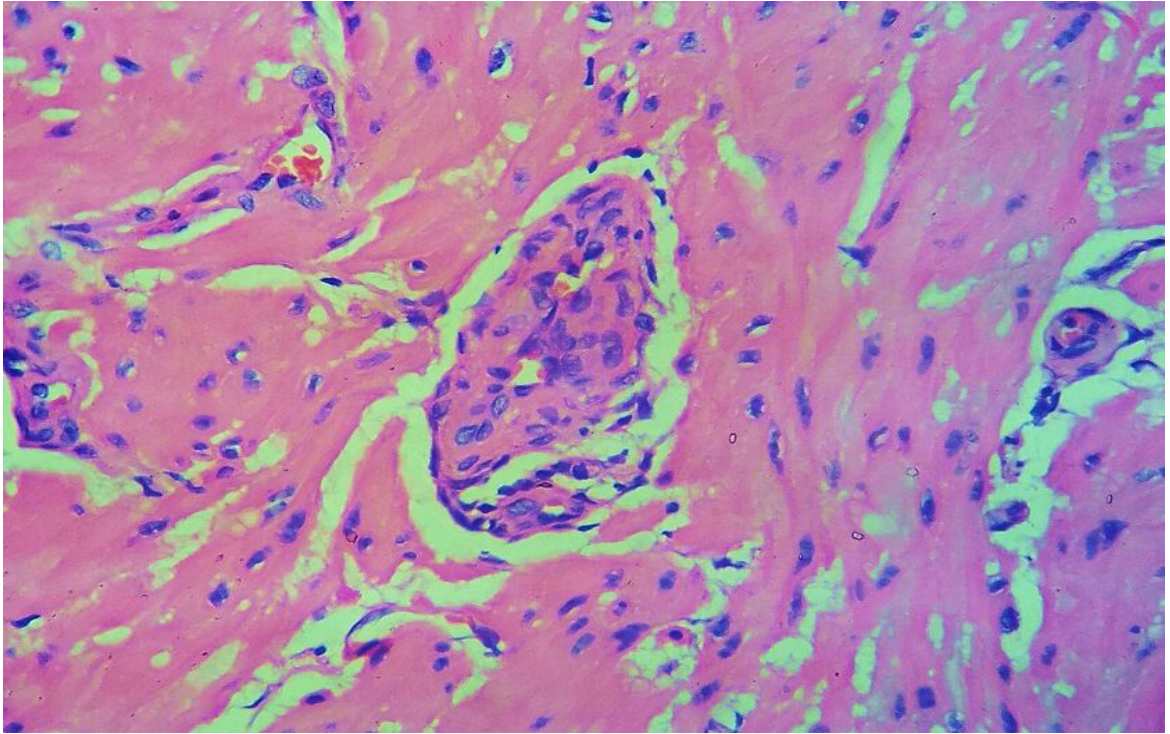**Figure 4.2. Description of the study population by lymph node site.**
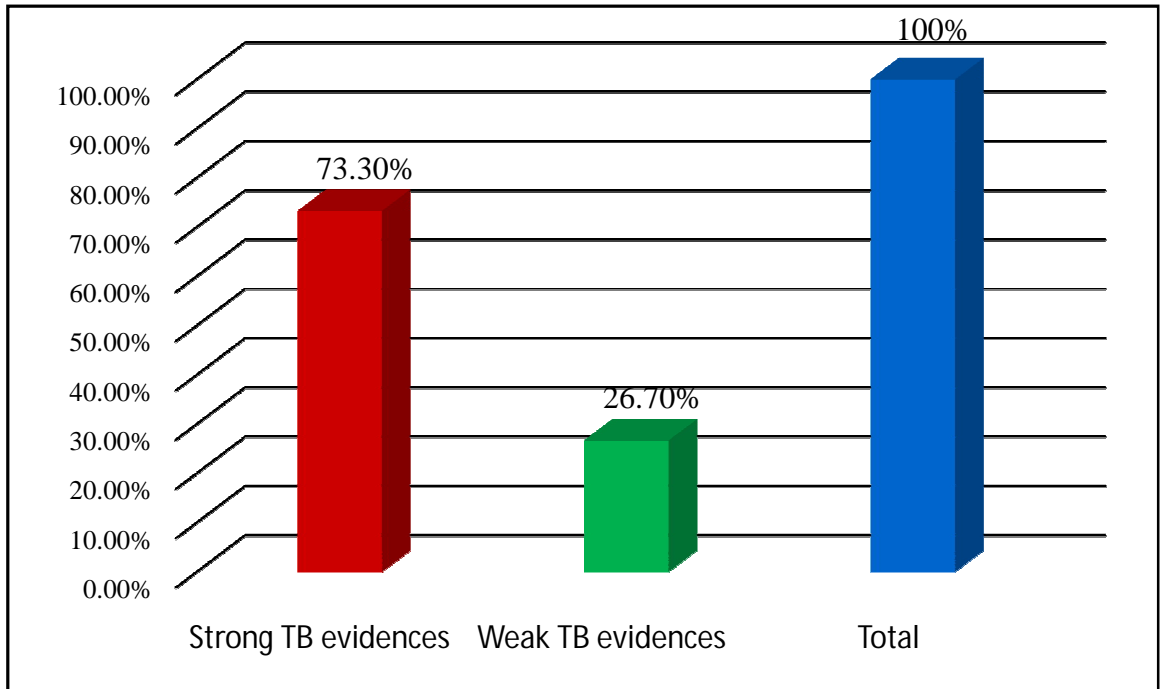
Figure 4.3. Description of the study population by strong and weak MTB evidences.

Photomicrograph 4.1. H and E section showingstrong MTB histopathological pattern containing (Multinucleated giant cells, granuloma and caseation)

Photomicrograph 4.1. H and E section showing weak MTB histopathological pattern containing(ill-defined aggregates of epithelioid histiocytes only, granulomas without necrosis and giants cells)
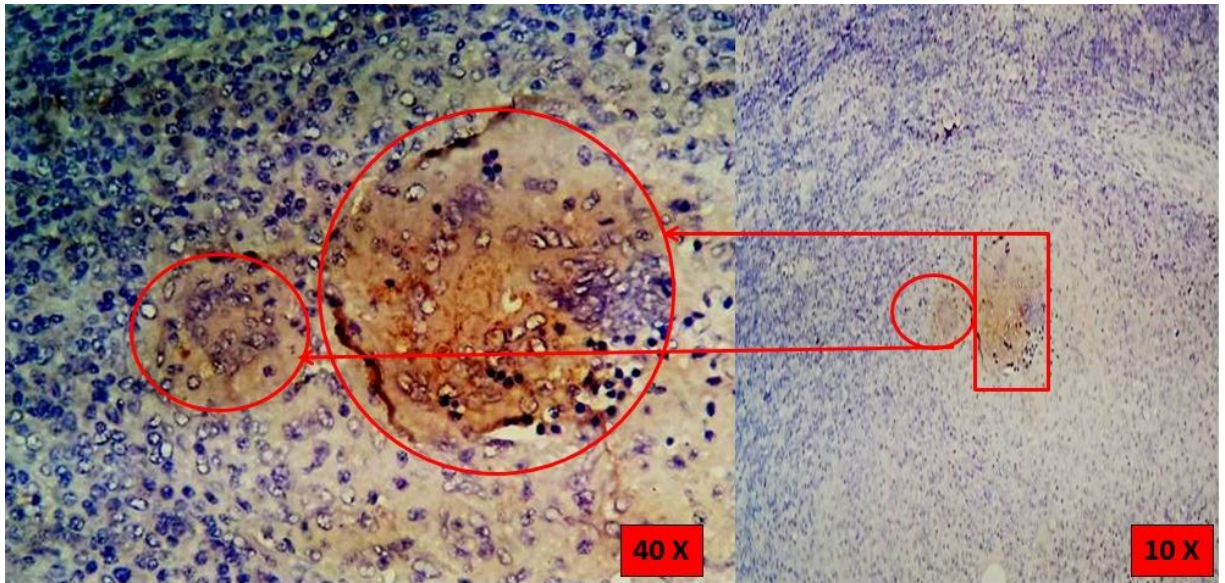
Photomicrograph 4.3. Strong and granular IHC stain of anti-38 KD.
The 38-KD antigen of mycobacterium tuberculosis was stained strongly in epithelioid cells and multinucleated giant cells and also was detected in necrotic area.
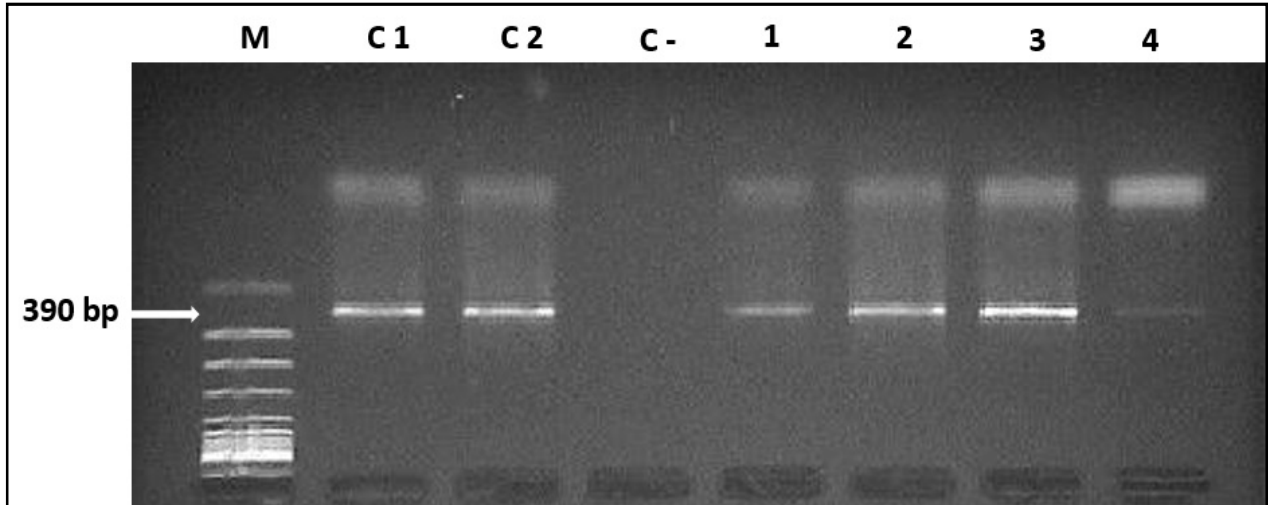
Photomicrograph 4.4. PCR products of the sequence IS6110 were analyzed on 2.0% agarose gel electrophoresis followed by ethidium bromide stain. Lane (M): Marker, lane (C1, C2): Positive control, lane (C): DNA buffer and lane (1, 2, 3 and 4) Positive samples**.**

5. Discussion

Sudan is a large country with miscellaneous population and history of civil conflict. Poverty levels are high with poor health delivery system. The country has a high burden of tuberculosis (TB) with an estimated 50,000 incident cases during 2009, when the estimated prevalence was 209 cases per 100,000 of the population (Ghada, *et al.* 2011). Lymph node is the leading cause of extra pulmonary tuberculosis, the most frequently extra pulmonary TB identified form is Lymph node tuberculosis (Kishore, *et al.* 2008).

The prevalence of tuberculous lymphadenitis has been reported to be higher in developing countries like Ethiopia compared to developed countries (WHO, Tuberculosis Fact sheets, 2014). The situation in Sudan is even grimmer, the incidence and prevalence of lymph node tuberculosis in Sudan is poorly documented. In 1992, Kheiry *et al.* drew attention to the fact that there were an increasing number of cases in Khartoum (Kheiry and Ahmed, 1992).

In this study, 161 lymph nodes tissue biopsies that were previously diagnosed by conventional histopathology as having tuberculosis depending on the presences of variable histological TB evidences were reassessed, and we found that, the prevalence of LNTB was 22.4% which is lower than other study done in Tanzania with prevalence of 69.5% (Perenboom, *et al.*1994). Other studies in Israel, Ethiopia and India showed higher figure

than ours with prevalence of 70%, 72.8% and 73.75% respectively (Weiler, *et al.* 2000; Kakkar, *et al.* 2000; Yassin, *et al.* 2003; Dagnachew, *et al.* 2013). Also our findings is much lower than findings of another Indian study with prevalence of 83% (Dandapat, *et al.* 1990).

Also in a study from Sudan, a total of 670 patients were registered at Kassala hospital with clinical, laboratory and radiological evidence proven TB. Pulmonary TB accounted for 73.4% while extra-pulmonary TB was reported in 26.6% of all TB patients (Tajeldin and Abdel Aziem, 2012). The majority of biopsies were from cervical LN (64.3%), these findings is identical to ours findings, and are in concordance with previously published results from this country carried by Aljafari *et al.* (2004) and study carried in USA by Talavera and Miranda, (2001). Also in Yemen Hussain, *et al.* (2011) reported similar results

In the present study out of 161 of studied subjects, 100/161 (62%) specimens were cervical lymph node. This is in correlation with study by Hussain, *et al.* (2011) which documented 74 (74%) cervical lymph node out of 100 lymph node specimens. Moreover, the study by Chen *et al.* (1992) have shown that, cervical nodes were affected in about 90% of cases.

In the present study, the rate of infection among females was 53% which is slightly higher than infection among males which was 47%. These results to some extend were in agreement with another study by Fatmi and Jamal (2002) which reported that out of (100) lymph node tuberculosis 66 patients (66%) were females and 34 (34%) were males, and the study Hussain, *et al.* (2011)which reported 70 (70%) females and 30 (30%) males.

Furthermore, Hussain, *et al.* (2011)reported that the highest rate of TB infection is an at age ranged from 22-38 which in agreement with our results that showed the tuberculosis infection can affect adults and children at any

time and age in which the disease appeared among the patients at ages starting from 4 to 80 years, but statistically our results showed that the higher frequency of study population were showed in age group (19-30) which represent 63 (39%) of study population, followed by age group (8-18) and (31-40) which represent 29 (18%), respectively, those patients are the most harbor the disease. Therefore, our results are in support to the study Nomani, *et al.* (2007) which reported that the maximum incidence was found to be in the age group 10–30 years.

Moreover the findings of this study showing high frequency of pediatric tuberculous lymphadenitis among patients attending with lymphadenopathy. In the present study out of (n=161) enlarged lymph node, 42 (26%) were pediatrics, the remaining were adult, these findings similar to previously published study from same country carried in 2003 by Aljafari, *et al.* (2004).

However, extra pulmonary tuberculosis is frequently making challenges and there is still difficulty to differentiate it from other diseases and is often misdiagnosed (Bayazıt, *et al.* 2004). Fukushima and collaborators in Japan reported that, various methods for diagnosis used in different countries could be one reason for the high levels of LNTB found (Fukusima, *et al.* 2011). Availability of a rapid, sensitive, specific and reliable diagnosis is an essential element in the management of LNTB.

In developing countries the laboratory diagnosis of LNTB is largely based on histopathology morphological evidence (it cannot differentiate changes caused by M. tuberculosis, non tuberculous mycobacteria or other granulomatous diseases) which is not good, followed by Z.N stain to confirm the presence of bacilli, but ZN staining has very low sensitivity and often is less specific in histological section than molecular tests (Dimoliatis and Liaskos, 2008).

Out of the 161 TB patients, 118 (73%) were found with strong histological evidence of TB. Such findings have been previously reported, separately counting different features (Drobniewski, *et al.* 2000).

There are two specific pathologic criteria for identifying tuberculosis lymphadenitis, caseation and granuloma formation. Caseation has been found to be more specific and sensitive (Finfer and Burstein, 1991). Accordingly, the study subjects were divided in to two group, strong evidence group containing (giant cell, granuloma, caseation), and weak evidence group containing (ill-defined granuloma, granuloma with or without necrosis, epithellioid cell only).

In thisstudy, 118 (73%) lymph nodes were of caseating granuloma type (strong evidences) and 42 (27%) were of non caseating granuloma type (weak evidence). This is in correlation with Woodard, *et al.* (1982) they found 67% caseating granuloma. Other workers reported 76% caseating granuloma and 24% noncaseating granuloma (Lake and Oski, 1978), (Kumar, *et al.* 1998). Fatmi and Jamal, (2002) reported 62% caseating granulomas and 38% non caseating granuloma, also Hussain, *et al.* (2011)reported 32 (32%) caseating and 68 (68) non caseating granulomas, such findings have been previously reported by Drobniewski *et al.* (2000) and Vincent *et al.* (2003).Statistically no significant association between lymph node site and H and E strong or weak evidences *P- Value=0.998*. Similar results were published by Woodard, *et al.* (1992). Another study from Yemen conducted by   Hussain, *et al.* (2011) showed similar findings.

Furthermore, this study came out with the fact that; the acid fastness of MTB in histological section is very poor. Of (n=161) patients only 4 (2.5%) patients were found to be TB positive with ZN. These findings is in agreement with Hussain, *et al.* (2011). Different studies carried out by

Kamboj *et al.* (1994), have reported a wide range of ZN positivity ranging from as low as 0% to as high as 75%. Also our study is in agreement with another study carried in Norway and Tanzania by Tehmina, *et al.* (2006), they used ZN stain to diagnosis LNTB, and they found that, ZN stain has very low sensitivity (12%). However, low AFB positivity could be due to the fact that only the intact bacilli take up the stain or due to intensive phagocytotic activity by macrophages in tuberculous granulomas, the morphological Characteristics of AFB often get distorted, formalin fixation also may play important role in low detectability of ZN staining (Goel and Budhwar, 2008) but another study in 2002 by Hajime *et al* refuse this suggestion, they reported that, the formalin is a water solution of formaldehyde and is not a lipid-soluble agent, the mechanism for its reduction in the sensitivity of acid-fast staining remains unknown (Hajime, *et al*. 2002). In regard to the association between ZN results and LN site. Statistically, there is no significant association between both variable, the *P-Value=0.221*. This finding looks similar to report obtain by Ove (1994). Recently, many complementary diagnostic tools have increasingly been developed for the most important species of mycobacteria (Park, *et al*. 2003). MTB IS6110 oligonucleotides is an attractive target for PCR amplification, and has been tested in intestinal TB and Crohn's disease (Jin, *et al*. 2010). The 38-KD protein is one of the most important antigens of Mycobacterium tuberculosis, it actively secreted and attached to the surface of the mycobacterial cell by a lipid tail that may also be responsible for binding of carbohydrate to the protein, this protein recently used to produce Monoclonal anti 38KD for IHC assay for demonstration of LNTB (Morten and Harald, 1992).

There have been several reports describing the role of IHC in the diagnosis of tuberculosis (Tehmina, *et al*. 2006). The present study illustrated the importance of IHC using species specific monoclonal antibody to 38 KD in identification of archival formalin-fixed, paraffin embedded tissue sections of LNTB and was compared with conventional ZN staining, H&E and PCR. So far and to the best of our knowledge no published study in Sudan has shown the diagnostic utility of IHC in diagnosis LNTB.

In this study IHC results showed positive Anti 38-KD staining with MTB antigens that included whole organisms, their fragments and debris in histological sections of 129/161(80.1%) of cases, while the remaining 32/161(19.9%) were found negative for anti 38-KD. Of these 129 positive IHC cases, only 4 cases were positive with ZN stain. Statistically there is no significant association between ZN stain and IHC results. *P-Value=0.365*. These findings are consistent with study from Norway carried by Ove (1994) when they used ZN and IHC stain to diagnose MTB in 47 samples of EPTB. Also our findings support the result of study from New Delhi by Ashok (2002). Also such findings it looks similar to study those obtained by Tehmina *et al.* (2006). Furthermore, relatively similar report was found in the study by Goel and Bufhwar (2007), when they assesses the advantage of immunostaining over conventional ZN staining. They used IHC staining using species-specific monoclonal anti-body to 38 kDa protein of Mycobacterium tuberculosis complex and ZN staining for acid fast bacilli, the tests were done on 69cases, 36 cases of confirmed EPTB and 33 non-tuberculous cases, in archival formalin fixed paraffin embedded tissue sections. AFB positivity was observed in only 36.1% of tuberculous granulomas while IHC staining was positive in 100% of tuberculous

granulomata with zero false positivity and negativity. Also our findings is in consistent with reports of study in 2008 carried by the same workers when theyassessed the 38-KD antigen as diagnostic adjunct to conventional cytomorphology and its advantage over ZN stain (Goel and Bufhwar, 2008). IHC was positive in 110/113 (97.3) of cases and AFB was observed in 48/113(44.2%) of cases. Also our study is agreement with review of published English literature shows reports of occasional application of immunohistochemistry using different types of antibodies like anti CBG, anti MPT64 and anti ESAT-6 antibody with ZN positivity ranging from zero percent to 44% and IHC positivity from 69% to 100% (Padma, *et al.* 2005; Ulrichs, *et al.* 2005; Sumi and Radhakrishnan, 2009; Tadele, *et al.* 2014; Tehmina, *et al.* 2014).

In 2012 direct visualization of MTB and its products in EPTB specimens, was done in India by Puja and amita using IHC monoclonal anti 38-KD in fresh and archival tissue granuloma. They reported that there was no significant difference in the diagnostic indices of ZN staining and/ or IHC in fresh or archival material (Amita, *et al.* 2012), this report refuse our findings.

When Compare IHC with H and E strong and weak evidence, and found that 100(62.1%) of 129 IHC positive cases were at strong evidence level and the remaining 29 (18%) cases were at negative level. statistically there is significant association between IHC result and H and E result *P-Value=0.015*. This finding support recent study findings obtain by Shirin which carried out in Tehran, Iran, when they assess role histopathological findings and immunohistological staining in diagnosis granulomatous tissue reaction associated with tuberculosis (Shirin, 2014).

In recent years, PCR methods have increasingly been developed for the most important species of mycobacteria. Some tests are already commercially available in Sudan but are not used as a complementary diagnostic tool (Park, *et al.* 2003; Chakaravorty, *et al.* 2005; Therese, *et al.* 2005).

In the present study IS6110 oligonucleotides DNA 390 bp with internal control 750 bp for PCR assay were used, following the same sequence as those used in the PCR test described by Lefmann, *et al.* (2006) and Naser, *et al.* (2002) who reported high sensitivity and specificity of these oligonucleotides in detection of LNTB . Our PCR results showed higher sensitivity and specificity in the detection of MTC in comparison to ZN staining such finding have been previously reported by Rodriguez, *et al.* (2012) who used IS6110 oligonucleotides DNA in detection of LNTB. Fukusima, *et al.* (2011), also reported same sensitivity and specificity for these oligonucleotides, such finding have been previously reported by Pahwa, *et al.* (2005), Sharma, *et al.* (2010) and Cortez, *et al.* (2011) when they used gene target IS6110 in detection of LNTB.

In this study out of (n=161) cases, 135 (84%) of cases were positive with PCR, while remaining were found negative. such findings have been previously reported by Rodriguez, *et al.* (2012). The distribution of PCR positive results among study subjects were found as follow, cervical lymph node express the highest frequency of positive result 83 (62%) of all 135 positive cases, followed by axillary lymph node 14 (10%), mediastinal, mesenteric, inguinal, and submandibular lymph node 7 (5%), 5 (4%), 5 (4%) and 4 (3%) respectively, the remaining was unknown site 15 (11), these findings are in agreement with Verma, and Kumar, they reported that out of 100 cases of lymphadenopathy, 78 (78%) cervical lymph node, 10 (10%)

axillary lymph node expressed positive results with PCR (Verma, *et al.* 2010).

In this study statistically strong agreement between PCR results and IHC results were observed *P-Value=0.000*, as well as our PCR result statistically showed significant association with H&E results *P-Value=0.001*. But statistically there is no significant association between PCR results and ZN stain result P-Value=0.374, this findings is also in agreement with another study carried in Norway by Manju (2007). Similar findings were obtain in study from japan by Yasush, *et al.* (2012).

Here PCR was used as gold standard for comparing the other variables. Accordingly in our study we found that, the Caseation to some extend has been found to be sensitive (78.5%) but less specific (46.1%) with PPV (100%) and NPV (32.5%) comparing with PCR results which used as gold standard in this study (Finfer and Burstein, 1991).

The sensitivity and specificity of ZN stain were 3% and 100% respectively with PPV 100% and NPV 16.7%, these findings support several studies by Gutierrez and Garcia (1993) and Hussain, *et al.* (2011). Study carried by Kohli, *et al.* 2015) aimed to assess the advantage of immunostaining over conventional ZN on diagnoses of TB tissue granuloma, and they found that ZN stain which had the sensitivity, specificity, positive predictive value and negative predictive values of 30.56%, 96.43%, 95.65% and 41.56% respectively these findings are to some extend looks similar to our results.

In contrast we found that immunohistochemistry has sensitivity 95.5% and specificity 100% with NPV 81%, similar results obtained in study elsewhere by Haines and Chelack, (1991). Also such findings have reported by Goel and Budhwar (2008) and Yasushi, *et al.* (2012) also their results,

combined with findings from a previous study carried by Pooja, *et al.* (2014).

Lymphadenopathy due to tuberculosis was found in (84%) of Sudanese in this study using PCR and IHC which is much over than the report in 2012 from the same country by Bilal and Elshibly, (2012) who reported a rate of (10%), and even much over than report of similar study from Greece by Papadopouli, *et al.* (2009) who reported (12%) in 2009. This may be attributed to the low specificity and sensitivity of other technique and strategies for the diagnosis of the lymphadenopathy, that may have a false negative rate of would have been more yielding if combined with PCR, a costly tool of diagnosis in a developing country (Goel, *et al*. 2001).

Conclusion

The presnt study suggest the following conclusion:
- The H and E morphological criteria used for diagnosis of LNTB to some extent have low sensitivity ((87.5%) and it cannot differentiate changes caused by *M. tuberculosis*, non-tuberculous, mycobacteria or other granulomatous diseases and may lead to over-diagnosis, especially in countries with high endemic rates of tuberculosis.
- ZN stain has very low sensitivity (3%) in detection of LNTB in histological section.
- IHC is a rapid, sensitive, and specific method for establishing an etiological diagnosis of tuberculosis in histologic specimens.
- IHC with species-specific monoclonal antibodies to 38 KD protein may be an efficient diagnostic adjunct to conventional ZN staining for the diagnosis of tissue granuloma of extra-pulmonary *tuberculosis*.
- Moreover anti 38-KD IHC of archival lymph node biopsy with MTB would be proven to be good adjunct to morphologic diagnosis.

- Immunohistochemistry has the advantages over PCR of being robust, quicker, and cheaper, and it can be used in high-endemic countries.

Recommendation

- Further studies are needed to clarify the role of IHC in detection of other types of extrapulmonary *tuberculosis* using different types of antibodies of monoclonal origin.
- Further, deeper studies are required for more precise measures of the relationship between PCR reports and presence of histopathological evidence for diagnosis of tuberculous lymphadenitis.
- IHC for diagnosis of MTB can be standardized and performed by trained technicians in routine laboratory.

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