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Molecular Epidemiology and Comparison of Diagnostic Methods of Tuberculous Lymphadenitis, Addis Ababa, Ethiopia

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Molecular Epidemiology and Comparison of Diagnostic Methods of Tuberculous Lymphadenitis, Addis Ababa, Ethiopia

MSc. Thesis

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Dedication

Dedicated to,

in loving memory of my Mom, Tolashi.

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List of Abbreviations

AFB	Acid Fast Bacilli
BAL	Broncho Alveoli Lavage
BCG	Bacillus Calmette-Guerin
BTB	Bovine Tuberculosis
CD	Cluster of Differentiation
CDC	Center of Disease Control
CI	Confidence of Interval
DNA	Deoxy Ribonucleic Acid
DOT	Directly Observed Treatment
DR	Direct Repeat
EDTA	Ethylene Diamine Tetra Acetic Acid
EPTB	Extra-pulmonary tuberculosis
EZN	Erlich Ziehl Neelsen
FNA	Fine Needle Aspiration
FNAC	Fine Needle Aspiration Cytology
HBTCs	High Burden Tuberculosis Countries
Hcl	Hydrochloric Acid
HIV	Human Immunodeficiency Virus
HLDR	Human Leucocyte D-Region
IL	Interleukin
LAM	Latino-American Mediterranean
LJ	Löwenstein-Jensen
MDR	Multi Drug Resistance
MHC	Major Histocompatibility Complex
MTC	Mycobacterium Tuberculosis Complex
NK	Natural Killer
NPV	Negative Predictive Value
NTBLN	Non Tuberculosis Lymphadenitis
NTLC	National TB and Leprosy Control Program
NTSS	National Tuberculosis Surveillance System

PCR	Polymerase Chain Reaction
PI	Principal Investigator
PPD	Purified Protein Derivative
PPV	Positive Predictive Value
PTB	Pulmonary Tuberculosis
RD	Region of Difference
rRNA	ribosomal Ribonucleic Acid
SDS	Sodium Dodecyl Sulfate
SIT	Spoligotype International Type
TASH	Tikur Anbessa Specialized Hospital
TB	Tuberculosis
TBLN	Tuberculosis Lymphadenitis
TGF-β	Tumour Gross Factor betta
Th-T	T-helper
TLCP	TB and Leprosy Control Program
TNF	Tumor Necrosis Factor
WHO	Word Health Organization
XDR	Extensive Drug Resistance
ZN	Ziehl Neelsen

ABSTRACT

Extrapulmonary tuberculosis is a significant health problem worldwide because of difficulties in its diagnosis and in monitoring its treatment, in which tuberculous lymphadenitis is high prevalent. To this effect, adequate knowledge on the species and strains of mycobacteria which circulate among the human population in specific geographic location is required.

Objective: The objective of this study was to determine the prevalence of TBLN and to compare diagnostic performance of laboratory methods in diagnosis of TBLN among clinically suspected TBLN patients; identify the dominant species/ strain of *M.tuberculosis* responsible for TBLN.

Materials and Methods: A cross-sectional study was conducted between February,2013 to October, 2013. Structured questionnaire, fine needle aspiration cytology and histopathology, Ziehl Nelson staining, mycobacterial culture, region of difference (RD)-PCR, spoligotyping and flowcytometry were used for undertaking this study. SPSS version 20 was used for data entry and analysis.

Result: Of the 206 TBLN suspected cases, 166 (80.6%) were positive for TBLN by FNAC and histopathological examinations. On the other hand, only 36% (74/206) were positive by mycobacterial culture. Acid-fast bacilli (AFB) were detected in 28.6% of the 133 TBLN suspected individuals while 79.3% of the 121 TBLN suspected cases were positive for mycobacteria by PCR. Majority (98.6%) of the causative agent of TBLN was *M.tuberculosis*. Further characterization of 74 isolates to strain level by spoligotyping, 57 isolates were classified into one of the 26 shared international types (SITs) according to SpolDB4.0 and the remaining 16 isolates generated 13 different spoligotype patterns which had not been reported to the SpolDB4.0.The most prevalent strains of *M. tuberculosis* isolated in this study were SIT149, SIT53, SIT26 and SIT37 comprising 52.6% of the total strains. The strains were further classified into families in which the most prevalent were T, CAS and Haarlem comprising of 81.1% of the isolates. Classification of the strains into lineages leads to indicated modern lineage was the most prevalent comprising 66.2%.

Conclusion: In this study it has been shown that, several clusters and new strains of *M. tuberculosis* circulate in TBLN patients in Ethiopia. As mapping the population structure of *M. tuberculosis* is vital to understand the transmission and disease dynamics of TB and set appropriate control measure.

Key Words: Tuberculosis lymphadenitis, MTC, RD9 and RD4 Typing, Spoligotyping, *Mycobacterium* family, Mycobacterium lineage, Phenotyping and Addis Ababa.

1. INTRODUCTION

1.1. Background

The global burden of TB remains enormous. Globally in 2012, there were an estimated 8.6 million incident cases of TB (range 8.3 million–9.0 million), 1.3 million people died from the disease (0.94 million deaths among HIV-negative cases and 0.32 million deaths among people who were HIV-positive) [1]. Most of the estimated number of cases in 2012 occurred in Asia (58%) and the African Region (27%) [1].The five countries with the largest number of incident cases in 2012 were India (2.0 million– 2.4 million), China (0.9 million–1.1 million), South Africa (0.4 million–0.6 million), Indonesia (0.4 million–0.5 million) and Pakistan (0.3 million–0.5 million). India and China alone accounted for 26% and 12% of global cases, respectively. Of the 8.6 million incident cases, an estimated 0.5 million were children and 2.9 million (range, 2.7–3.1 million) occurred among women [1].The infection rate with this bacteria increasing and one of the reasons for this is long process of laboratory identification, therefore establishing new diagnosis methods could decrease disease rate. WHO estimates that if the current tools for the diagnosis and treatment of TB are not improved, nearly one billion additional people will become infected by *M. tuberculosis* between the 2000 and 2020. Among these, 200 million will develop the active disease and 35 million will die from TB [2].

1.2. Etiologic Agent

M. tuberculosis belongs to the family of Mycobacteriaceae, order Actinomycetales and genus Mycobacterium. There are three species under this genus and these include *M.tuberculosis* complex, the non-tuberculosis mycobacteria and *M. leprae* [3]. Human and animal tuberculosis are caused by different members of the *Mycobacterium tuberculosis* complex (MTC), of which *M. tuberculosis* and *M. bovis* are best known and share 99.9 % of the same genome [4]. A recent study suggest using phylogenetically informative spacers, in combination with previously identified single nucleotide mutations and chromosomal deletions to identify different clades in the RD9 deleted lineage each with a separate host preference [5]. Of the

pathogenic species belonging to the *M. tuberculosis* complex, the most frequent and important agent of human disease is *M. tuberculosis* [6]

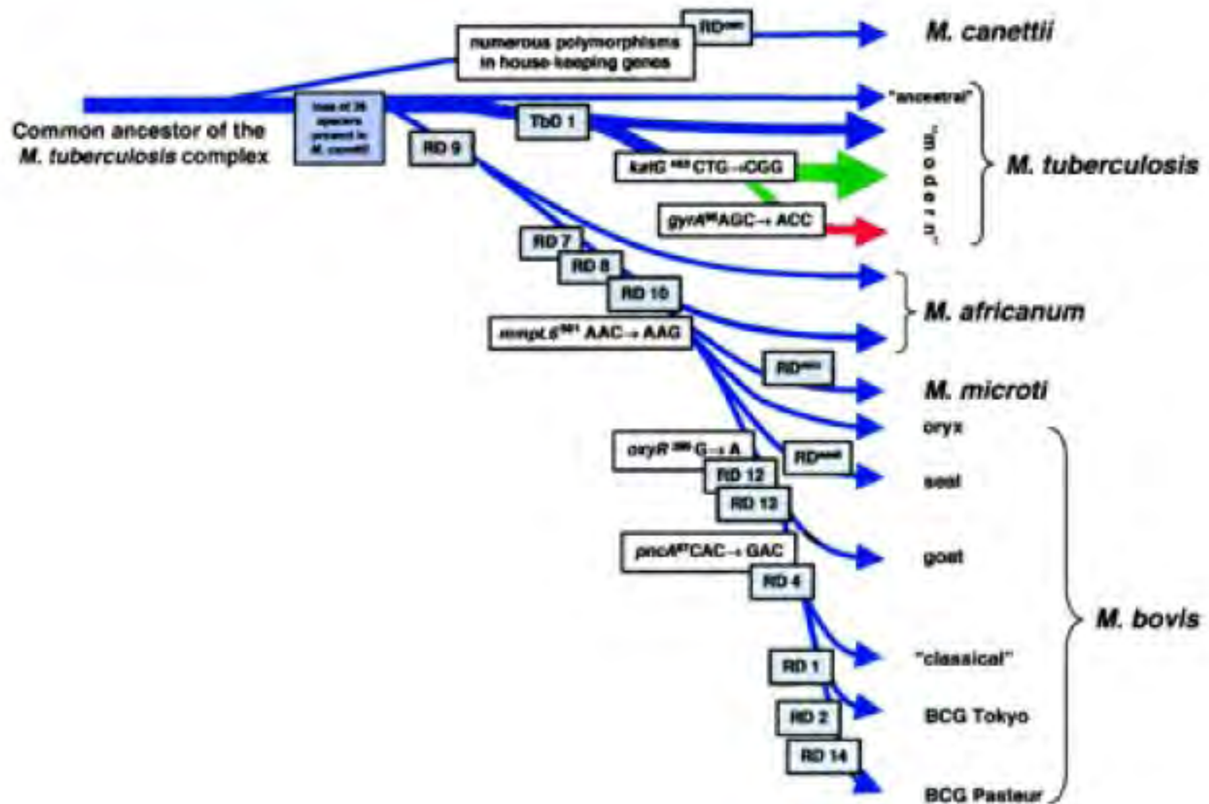


Fig.1: *Scheme of the proposed evolutionary pathway of the tubercle bacilli illustrating successive loss of DNA in certain lineages (grey boxes). The scheme is based on the presence or absence of conserved deleted regions and on sequence polymorphisms in five selected genes (Source: Brosch et al., PNAS, 2002)*

Today's observed pattern of phylogeographical diversity of MTC is undoubtedly the result of both a deep ecological differentiation and of a more recent demographic and epidemic history. It is hypothesized that strains comprising TB clades have evolved different properties which may influence a local strain population structure [7]. TB-lineage can be used to predict major lineages as defined by the United States Centers for Disease Control and Prevention (CDC). It can predict the following 6 major MTC genetic lineages as defined by CDC. Modern Lineages(East-Asian (Beijing), Euro-American, East-African Indian) and Ancestral Lineages(Indo-

Oceanic, *M.africanum* (West African 1 and West African 2),*M. bovis*) [8].Each of the six main lineages was associated with particular geographical areas. The East-Asian lineage is dominant in many countries of the Far East, and the Indo-Oceanic lineage occurs all around the Indian Ocean. The Euro-American lineage is clearly the most frequent lineage in Europe and the Americas. Though most other areas were associated with only one or two lineages, all six main lineages were represented in Africa. These included the two West-African lineages that did not occur elsewhere, as well as the Indo-Oceanic lineage, the most ancestral of the six lineages, which was associated with East Africa[9].

1.3. Transmission of Tuberculosis

M. tuberculosis infections occur by airborne transmission. The route of entry of the tubercle bacillus into the body is via the respiratory tract through the inhalation of respiratory droplet nuclei, which are few viable, virulent organisms, small enough in size to allow passage into the lower respiratory tract [10]. Infection of human beings with *M. bovis* almost always occurs by inhalation of aerosols or consumption of milk/meat containing the bacillus [11].

Once organisms have made their way into the lung, they have four potential fates [12]. The initial host immune reaction can be completely effective and kill all bacilli, such that the patient immune system completely controls the bacilli and has no chance of developing tuberculosis at any time in the future; if the immune system of host is undermine, the organisms can begin to multiply and grow immediately after infection, causing clinical disease known as primary tuberculosis; bacilli may become dormant and never cause disease at all, such that the patient has what is referred to as latent infection, or the latent organisms can eventually begin to grow, with resultant clinical disease, known as reactivation tuberculosis [13].TBLN is assumed to result from lympho-haematogenous spread of tubercle bacilli from the site of primary infection in the lungs, as reactivation of previously contained foci . Hilar and mediastinal lymph nodes are initially involved. The infection then spreads through the lymphatic to the draining cervical lymph nodes [14].

1.4. Immunopathogenesis of Tuberculosis

Phagocytosis of *M.tuberculosis* by alveolar macrophages is the first event in the host pathogen relationship that decides outcome of infection. Phagocytized microorganisms are subject to degraded by intralysosomal acidic hydrolases upon phagolysosome fusion. This highly regulated event constitutes a significant antimicrobial mechanism of phagocytes [15]. After entry of *M. tuberculosis* into alveolar macrophages, it produces inflammatory cytokines and chemokine that serve as a signal for infection. The monocytes, neutrophils, and lymphocytes migrate to the focal site of infection, but they are unable to kill the bacteria efficiently. During this time, the bacilli resist the bactericidal mechanisms of the macrophage (phagolysosome) by preventing phagosome-lysosome fusion, multiply in the phagosome, and cause macrophage necrosis. The released bacilli multiply extracellularly, and phagocytized by another macrophage that also fails to control the growth of *M.tuberculosis*, and likewise are destroyed [16, 17].

Within 2 to 6 weeks of infection, the specific immune response produces primed T cells which migrate back to the focus of infection, guided by the chemokines produced by the infected cells, and the accumulation of macrophages, T cells, and other host cells (dendritic cells, fibroblasts, endothelial cells, and stromal cells) leads to the formation of granuloma at the site of infection. The bacilli are contained in the caseous centers of the granuloma. The bacilli may remain forever within the granuloma, get reactivated later or may get discharged into the airways after enormous increase in number, necrosis of bronchi and cavitation. Fibrosis represents the last-ditch defense mechanism of the host, where it occurs surrounding a central area of necrosis to wall off the infection when all other mechanisms failed [15, 18].

The granuloma formation walls off tubercle bacilli from the rest of the lung tissue, limits bacterial spread, and provide microenvironment for interactions among macrophages and other cells of the immune system and the cytokines produced by these cells. The CD4+T cells producing interferon- γ (IFN- γ) recognize infected macrophages, presenting antigens from *M. tuberculosis* and kill them [19].

If the infection is successfully contained at granuloma formation stage, it shrinks and may eventually disappear, leaving a small scar or calcification and the patient‟s T cells become responsive to *M. tuberculosis*-derived antigens. If, however, the immune response does not successfully control the bacterial replication, the granulomas increase in size and cellularity. Eventually, cell death in the granuloma leads to necrosis. In this case, if the granuloma is close to the surface of the lung, the tissue destruction caused by necrosis can breach the mucosal surface and the granuloma contents leak into the lumen of the lung – a process referred to as cavitation. This gives rise to the prototypic symptom of TB – a persistent cough with blood in the sputum. At this point, the patient is highly infectious, spreading the bacteria by aerosol, even the bacilli can disseminated to other organ, like lymph node. Tissue destruction in TB is not mediated by the activities of the bacteria alone, it is primarily immunopathological in nature and the crucial point to understand is that an inflammatory immune response is critical for the survival of both the host and the bacteria [20].

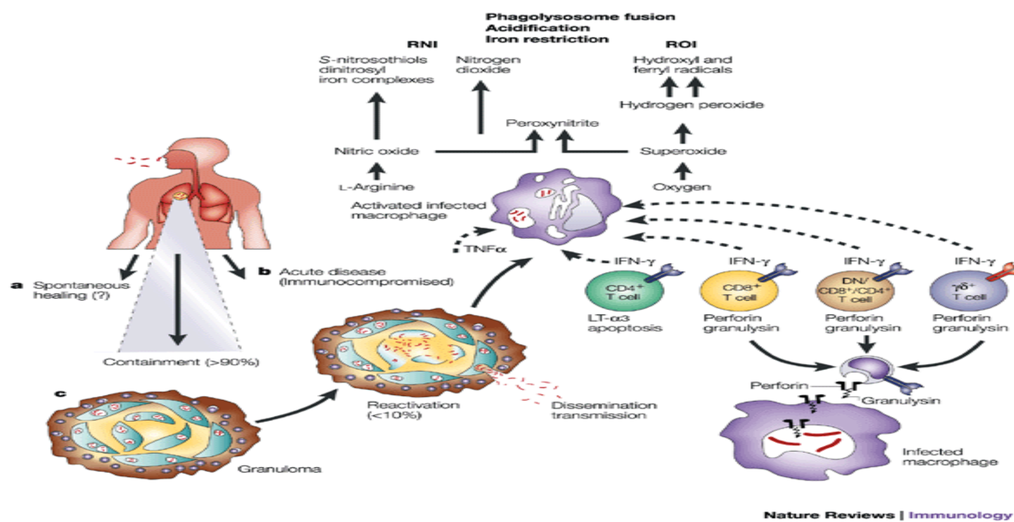


Figure 2. *Different outcomes of infection with M. tuberculosis, different T cell populations involved in protection and major anti-mycobacterial effector mechanisms of macrophages. This scheme firstly depicts the different outcomes of tuberculosis in healthy and immunocompromised subjects. Secondly, the figure shows the different T cell populations and their major T cell effector mechanisms in the control of disease. Thirdly, the figure shows anti-mycobacterial effector mechanisms of activated macrophages. (Kaufmann H.E. How can immunology contribute to the control of tuberculosis. Nature Reviews Immunology 2001; 1:20–30.)*

1.5. Immunology of Tuberculosis

Almost 20 people develop tuberculosis and four people die from the disease every minute, somewhere in the world. Tuberculosis thus remains a major disease in terms of mortality and morbidity. In the majority of infected people, the immune response is able to adequately control the infection, and consequently only 5-10% will develop clinical sign and symptom of disease during their lifetime. The host which is latent state for the bacterium and, if they become immune depressed, they may present a reactivation of the disease, leading to the dissemination of the infection. Cell mediated immunity of the host response to *M. tuberculosis* is important in preventing clinically evident disease following infection [21, 22].

Understanding the components of host immune response at a basic level is likely to lead to a better understanding of the pathogenesis of tuberculosis in humans and to result in better and novel approaches to prevention and therapy of this disease.CD4+T, CD8+T cells, macrophages have an essential role in the response to TB. They are generally involved in amplifying immune responses by secreting various cytokines, serves cytotoxic effector cells capable of lysing target cells directly, express the co-stimulatory molecules necessary for activating T cells, balance central in maintaining the homeostasis of the immune system, prevent autoimmunity and maintain self-tolerance and they can be minimize excessive tissue destruction from adaptive immune responses [23].

Pathogenic mycobacteria can spread into neighboring resting macrophages and other cells considered for replication. Macrophages, activated by exposure to inhaled particulates, possess potent microbicidal activity which can kill bacilli and arrest TB infection, but usually they cannot eliminate infection entirely [24, 25]. Its activation is achieved by T lymphocytes which are the principle mediator of cell mediated immune response against MTB. CD4+ T cells are primarily helper T cells which secrete different types of interleukins involved in the activation of macrophages. IFN- γ specifically activates macrophages and stimulates them to ingest and kill mycobacteria more effectively [26, 27].

The host immune response to *M. tuberculosis*, especially cell-mediated immunity involving macrophages, T lymphocytes, and natural killer (NK) cells, are particularly important in preventing clinically evident disease following infection. CD4+T cells are involved in amplifying immune responses by secreting various cytokines, but some CD4+ T cells also serve as cytotoxic effector cells capable of lysing target cells directly. They are capable of secreting cytokines such as Interferon-γ (IFN-γ) and interleukin-4 (IL-4) and thus may play a role in regulating the balance of Th1 and Th2 T-lymphocytes in the TB patients [28, 29]. $CD8^+$ T-lymphocytes are also important for an effective T-cell immune response in their roles as cytotoxic effector cells capable of lysing target cells directly, or supporting the immune response of $CD4^+$ T-lymphocytes [30].

Lymphocyte sub-populations proliferate following exposure to the mycobacterium antigen and start secreting specific cytokines. Cytokines are, in fact, messengers of the immune system, leading the system to a stronger defense against the disease [31, 32].The CD4+ T helper cells can be differentiated into Th1, Th2, Th17 and Treg. cells. The Th1 cells produce cytokines, notably IFN-γ, TNF-α, IL-2, lymphotoxin and granulocyte-macrophage colony-stimulating factor (GM-CSF), which prompts stimulation of Th1 cells, CTL, and maturation and activation of macrophages as well as granulocytes. The Th2 cells produce B cell stimulation factors such as IL-4, IL-5, IL-10 and IL-13, which promote antibody production but suppress the Th1 type immune response. The Th1, Th2 and Th17 subsets may be modulated by Treg cells, of which there are several types and the list is growing [33, 34]. The FoxP3-expressing Treg cells are expanded during TB infection, and inhibit human memory γ -δ T cells to produce IFN- γ in response to *M. tuberculosis* antigens [35, 36].

The different T cell populations produce interferon γ (IFN γ) and hence are of the T helper 1 (Th1) type. This cytokine is the central mediator of macrophage activation. IFN γ synergizes with tumor necrosis factor α (TNF α) in activating macrophages. CD4+T cells also produce lymphotoxin α (LT α), which participates in protection against tuberculosis. At least some of the

CD8 T cells, γδ T cells, and CD1 restricted T cells secrete perforin and granulysin which directly kill mycobacteria within macrophages [37].

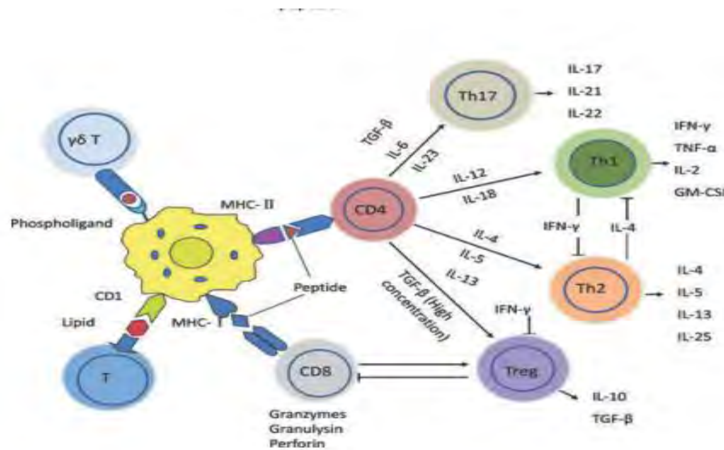


Figure 3.Adaptive immunity to tuberculosis infection.

The infected macrophages and dendritic cells secrete cytokines that include IL-12, IL-23, IL-7, IL-15 and TNF-a, and present antigens to several T-cell populations including CD4+ T cells (MHC class II), CD8+ T cells (MHC class I), CD1-restricted T cells (glycolipid antigens) and γδ T cells (phospholigands).These T cells produce the effector cytokine IFN-γ, which activates macrophages in conjunction with TNF-α to effect killing of intracellular mycobacteria through reactive oxygen and nitrogen intermediates. In addition, CD8+ cytotoxic T cells can kill intracellular mycobacteria through granulysin and perforin-mediated pathways. However, CD4+ Th2 cells produce immunosuppressive cytokines such as IL-4, and CD4+CD25+FoxP3+ regulatory T (Treg) cells produce IL-10 and TGF-β that may suppress mycobactericidal effector mechanisms.(*Adapted from: Dheda K.et al. The immunology of tuberculosis: From bench to bedside. Respirology 2010;15: 433–450.*)

Studies in animal models and in humans have demonstrated that a wide range of immune components are involved in an effective immune response against *M.tuberculosis*. These include, beside macrophages and dendritic cells, αβ -T cells (both CD4 + and CD8 +), CD1 restricted T cells, γδ -T cells, and cytotoxic T cells, as well as the cytokines produced by these immune cells [37-40].Although CD4+ T cells along with CD8+ T cells and the natural killer (NK) cells are the major producers of IFN- γ, studies carried out in CD4+ deficient mice have shown that it is the early production of IFN- γ by CD4+ T cells and subsequent activation of macrophages that determine the outcome of infection. The CD4+ T cells also play other roles in the defense against infection that is independent of IFN- γ production [41, 42]. The CD8+ T-cells, in addition to

producing IFN- γ and other cytokines, may also are cytotoxic for *M. tuberculosis* infected macrophages, and thus play an important role in providing immunity to TB. The CD8+ T-cells can directly kill *M. tuberculosis* via granulysin, and facilitate the control of both the acute as well as chronic infection [42, 43].

The increased incidence of tuberculosis, specifically extrapulmonary tuberculosis, among individuals with HIV infection, demonstrates the importance of cell-mediated immune responses for the containment of *M. tuberculosis* infection [44]. Activated effector T-lymphocytes migrate to granulomas, and presumably control infection through the release of cytokines and cytolytic function. These immune responses appear to be modulated through the recruitment of regulatory T -lymphocytes (Treg) to the sites of active infection. This suggests Treg may play a significant role in the host immune response to *M. tuberculosis* infection, and specifically a role in determining the site of tuberculosis disease [45, 46].

It is known that the cellular immune response, particularly that produced by lymphocytes, plays a central role in controlling *M.tuberculosis* replication. The immune response after *M. tuberculosis* disease may be visualized by the measurement of T-lymphocyte phenotypes in human peripheral blood. Different researchers showed that, diminished numbers of CD4+ and increased number of CD8+ T-cells have been described in patients with active tuberculosis. Expanded immunophenotyping demonstrated the loss of both naïve and memory/effector subpopulations of CD4+ T-lymphocytes, and increased CD8+ T-lymphocyte activation. Here it also a profound depletion in T-cells subpopulations in patients with severe tuberculosis, in the absence of other identifiable causes of immunodeficiency [47].

The study done in Iran on different markers (CD3, CD4, CD8 and CD4/CD8 ratio) in peripheral blood TB patients before chemotherapy indicated that, altered T lymphocytes are seen. In the patients group, increase in T-CD8+ lymphocytes along with decrease in T-CD4+ lymphocytes before antibiotics, resulted in a decrease of CD4/CD8 ratio. There is some study

which showed change of peripheral T lymphocytes previous EPTB had significantly lower median $CD4^+$ lymphocyte levels than persons with pulmonary tuberculosis or latent *M tuberculosis* infection [48, 49].

T-lymphocyte subsets, both $CD4^+$ and $CD8^+$ T-cells, play a crucial role in immunity against mycobacterium infections. They have many effector functions such as cytolysis and release of potent anti-mycobacterial cytokines like IFN-γ and TNF-α. The study done in China by Jing X et al., showed a clear reduction in the percentages of $CD3^+$ T lymphocytes in TB patients compared with controls, and of T lymphocytes and $CD3^+CD4^+$ T helper cells in extra-pulmonary TB patients compared with pulmonary TB patients[50].

Study from India, on immunophenotypic characteristics and intracellular cytokine levels of T-lymphocytes and monocytes showed that, the CD4+ percentage was lower in patients with TB when compared to healthy individuals. Study in Turkey also indicated, the percentages of total CD3+ lymphocytes in APTB patients had lower CD4+ T cells, CD3 + CD4 + levels than healthy control . The percentages of CD3 + and CD3 + CD4 + cells, and the ratio of CD3 + CD4 + to CD3 + CD8 + cells, were significantly lower. There was also a decrease in total T-lymphocytes (CD3+ cells) and CD3 + CD4 + cells in TB patients compared with control subjects according to research conducted in China[51-53].

Extrapulmonary tuberculosis is likely a marker of underlying immune suppression and has been associated with decreased in vitro secretion of gamma interferon (IFN-γ) in response to mycobacterial antigens compared to that of *M. tuberculosis* infected controls. It was shown by Almeida et al. that subjects with prior extrapulmonary tuberculosis had the highest frequency of Treg cells compared to those with prior pulmonary tuberculosis, individuals with latent *M. tuberculosis* infection, and uninfected controls. These findings suggest that immune dysregulation may be a feature of persons who develop extrapulmonary tuberculosis [54].

1.6. Global epidemiology of Tuberculosis

M.tuberculosis is harmful human pathogen that causes considerable morbidity and mortality in the worldwide. Globally, 8.4 million people are estimated to develop tuberculosis (TB) each year, and nearly 2 million deaths result from the disease. There were an estimated 12 million prevalent cases (range, 11 million–13 million) of TB in 2012 equivalent to 169 cases per 100 000 population. In the African Region, estimates of TB prevalence rates are far from the target level, and halving the 1990 rate by 2015 appears unlikely. Overall, one-third of the world"s population is currently infected with the tuberculosis bacillus, over 90 percent of them in developing countries [1]. The African region accounted for most HIV-positive TB cases (79%), followed by the South East Asia region which had 11% of total cases. The prevalence of HIV infection among patients with TB ranges from 50% to 80% in many settings in Sub- Saharan Africa, but in other parts of the world it varies from 2% to 15% [55].

WHO estimates that if the current tools for the treatment and prevention of TB are not improved, nearly 1 billion additional people will become infected by *M. tuberculosis* between the 2000 and 2020. Among these, 200 million will develop the active disease and 35 million will die from TB [2].Extrapulmonary tuberculosis accounts for approximately 10–15% of all tuberculosis infections and occurs in up to 50% of patients with human immunodeficiency virus (HIV)-tuberculosis co-infection. The annual incidence rates of EPTB have increased not only in developing countries but globally over the last few years [56, 58].

1.7. Epidemiology of tuberculosis lymphadenitis (TBLN)

EPTB is a significant health problem in both developing and developed countries. The reported prevalence of EPTB in India varies between 8.3% and 13.1% in different districts. The reported proportions of EPTB among all TB cases in other developed countries ranged from 12% to 28.5% [59].*M. tuberculosis* amounted to 68% as a causative agent of lymph node tuberculosis in the area with 19% of the patients being HIV positive. In India a finding that closely agrees with the above results revealed that *M. tuberculosis* is still the most common cause of tuberculous

lymphadenitis and mycobacteria other than tuberculosis are responsible for very few cases [60]. Tuberculous lymphadenitis was found to be predominantly cervical in location and most patients responded well to chemotherapy. The relationship of HIV to EPTB has been clearly documented but it is evident that extra pulmonary tuberculosis especially lymph node tuberculosis does occur in the absence of HIV infection in young patients [61]. As study conducted in a tertiary care hospital in India revealed, mycobacteria were grown on 74 cultures. Seventy two cultures (97.3%) were positive for *M.tuberculosis, and,* and only 2 (2.7%) were positive for *M.kansasii*. In 89 histopathologically proven patients of TBLN, 56(62 %) cultures were positive and AFB smear positive in 21(23.6%) patients [62].

Epidemiological survey of EPTB in the United States indicated that ,among 253,299 total tuberculosis cases reported from 1993 through 2006 to the National Tuberculosis Surveillance System (NTSS), 47,293(19%) were EPTB,186,540(74%) were PTB,4478(2%) were disseminated tuberculosis, and 14,910(6%) were concurrent EPTB. The disease site constituting the largest proportion of EPTB cases was lymphatic tuberculosis, followed by pleural tuberculosis. More than 60% of lymphatic tuberculosis was cervical lymphatic tuberculosis [63].

Study conducted in Nigeria with peripheral lymphadenopathy patients revealed that 48 were subjected to FNAC. Aspirates were mainly from lymph node 43(89.6%), breast 4(8.3%) and periumblical mass 1(2.1%).AFB were positive in 23 (47.9%). Generally the study showed that EPTB consists of 10%-27% tuberculosis and it account for significant proportion of TB impact on health [64].

The study from Tanzania on 128 (99 HIV-seropositive) patients indicated that, 89 (67 HIV-positive) patients TB lymphadenitis could be proven. Histology and LJ culture of a lymph node biopsy had the highest diagnostic yield, 85% and 88% respectively, followed by detection of acid-fast bacilli (AFB) in biopsy smear (53%) and in fine-needle aspirations (35%).Again in same country, 191 patients admitted to hospital with suspected EPTB tuberculosis, TB was

diagnosed in 158 patients. *M. tuberculosis* was detected in the blood of 25 patients, in 92% by a polymerase chain reaction (PCR) technique and in 52% by culture of buffy coat cells [65, 66].

In Sudan, *M. tuberculosis* DNA was detected in the blood samples of 30/39 (77%) patients with tuberculosis lymphadenitis, but in none of the cases with reactive or malignant lymphadenopathy. The presence of *M. tuberculosis* DNA correlated strongly to multiple lymph-node involvement and to caseating-granulomatous and predominantly necrotic cytomorphological categories [67].

1.8. Epidemiology of tuberculosis in Ethiopia

TB is also a major public health problem in Ethiopia. Ethiopia is among the High burden tuberculosis countries (HBTCs) with regard to the number of tuberculosis patients. The WHO TB control program estimate that the proportion of extrapulmonary TB among the total number of new TB cases (143, 503) is about 33% [1].The proportion of EPTB, with majority of TBLN among newly diagnosed TB patients has been increasing for the last two decades. In spite of these high numbers, diagnosis of tuberculosis lymphadenitis remains a challenge [68]. Over a third of the population has been exposed to tuberculosis and the annual risk of tuberculosis is estimated at 2.2 %. According to the Ministry of Health statistics, tuberculosis is one of the leading causes of morbidity, the fourth cause of hospital admission, and the second cause of hospital death in Ethiopia [68, 69].

Study done in Ethiopia by Deresse et al (2012), of total 134, 38% of the samples were positive for TBLN by culture, 11.8% by Erlich-Ziehl-Neelsen (EZN) staining, 23.4% by PCR, and 59.8% by cytology. Cytology had the highest sensitivity (81%) and EZN stain the least (22.9%). The specificity of EZN stain was the highest (92.4%) while cytology was the lowest (50%). In this study, out of 50 culture-positive samples, 21 (42%) were positive by PCR while 8 (10.8%) out of 74 culture-negative samples were positive by PCR [70].

A cross-sectional study from Afar region of Ethiopia regarding the forms of TB, 137 (63.4 %) had PTB and the rest were diagnosed with EPTB. Among PTB patients, the majority (61.3 %) of them were diagnosed as smear negative PTB. Patients with PTB reported persistent cough (100 %), fever (93.4 %), weight loss (92 %), loss of appetite (89.1 %), night sweating (84.7 %), and hemoptysis (26.3 %). On the other hand, patients with EPTB reported fever (89.9 %), swelling (mainly neck and axillary areas) (73.4 %), loss of appetite (77.2 %), weight loss (77.2 %), night sweating (64.6 %), chest pain (25.3 %), and cough (17.7 %) [71].

A cross sectional study done in 27 health centers of Addis Ababa, Ethiopia, revealed that the prevalence of both pulmonary and extra pulmonary TB was 46.0% (233/506).Of the TB patients, 100(42.9%), 96(41.2%) and 37(15.9%) had smear positive, smear negative and extra pulmonary TB respectively [72].

1.9. Clinical manifestation of Tuberculosis

Tuberculosis lymphadenitis usually affects children and young adults with high female predilection. Patients usually present with gradual enlarging of lymph nodes and may otherwise be asymptomatic. Isolated cervical lymphadenopathy is most commonly seen in about 67 % HIV negative patients. The enlarged lymph nodes may be of varying size, are usually firm, painless and may be discrete or matted [73-75].

Cervical lymphadenitis is the most common manifestation of mycobacterial infections encountered in the otolaryngologic practice. Cervical lymphadenopathy is generally described as a painless, slowly growing neck mass or masses developing over weeks to months. The lymph nodes are usually multiple and matted but may be single, mobile, or fluctuant or with discharging sinuses. Some patients with lymph node tuberculosis may manifest systemic symptoms and these

include fever, weight loss, fatigue and occasional night sweats. Cough is a less prominent feature, seen in approximately 10 percent of patients [73, 76].

1.10. Diagnosis and identification of Tuberculosis

Mycobacterial cervical lymphadenitis remains a diagnostic challenge in developing countries for many clinicians because of the low sensitivity of conventional methods in detecting tubercle bacilli in clinical specimens; it mimics other pathologic processes and yields inconsistent physical and laboratory findings [76].

1.10.1. Ziehl Neelsen (ZN) staining

The primary method for the diagnosis of tuberculosis is Ziehl Neelsen staining. Although the method is specific and rapid; the technique has low sensitivity in the detection of tubercle bacilli in various clinical specimens. Smear negative and culture positive results can occur since a significant number of bacilli must be present in the given specimen to be detected by acid fast smear examination. For instance, 5, 000 -10, 000 acid-fast bacilli per ml of sample must be present to permit detection by acid fast staining [77-80].

1.10.2. Fine Needle Aspiration Cytology (FNAC) and Histopathology

Fine-needle aspiration (FNA) has become a widely used diagnostic tool and it remains one of the most rapid and cost-effective diagnostic methods of tuberculous lymphadenitis where it is difficult to perform molecular techniques. It is being increasingly used as the main diagnostic procedure for establishing the diagnosis of tuberculous lymphadenitis. In areas where mycobacterial infections are prevalent, a diagnosis of tuberculosis can be made confidently when its cytomorphological features are met. In one study, the sensitivity and specificity of FNAC in the diagnosis of tuberculous lymphadenitis was stated as being 88% and 96% respectively [81,82].Histopathologic examination is one of the most important means for diagnosing mycobacterial cervical lymphadenitis. In histological diagnosis of surgical lymph node biopsy specimens, diagnosis is based on the presence of granulomas, central

necrosis (suggestive of lymph node tuberculosis), and if possible, demonstration of acid-fast bacilli by staining of tissue sections [83-85].

1.10.3. Mycobacterial culture

The identification of tubercle bacilli by culture is required for the ultimate proof of mycobacterial infection. However, due to unavailability of laboratory equipment and safety procedures, the method is not practiced in resource poor settings. Specimens collected from normally sterile body sites may be placed directly onto the culture media or can be mildly decontaminated prior to inoculation. Such specimens include FNA and biopsy materials from lymph nodes. The detection rate for *M. tuberculosis* from fine needle aspirates is low by microbiological techniques. Different media (egg based, agar based and liquid media) have been devised for cultivating of tubercle bacilli. Among the aforementioned media, bacterial culture on egg - based Lowenstein Jensen (LJ) media are commonly used in most laboratories. Lowenstein Jensen medium enriched with sodium glycerol favors the growth of *M. tuberculosis*, while LJ medium without glycerol but containing pyruvate encourages the growth of *M. bovis*. However, bacterial culture takes at least 3 to 8 weeks to observe a positive growth after incubation at 37 ^{o}C [86-88].

1.10.4. Polymerase Chain Reaction (PCR)

In order to reduce the time needed for and improve the sensitivity of identification methods, combination of the above cited techniques and PCR methods have been recommended. However, the diagnosis of EPTB remains an important clinical challenge, primarily because of the inadequate sensitivity of conventional bacteriologic methods for detecting *M. tuberculosis* and *M. bovis* in the extrapulmonary specimens. Nucleic acid methods are currently employed for rapid identification of mycobacteria from both cultured and uncultured clinical specimens. Key mycobacterial targets for PCR amplification are: the insertion sequence IS6110, Region of Difference (RD), 65KD heat shock protein, 38KD protein, and ribosomal RNA [89-92].

Spoligotyping method is based on PCR amplification of a highly polymorphic DR locus in the *M. tuberculosis* complex, which contains DR sequences interspersed with variable spacer sequences, followed by reversed line blot hybridization. It relies on determination of the presence or absence of spacers in the in vitro-amplified DNA by hybridization to multiple synthetic spacer oligonucleotide covalently bound to a filter. Amplification differences in RD regions reflecting variable deletions in IS6110, 65KD heat shock protein, 38KD protein, and ribosomal RNA region could also be used as a tool for differentiation of members of the mycobacterium tuberculosis [93, 94].

1.11. Treatment and prevention

M. tuberculosis is susceptible to several effective anti-microbes. Isoniazid, Ethambutol, Rifampin, Pyrazinamide, Streptomycin, and combinations of these agents constitute the primary drugs of choice for treatment of tuberculosis. Isoniazid and rifampin are active against both intra- and extracellular organisms, and pyrazinamide, a nicotinamide analog, acts at the acidic pH found within cells. Streptomycin does not penetrate into cells and is thus active only against extracellular organisms [95]. *M. tuberculosis* is also susceptible to other drugs, second-line drugs, which are less efficacious and more toxic than the first-line drugs. Second-line drugs are Capreomycin, Kanamycin, Ethionamide, Thiacetazone, Aminosalicylic acid, and Cycloserine. Fluoroquinolones and Ofloxacin that may be used to replace those of the primary group if they are inappropriate because of resistance or drug toxicity [95, 96]. At present, the bacillus Calmette and Guérin (BCG) is the only available vaccine. It has been used for prophylaxis of tuberculosis in various countries since 1923; administration is usually intradermal. It is a live vaccine derived originally from a strain of *M. bovis* that was attenuated by repeated subculture. Although BCG can be effective in reducing the incidence of childhood TB, particularly meningitis, it is relatively ineffective in protecting against adult TB, and doesn‟t prevent infection with the organism [95].

1.12. Statement of the problem

M.tuberculosis, one of the most harmful human pathogens worldwide, causes considerable morbidity and mortality [97]. The 22 HBTCs that have been given highest priority at the global level accounted for 80% of all estimated cases worldwide [1].TBLN, the most common presentation of EPTB, is a significant health problem in both developing and developed countries. Recently different reports showed that TBLN infection is an increasing problem. The problem is compounded due to HIV pandemic, TB-HIV co-infection, and MDR and XDR tuberculosis [1].Tuberculosis is also a major public health problem in Ethiopia which ranks 7th among the 22 High Burden Tuberculosis Countries (HBTCs) and ranks 2nd EPTB cases globally [1].Even though early case detection and treatment is one of the pillars of the TB control program, extrapulmonary tuberculosis is a significant true challenge in its different clinical presentation. The challenges are due to significant proportion of clinical samples, low number of bacilli in EPTB specimens and their slow growth rate which reduce the sensitivity of conventional diagnostic methods [2].

Though Ethiopia is one of the countries with high prevalence of TB, information about genotypic characteristics of *M. tuberculosis* complex species and strains found in the country, analysis on timely updated of the MTC species/strains distribution, emergencies of drug resistance and information relevant to the development of new diagnostic, drug and vaccine are limited [98]. But this information is very vital in order to study phylogenic characteristics of the organism which in turn will provide a new insight into the natural history of the disease in addition to its use in designing more effective control measures [98].

Extrapulmonary tuberculosis (EPTB) appears to be a marker of an underlying immune defect. The risk of extrapulmonary disease is increased in HIV-infected persons; it occurs in 10% to 20% of HIV-seronegative persons but in 40% to 80% of those infected with HIV. The increased risk in HIV-infected persons has been associated with advanced immune suppression. Thus, if there is a predisposition to developing tuberculosis, the immunologic defects associated with this predisposition should be most readily identified among persons with EPTB disease [49].

1.13. Significance of the study

Based on the above figure and facts, it is possible to hypothesize that the diagnostic of TBLN only by routine method is the problem of controlling the TB disease. Therefore it is essential to have reliable and affordable diagnostic technique which is timely and cost effective for early detection and management of TB patients. Besides, nucleic acid amplifications techniques like PCR, has a considerable impact on disease diagnosis on account of their speed, specificity and enhanced sensitivity.

The genetic diversity of the mycobacterium strains circulating within an endemic and epidemic area may give rise to an array of pathogenic characteristics. There is limited information about genotypic characteristics of mycobacteria species and strains circulating in Ethiopia. But there is an increasing demand of knowledge on the phylogenetic characteristics of this microorganism to have a good understanding towards the dynamics of mycobacteria species and strains circulation in the population. And hence; there is a need to undertake molecular typing study throughout the country for the implementation of mapping the strains circulating in the country.

Understanding the components of host immune response at a basic level is likely to lead to a better understanding of the pathogenesis of tuberculosis in humans and to result in better and novel approaches to prevent and therapy of this disease[99]. Therefore, an evaluation of change of lymphocyte percentage can reflect the state of a disease pathogenesis and the immune response of *M. tuberculosis* disease may be visualized by the measurement of T-lymphocyte phenotypes in human peripheral blood.

2. OBJECTIVES

2.1. General Objective

The objective of this study was to determine the prevalence of TBLN and to compare diagnostic performance of laboratory methods in diagnosis of TBLN among clinically suspected TBLN patients; identify the dominant species/ strains of *M.tuberculosis* complex responsible for TBLN in Addis Ababa, Ethiopia

2.2. Specific Objectives

- To determine the prevalence of TBLN
- To identify the dominant species and strains of mycobacterium causing TBLN
- To compare the diagnostic performance of FNAC/histology, ZN staining, and direct PCR in the diagnosis of TBLN against culture
- To evaluate the phenotype of some peripheral T lymphocytes in TBLN patients

3. MATERIALS AND METHODS

3.1. Study period and area

The study was conducted from February to October, 2013 in Addis Ababa, Ethiopia at Tikur Anbessa Specialized Hospital (TASH) and Alem Tena Higher Clinic (ATHC).

3.2. Study design

Cross-sectional study was used during the study period to achieve the objectives.

3.3. Study Participant

Adult patients clinically suspected for TBLN and referred to Pathology laboratory of TASH and ATHC for analysis of FNA/ biopsies were included. Twelve TBLN confirmed cases and 10 apparently healthy individuals among the staff and students of Black lion hospital and Alem Tena Higher Clinic without any history of immunosuppressive disease and immunosuppressive therapy, tuberculosis or history of exposure to tuberculosis, who are age and sex matched with cases were recruited for characterization of Immunophenotype of peripheral T lymphocytes.

3.4. Patient requirement

3.4.1. Inclusion criteria

- Patients clinically suspected for TBLN for whom biopsy and FNA were collected
- Patients who were gave their consent
- Patients who were 18 and above years of age

3.4.2. Exclusion criteria

- Non-volunteer patients
- Patients who were critically ill
- Patients who are taking antitubeculosis

3.5. Sample size determination

Patients clinically suspected for TBLN and referred to Pathology laboratory for FNA or biopsy in Addis Ababa, Ethiopia, and who were fulfilling the selection criteria were included. Using

16% prevalence of EPTB and single population proportion formula the sample size was calculated to be 206.

$n=Z_{(\alpha/2)}^2 \; \underline{P(1-P)/} \; d^2$ (Where n-sample size, d-margin of error, 0.05 at 95% CI, p-prevalence of EPTB, $Z\alpha/2$-half of confidence interval)

P=0.16, Zα/2=1.96,d=0.05, P = 0.16 → $n = (1.96)^2(0.16)(0.84)/(0.05)^2 = 206$

3.6. Sample collection, storage and processing

Pre tested and structured questionnaire was used to collect demographic data. FNA/ biopsy were collected by pathologist/surgeon from the affected nodes.5mL of peripheral venous blood sample were collected by experienced laboratory personnel for flowcytometric analysis of T lymphocytes.

3.6.1. Fine Needle Aspiration (FNA) and Biopsy

FNA was performed using 21 gauge needle using standard procedure. The overlying area was cleaned with 70% alcohol. The enlarged lymph node was fixed and maintained in stable position by the left hand of the physician. Then the node had been entered with a negative pressure applied to the syringe. Multiple (average six) in and out passes was made by the needle without exiting the node. After removing the needle a drop of sample was placed on clean slide. The drop was spread out to make a smear by laying another slide on top of it. Two smears were prepared: one for cytomorphological examination and the other for ZN-AFB staining. For biopsy, following examination and selection of an appropriate lymph node for excision, a local anesthesia (2% Lidocaine) was applied under aseptic conditions to the overlying skin. The affected node was accurately dissected out from the surrounding tissue with a surgical blade. The node was cut into two halves. One half was put in a bottle of formalin and the rest in physiological saline. The surgical incision was sutured with 2/0 catgut and the wound was dressed with a piece of gauze. After a naked eye examination of the cut surfaces of the node, smears was made on a slide for AFB staining. The epidemiological and clinical data were also documented at the same time for each patient.

The formalin bottle biopsy and FNAC slides were transferred to the Pathology Laboratory. Smears prepared from FNA and biopsy samples were stained by Ziehl-Neelsen staining for acid-fast bacilli. Finally the pathologist reads the slide and reports it. Thereafter, leftover FNA and biopsy sample was added by assistant nurse aseptically into sterile universal bottles with 5 ml of 0.85 percent saline solution, for the research purpose. After collected in physiological saline, FNA and biopsy samples were stored at 4^0C until it was transported to Aklilu Lemma Institute of Pathobiology (ALIPB) laboratory using ice pack for further analysis.

3.6.2. Culture

In the TB laboratory at ALIPB, FNA and biopsy specimen were homogenized and mixed with 0.85% normal saline. Briefly; the specimen was decontaminated by an equal volume of 4% NaOH and was centrifuged at 3000rpm for 15 minutes. Then the supernatant was discarded and the sediment was neutralized with 2N HCl. The neutralization was achieved when the color of phenol red indicator was changed from red to deep red. Then thoroughly mixed and decontaminated sediment which contain specimen was inoculated onto Lowenstein Jensen media (separate tubes of pyruvate and glycerol media) and incubated at 37^0C for at least 6 weeks with weekly observation for the presence of mycobacterial colonies. Microscopic examinations of the colonies were performed by using Ziehl-Neelsen staining method so as to select AFB positive isolates. Heat killed cells were prepared from AFB positive isolates by mixing two loopful of colonies in 200µl distilled water and heating at 80^oC for one hour. The same amounts of colonies were kept in glycerol stock at -20° C as backups for future possible related experiments. Finally the heat killed cells were used for molecular characterization.

3.6.3. DNA extraction

Mycobacterial genomic DNA was extracted as previously described by Van sooligen et al [6] with minor modifications. Briefly, 0.2ml of the FNA material was centrifuged at 1200 rpm for 20 min and the supernatant was discarded. Thereafter the pellet was re-suspended in 500µl TE. 50 µl of 10 mg/ml of lysozyme (Boehringer Mannheim, Mannheim, Germany) was added and mixed well before incubation for 1 h at 37°C. The lysozyme-treated samples were incubated at 65°C for 10 min in the presence of 6µl of 10 mg of proteinase K /ml (Boehringer Mannheim) and 70µl of 10% sodium dodecyl sulfate (Boehringer Mannheim, Mannheim, Germany).100 µL

of 5 M NaCl and 80 µL of pre-warmed CTAB/NaCl (Cetyl trimethyl ammonium bromide in sodium chloride) (Merck, p.a) solution was added, and vortexed until the liquid content become "milky" followed by incubation at 65°C for 10 min. Extraction was performed by adding approximately 700 - 800 µl of chloroform/isoamyl alcohol (24:1) (Merck, p.a) and then centrifuged for 10min at 12,000 rpm. The aqueous phase was transferred to a fresh micro centrifuge tube. The DNA precipitate was obtained by adding 450µl of isopropanol (Merck, p.a) to the aqueous phase. After storage for 1 h at -20°C, the DNA was collected by centrifugation at 12,000 rpm for 15 min, washed with cold 70% ethanol, and centrifuged for 5min at 12,000 rpm. Most of the supernatant was removed and placed at room temperature for 15min to evaporate all the ethanol. Finally the pellet was resuspended in 1xTE and stored at -20^{0}c until the PCR assay was performed.

3.6.4. Region of Difference Based Deletion Type Multiplex PCR

Heat killed culture positive samples were investigated by multiplex PCR based deletion typing for the presence or absence of RD4 and RD9 using primers used in table 1 [4]. The PCR amplification mixture used for RD4 and RD9 typing was as follows: reactions PCR amplification of mixtures used for RD9 typing was performed using Thermal Cycler PCR machine (VWR International, UK) according to the standard procedure described by Brosch et al [4]. In short: reaction mixtures were made in a total volume of 20 µl consisting of 10 µl HotStarTaq Master Mix (Qiagen, UK), 7.1 µl distilled water, 0.3 µl of each of the three oligonucleotide primer (100 µM), and 2 µl DNA template samples or controls (heat killed cells). *M. tuberculosis* H37Rv and *M. bovis*, and water Qiagen (Qiagen, UK) were used as positive and negative controls, respectively. The reaction mixture was then heated using Thermal Cycler PCR machine (VWR International, UK) using the following amplification program: 95°C for 10 minutes for enzyme activation; 95°C for 1 minute for denaturation; 61°C for 0.5 minutes for annealing; 72°C for 2 minutes for extension, involving 35 cycles all in all; and final extension at 72°C for 10 minutes. The product was electrophoresed by Agarose Gel Electrophoresis System (BIO RAD, UK) in 1.5% agarose gel in 1X TAE running buffer. Ethidium Bromide at a ratio of 1:1000, 100 bp DNA reference ladder and orange 6x loading dye were used in agarose gel electrophoresis. The gel was visualized using MultiImage Light Cabinet (Alpha Innotech

Corporation, UK) and photograph was taken. After running electrophoresis in 1.5% agarose gel, the result was interpreted as *M. tuberculosis* (RD9 present) when a band of 396bp was observed while the absence of RD9 (either *M. bovis* or *M. africanum*) when a band with 575bp was observed [100].The absence of RD9 was further differentiated by RD4 typing; interpreted as *M. bovis* (RD4 deleted) when the band size of 446 was observed whereas presence of RD4 (*M. tuberculosis* or *M. africanum*) when the band size of 335 was detected [101].

Table 1. Oligonucleotide primers used for molecular typing of *Mycobacterium* isolates and sizes of the expected PCR products.

Locus	Primer name	Primer sequence	Present	Absent
RD4	RD4_FlankF	CTCGTCGAAGGCCACTAAAG	335	446
	RD4_FlankR	AAGGCGAACAGATTCAGCAT		
	RD4_InternalF	ACACGCTGGCGAAGTATAGC		
RD9	RD9_FlankF	AACACGGTCACGTTGTCGTG	396	575
	RD9_FlankR	CAAACCAGCAGCTGTCGTTG		
	RD9_IntenalF	TTGCTTCCCCGGTTCGTCTG		

3.6.5. Spoligotyping

Spoligotyping was performed at ALIPB, following the procedure described by Kamerbeek *et al* [93]. In short; the DR region was amplified with primers DRa (biotinylated at the 5‟end) and DRb, by Thermal Cycler PCR machine (VWR International, UK) and the amplified DNA was hybridized to inter-DR spacer oligonucleotides covalently bound to a membrane. A total volume of 25 µl reaction mixtures was used for PCR: 12.5 µl of HotStarTaq Master Mix ((Qiagen, UK): this solution provided a final concentration of 1.5 mM $MgCl_2$ and 200 µM of each deoxnucleotides triphosphates), 2 µl of each primer (2 Pico mol each), 5 µl suspension of heat killed cells (approximately 10 to 50 nano gram), and 3.5 µl distilled water. Initial heating of the mixture was done at 96°C for 15 minutes and then subjected for amplification by holding 1 minute at 96°C, one minute at 55 °C, and 30 seconds at 72°C for 30 cycles. Final elongation was performed by holding at 72°C for 10 minutes. The amplified PCR product was denatured at 96°C for 10 minutes and then cold shocked on ice to get a single stranded DNA of PCR product. Then this single stranded PCR product was discharged into cellulose membrane using Mini Blotter 1 (Ocimum Biosolutions, UK). Finally, the single stranded PCR products

were hybridized using HB-100 Hybridization Oven (VWR International, UK) to a set of 43 immobilized oligonucleotides on the cellulose membrane. After hybridization, the membrane was washed twice for 10 minutes in 2X SSPE (1X SSPE is 0.81 M NaCl, 10 mM NaH_2 PO 4 , and 1 mM EDTA (pH 7.7), and 0.5% sodium dodecyl sulphate at 60^oc and then incubated in 1: 4000 diluted streptavidin-peroxidase (HotStar, UK) for 45 to 60 minutes at 42^oc. The membrane was washed twice for 10 minute in 2X SSPE and 0.5% sodium dodecyl sulphate at 42^oc and rinsed with 2X SSPE for 5 minute at room temperature. Hybridizing DNA was detected by the enhanced chemiluminescence method using hyper cassette (Amersham Biosciences, UK) and by exposed to x-ray film (Hyperfilm ECL) as specified by the manufacturer.

3.6.6. Flow cytometric analysis of peripheral blood

BDMultiTEST^{TM} CD3 fluorescein isothiocyanate (FITC,clone SK7),CD8 phycoerythrin (PE,clone SK1),CD45 peridin chlorophyll protein (PerCP,2D1(HLe-1) and CD4 allophycocyanin (APC,clone SK3) monoclonal antibodies and MultiTEST CD3 FITC, CD8 PE, CD45 PerCP and CD4 APC with TruCOUNT Tubes were obtained from Becton Dickinson Immuno cytometry Systems (BDIS, San Jose, CA, USA). 5mL blood was collected into EDTA vacutainer tubes from 12 confirmed cases for TBLN and 10 apparently volunteer‟s healthy individuals. Then it was transported to the TASH serology laboratory within one hour where it was processed. The blood samples were stained and fixed on the same day immediately. In short 20µL of MultiTEST CD3/CD8/CD45/CD4 reagent was added in the bottom of the TruCOUNT Tube, and then 50µL of well mixed anti- coagulated whole blood was added in to the bottom of the tube. After capping it was vortexed gently and incubates for 15 minutes in the dark room at room temperature, then a 450 µL 1x FACS (BDIS, San Jose, CA, USA) lysing solution was added, again after capping gently and it was mixed well before incubation for 15 minutes in dark room at room temperature. Peripheral T lymphocyte subsets population analyses were performed using a FACS Calibur (BD Biosciences, California USA). A total of 10,000 cells were counted, cell samples were analyzed on a FACSCalibur flow cytometer (BDIS) equipped with an argon and a diode laser for four color detection. Acquisition and analyses were performed using MultiTEST^{TM} software (Becton Dickinson).Fluorescence voltages and compensation values were determined using singly fluorochrome stained cells from a CaliBRITE Beads which was afforded with the reagents kit.

3.7. Quality control

The qualities of laboratory works were assured by using well known instruments, known reference strain, reagents and chemicals from recognized companies on top of following recent and standard procedures. Beside, known positive and negative controls were used along with each test procedure. Appropriate data collection procedures and analysis software‟s were implemented to make sure that the data was well organized.

3.8. Data collection and analysis

For each individual patient, information relevant to epidemiological investigation and clinical data were recorded on a data sheet. Presence or absence of tuberculosis, and the species and strain of mycobacterium involved were recorded. The database was established in Microsoft® Excel for Windows7.Descriptive statistics was used to assess immunophenotyping characterization of peripheral T lymphocyte with positive TBLN. Specificities, sensitivities and kappa value of different method of diagnosis were assessed. The spoligotyping patterns which were prepared in binary and octal were entered and determined by comparing the spoligotyping results with already existing designations in the international spoligotyping database, SpolDB4.0 (http://www.pasteur-guad-eloupe.fr:8081/SITVITDemo/).Confidence level of 95% was used for statistical significance. Data entry and statistical analyses was performed by SPSS version 20 (SPSS INC. Chicago, IL).

3.9. Ethical Consideration

Ethical permission was obtained from the Ethical and research committee of Department of Microbiology, Immunology and Parasitology, School of Medicine College of Health Science, Addis Ababa University. The support letter from the department of Pathology and Minor surgery at TASH, ATHC and ALIPB were obtained. The purpose, merit and demerit, if any, of the study was clearly stated to the patients in the languages they understood and written informed consent was obtained from each participant before collection of samples. Result of the laboratory investigation was reported to attending physician.

4. RESULT

4.1. Socio-demographic characteristics of the study subjects

A total of 206 clinically suspected TBLN patients who fulfilled the inclusion criteria were included in our study. The study participants" age ranged from 18 to 71 years old with the mean age± SD (31.4± 12.2). Majority of the TBLN cases were found in the age group of 18 to 27 years. There were 94(45.6%) males and 112(54.4%) females suspected TBLN cases making male to female ratio 1:1.2 (**Table 4.1**).

Table 4.1. Distribution of the study population by socio-demographic characteristics in Addis Ababa, Ethiopia, February- October, 2013 (N=206).

Variables		Frequency(No)	Percentage (%)
Gender	Male	94	45.6
	Female	112	54.4
	Total	**206**	**100.0**
Age group	18-27	97	47.1
	28-37	51	24.8
	38-47	36	17.5
	48-57	10	4.9
	≥58	12	5.8
	Total	**206**	**100.0**
Residence	Urban	114	55.3
	Rural	92	44.7
	Total	**206**	**100.0**
Occupation	Merchant	23	11.2
	Student	36	17.5
	Housewife	33	16.0
	Gov. employee	12	5.8
	Farmer	50	24.3
	Unemployed	17	8.3
	Others	35	17.0
	Total	**206**	**100.0**
Educational status	Illiterate	59	28.6
	Prim. school	83	40.3
	Sec. school	41	19.9
	Diploma	17	8.3
	Degree	6	2.9
	Total	**206**	**100.0**
Marital status	Single	96	46.6
	Married	99	48.1
	Divorced	5	2.4
	Widowed	6	2.9
	Total	**206**	**100.0**

Majority of FNAC and histopathology confirmed cases were females 97/206(58.8%), which was statistically significant as compared to males (P≤0.05). More than half of cyto-histopathology proven cases were found in age group of 18-27(51.8%) years old and the urban dwellers (55.4%). Majority of TBLN confirmed by FNAC and histology were among farmers (25.3%) followed by students (17.5%) (**Table 4.2**).

Table.4.2 Socio-demographic characteristics of FNAC and Histopathologically diagnosed TBLN patients from Addis Ababa, Ethiopia, February- October, 2013 (N=206)

Variables		TBLN, n=166 (%)	NTBLN,n=40(%)	Total (%)	P- value
Gender	Male	69(41.6)	25(62.5)	94(45.6)	**0.027**
	Female	97(58.4)	15(37.5)	112(54.4)	
Age group	18-27	86(51.8)	11(27.5)	97(47.1)	**0.74**
	28-37	37(22.3)	14(35)	51(24.7)	
	38-47	28(16.9)	8(20)	36(17.5)	
	48-57	7(4.2)	3(7.5)	10(4.9)	
	≥58	8(4.8)	4(10)	12(5.8)	
Residence	Urban	92(55.4)	22(55)	114(55.3)	**1.000**
	Rural	74(44.6)	18(45)	92(44.7)	
Occupation	Merchant	18(10.8)	5(12.5)	23(11.2)	**0.253**
	Student	29(17.5)	7(17.5)	36(17.5)	
	Housewife	23(13.9)	10(25)	33(16.0)	
	Gov. employee	9(5.4)	3(7.5)	12(5.8)	
	Farmer	42(25.3)	8(20)	50(24.3)	
	Unemployed	17(10.2)	0(0)	19(9.2)	
	Others	28(16.8)	7(17.5)	35(17.0)	
Educational status	Illiterate	45(27.2)	14(34.1)	59(28.6)	**0.598**
	Prim. school	70(42.2)	13(32.5)	83(40.3)	
	Sec. school	31(18.8)	10(24.4)	41(19.9)	
	Diploma	15(9.1)	2(4.9)	17(8.3)	
	Degree	5(3.0)	1(2.4)	6(2.9)	
Marital status	Single	83(50.0)	13(32.5)	96(46.6)	**0. 231**
	Married	74(44.6)	25(62.5)	99(48.1)	
	Divorced	4(2.4)	1(2.5)	5(2.4)	
	Widowed	5(3.0)	1(2.5	6(2.9)	

As it was shown in table 4.3, the highest culture positivity rate 40/74 (54.1%) was recorded among the youngest age group (18-27) years old.38/74(51.4%) and 41/74(55.4%) of L-J culture positive patients were urban dwellers and single respectively. The culture proven diagnostic modality showed that 37/74(50%) and 37/74(50%) of L-J culture positives were males and females respectively. Furthermore, the largest proportion (27%) of L-J culture positive patients

was farmers followed by students (18.9%) in their occupation. The difference of the occupation among the study group was statistically insignificant (**p=0.488**)

Table 4.3. Socio-demographic characteristics of L-J culture positive TBLN patients in Addis Ababa, Ethiopia, February- October, 2013

Variables		**TBLN, n=74 (%)**	**NTBLN,n=132(%)**	**Total (%)**	**P- value**
Gender	Male	37(50)	57(43.2)	94(45.6)	**0.346**
	Female	37(50)	75(56.8)	112(54.4)	
Age group	18-27	40(54.1)	57(43,2)	97(47.1)	**0.149**
	28-37	15(20.3)	36(27.3)	51(24.6)	
	38-47	9(12.2)	27(20.4)	36(17.5)	
	48-57	3(4.1)	7(5.3)	10(4.8)	
	≥58	7(9.4)	5(3.9	12(5.8)	
Residence	Urban	38(51.4)	76(57.6)	114(55.3)	**0.474**
	Rural	36(48.6)	56(42.4)	92(44.7)	
Occupation	Merchant	8(10.8)	15(11.4)	23(11.2)	**0. 488**
	Student	14(18.9)	20(15.2)	34(16.5)	
	Housewife	7(9.4)	26(19.7)	33(16.0)	
	Gov. employee	3(4.1)	9(6.8)	12(5.8)	
	Farmer	20(27.0)	30(22.7)	50(24.3)	
	Unemployed	7(9.4)	12(9.1)	19(9.2)	
	Others	15(20.3	20(15.2	35(17.0)	
Educational status	Illiterate	22(29.7)	37(28.0)	59(28.6)	**0.105**
	Prim. school	37(50.0)	46(34.8)	83(40.3)	
	Sec. school	9(12.2)	32(24.2)	41(19.9)	
	Diploma	4(5.4)	13(9.8)	17(8.3)	
	Degree	2(2.7)	4(3.0)	6(2.9	
Marital status	Single	41(55.4)	55(41.7)	96(46.6)	**0.177**
	Married	29(39.2)	70(53.0)	99(48.1)	
	Divorced	1(1.4)	4(3.0)	5(2.4)	
	Widowed	3(4.1)	3(2.2)	6(2.9	

Table 4.4. Sex & Age distribution of L-J culture positive TBLN patients in Addis Ababa, Ethiopia, February- October, 2013

Sex	**Patients and Age group (in years) (%)**					**Total (%)**
	18-27	28-37	38-47	48-57	≥58	
Male	18(48.6)	11(29.7)	2(5.4)	2(5.4)	4(10.8)	37(100)
Female	22(59.5)	4(10.8)	7(18.9)	1 (2.7)	3(8.1)	37(100)
Total	40(54.1)	15(20.3)	9(12.2)	3(4.0)	7(9.5)	74(100)

4.2. Clinical Profile

More than half of the study participants (54.4%) and (4.4%) had a history of modern and traditional treatment respectively. One or more kinds of previous broad spectrum antibiotics treatment for neck swelling were reported in 91/206 (44.2%) of TBLN cases. Out of the total patients who were visited for neck swelling treatment, 62/206(30.1%) had previous history of tuberculosis, of which 58/62 (93.5%) and 4/62(6.5%) have completed previous anti- TB treatment once and twice respectively. From the total participants who took previous anti-TB 57/62 (92%) completed the course of treatment whereas 5/62 (8%) discontinued it. However, 12/62 (19.4%) of them confirmed TBLN by culture, had completed course of anti-TB drugs once. As shown in Figure 4, 66.5%,8.3%,11.2%,9.2% and 4.9% of FNAs and biopsies were collected from anterior and posterior cervical; supraclavicular, axillary, inguinal and others lymph node of the TBLN suspected patients respectively.

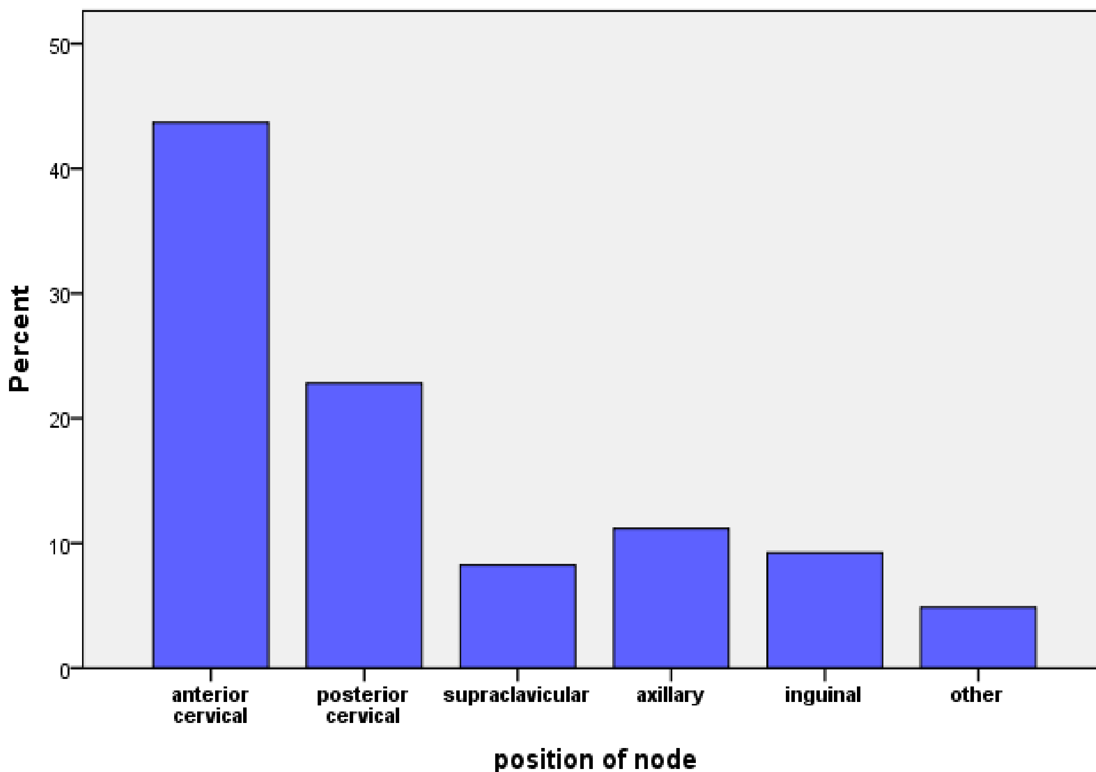


Figure 4. *Distribution of position of Lymph node from TBLN suspected cases in Addis Ababa, Ethiopia, February- October, 2013*

4.3 Laboratory result

One hundred ninety eight (168 from ATHC and 30 from TASH) fine needle aspirates (FNA) and 8 surgical biopsies from TASH were collected from eligible patients. As shown in table 4.6, of the 206 specimens analyzed 38/133 (28.6%) were ZN smear positive for AFB, 74/206 (36%) were L-J culture positive, 96/121 (79.3%) were direct PCR regions of difference (RD9) positive for *M. tuberculosis*. Moreover, cyto-histopathological analysis indicated that 166/206 (80.6%) of the suspected patients had tuberculosis lymphadenitis.

4.3.1. Macroscopic Examination of FNA and excisional biopsy

Macroscopic examination of gross FNA and histology were seen in 206 samples. The purulent (pus) FNA and biopsy constituted the largest aspirates which accounted 125/206 (60.7%), followed by 49/206(23.8%) and 32/206(15.5%) caseous and hemorrhagic (blood mixed) aspirate and excision biopsy respectively (**Figure 5**).

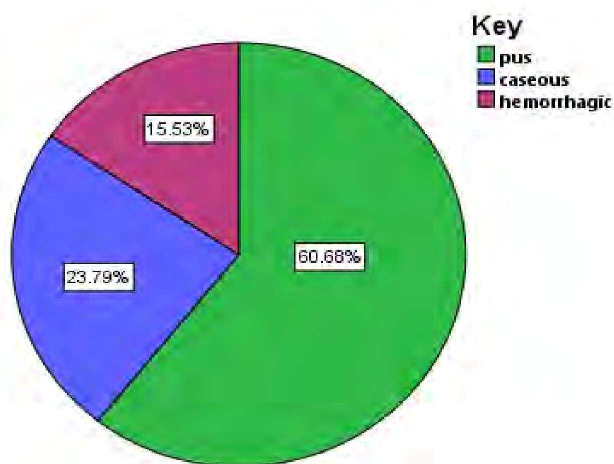


Figure 5. *Distribution of the types of aspirates obtained from TBLN suspected cases in Addis Ababa, Ethiopia February- October, 2013*

Tuberculosis lymphadenitis was confirmed in 107/125 (85.6%) of pus aspirate by cyto-histopathological examination. Growth of bacteria were proven on L-J culture in 33/125(26.4%) of the pus aspirates. Ziehl Nelson staining and direct PCR from pus aspirates identified TBLN in 15/87(17%) and 59/71(83.1%) respectively. Caseous materials were also analyzed in different

diagnostic yield of laboratory methods.FNAC and histopathology gave diagnostic yield of 46/49(93.9%) of caseous aspirates. Of 49 caseous materials, bacterial growth on L-J culture media were confirmed in 37/49 (75.5%). Ziehl Nelson staining demonstrated AFB and PCR gave the positive signal in 22/37(59.5%) and 33/38(86.8%) of the caseating aspirates respectively. A significant association between the presence of caseous materials and proven tuberculosis lymphadenitis was demonstrated in FNA and biopsy for the L-J culture,FNAC and histopathology, Ziehl Nelson staining and PCR of the different diagnostic modalities**(p=00).** In similar way blood mixed aspirates ruled out TBLN in 13/32(40.6%), 1/9(11.1%), 4/12(33.3%) and 4/32(12.5%) by cyto-histology, Ziehl Nelson, direct PCR from the FNA and culture respectively (**Table 4.5).**

Table.4.5.Comparison of diagnostic yield of different laboratory methods used for diagnosis of TBLN with type of aspirate in Addis Ababa, Ethiopia February- October, 2013

Type of Aspirate	Laboratory Methods											
	Culture (**N=206**)			FNAC+Histopathology (**N=206**)			ZN-AFB(**N=133**)			Direct PCR(**N=121**)		
	Positive (n, %)	**Negative (n, %)**	**Total**	**Positive (n, %)**	**Negative (n, %)**	**Total**	**Positive (n, %)**	**Negative (n, %)**	**Total**	**Positive (n, %)**	**Negative (n, %)**	**Total**
Caseous	37 **(75.5)**	12 (24.5)	**49**	46 **(93.9)**	3 (6.1)	**49**	22 **(59.5)**	15 (40.5)	**37**	33 **(86.8)**	5 (13.2)	**38**
Pus	33 (26.4)	92 (73.6)	**125**	107 (85.6)	18 (14.4)	**125**	15 (17.2)	72 (82.8)	**87**	59 (83.1)	12 (16.9)	**71**
Hemorr hagic	4 (12.5)	28 (87.5)	**32**	13 (40.6)	19 (59.4)	**32**	1 (11.1)	8 (80.9)	**9**	4 (33.3)	8 (66.7)	**12**
Total	74 (36)	132 (64)	**206**	166 (80.6)	40 (19.4)	**206**	38 (28.6)	95 (71.4)	**133**	96 (79.3)	25 (20.7)	**121**

4.3.2. Cytology and Histopathology Result

Cyto-histopathological diagnoses of FNA and biopsy smears were available for all specimens collected. The criteria for cyto- histopathological diagnosis was epitheloid cell granulomas with or without multinucleate giant cells, with or without necrosis and caseous necrosis without granuloma. Thus, cytomorphological features of FNAC and histopathology consistent with

tuberculosis lymphadenitis were reported in 166/206 (80.6%) of the examined smeared specimens, whereas the remaining 40/206(19.4%) smeared specimens showed different kinds of lymphadenopathies.

A comparison of FNAC and Histopathology and Ziehl-Neelsen (AFB) showed that both diagnostic modalities diagnosed TBLN in 38/133(28.6%), but FNAC and Histopathology alone dictated TBLN in 125/133(94%) suspected TBLN cases. When compared with culture both combined together dictated 71/206(34.5%) and the agreement of the two techniques in diagnosis of TBLN was slight (**kappa=0.2**), however FNAC and histology detected 71/74 (96%) cases of culture proven tuberculous lymphadenitis. FNAC and histopathology with direct PCR identify TBLN in about 77% and this two techniques have a moderate agreement in diagnosis of TBLN (**kappa=0.41**) (**table 4.6**). Moreover the sensitivity, specificity, PPV and NPV of the FNAC and histopathology were **96%, 28%, 42.8% and 92.5%** respectively when culture used as the gold standard (**Table 4.7**).

4.3.3. Ziehl-Neelsen staining

Of the total of 206 specimens collected only 133 samples were processed to detect the bacilli by Ziehl-Nelson staining. Of 133 processed FNA and biopsy, ZN smears detected 38/133 making the detection rate of AFB, 28.6%.ZN-AFB combined with direct PCR detected the bacilli in 29/104(27.9%).Moreover the agreement between these two techniques in diagnosis of TBLN was poor(**kappa=0.00**). Beside when combine with culture the diagnostic ability of the two techniques was 28/133(21%), with the (**kappa=0.30**) which was fair agreement. The overall FNA, ZN smears detected 28/64(43.8%) of culture proven TBLN cases. The diagnostic sensitivity, specificity PPV and NPV of ZN-staining was **43.8%,** 85.5%, 73.7% and 62.1% respectively (**Table 4.6 & Table 4.7).**

Table 4.6.Comparison and kappa values of diagnostic yield of different laboratory methods used for diagnosis of TBLN with each other's and prevalence of TBLN by different diagnostic methods in Addis Ababa, Ethiopia, February- October, 2013

Laboratory Methods		**Culture(n=133)**			**Prevalence n (%)**	**Kappa value**
		Positive	Negative	Total		
AFB(n=**133**)	Positive	28	10	38	**38(28.6)**	**0.30**
	Negative	36	59	95		
	Total	**64**	**69**	**133**		
		Culture(N=206)			**166(80.6)**	**0.20**
FNAC and Histopathology (N=**206**)		Positive	Negative	**Total**		
	Positive	71	95	166		
	Negative	3	37	40		
	Total	**74**	**132**	**206**		
		Culture(n=121)			**96(79.3)**	**0.28**
Direct PCR(n=**121**)		Positive	Negative	**Total**		
	Positive	53	43	96		
	Negative	3	22	25		
	Total	**56**	**65**	**121**		
		FNAC and Histopathology(N=206)				
AFB(n=**133**)		Positive	Negative	**Total**		**0.10**
	Positive	38	0	38		
	Negative	87	8	95		
	Total	**125**	**8**	**133**		
		FNAC and Histopathology(N=121)				**0.41**
Direct PCR(n=**121**)		Positive	Negative	**Total**		
	Positive	93	3	96		
	Negative	16	9	25		
	Total	**109**	**12**	**121**		
		FNAC and Histopathology(N=206)			**74(36)**	**0.20**
Culture (N=**206**)		Positive	Negative	**Total**		
	Positive	71	3	74		
	Negative	95	37	132		
	Total	**166**	**40**	**206**		
		AFB(n=104)				**0.00**
Direct PCR(n=**104**)	Positive	29	63	92		
	Negative	4	8	12		
	Total	**33**	**71**	**104**		

Table 4.7.Sensitivity, specificity, positive and negative predictive values of the laboratory methods against culture as gold standard used for the diagnosis of TBLN patients in Addis Ababa Ethiopia, February- October, 2013

	Laboratory Methods	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Microscopic Investigation	Ziehl Nelson(AFB)	43.8	85.5	73.7	62.1
	FNAC+Histology	96	28	42.8	92.5
	Direct PCR dictation	94.6	33.8	55.2	88

4.3.4. Culture (Microbiological Study)

A total of 198 FNA and 8 biopsy samples were processed for bacterial growth on Löwenstein–Jensen (L-J) culture media. From which 74/206 (36%) showed positive growth after 8 weeks of incubation and the rest were culture negative. Of positive culture 21/74(28.4%), 50/74(67.6%) and 3/74(4.0%) grew on both glycerol and pyruvate, on glycerol only and pyruvate only containing media respectively. Culture positivity was proven in 44/74(59.5%) and 30/74(40.5%) specimens within 4-6 and 7-16 weeks of incubation period respectively. Those all culture positive cases were checked by ZN- AFB for further molecular investigation.

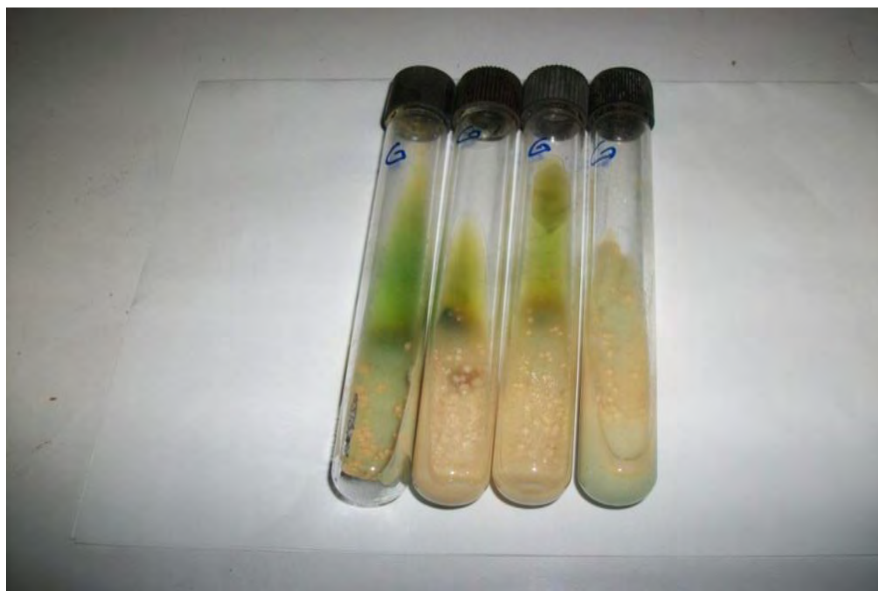


Figure 6. *Some of Culture Positive of TBLN cases on LJ media, Addis Ababa, Ethiopia, February- October, 2013*

4.3.5. Polymerase Chain Reaction (PCR)

RD9 and RD4 deletion typing (MTC Species identification)

M. tuberculosis complex species identification and comparative diagnostic modalities were done based on RD9 and RD4 (region of difference nine and four) sets of primers kits. Accordingly 73/74(98.6%) culture proven isolates were confirmed as *M. tuberculosis*, whereas, 1/74(1.4%) confirmed as *M.bovis.* FNA specimens were also directly amplified which 121/206(58.7%) were available for the procedure. Of which 96/121(79.3%) of them positive for PCR, all of them being *M. tuberculosis*. When direct PCR combined with culture they dictated TBLN in 53/121(43.8%) with (**kappa=0.28**). More over direct PCR dictated TBLN in 53/56(94.6%) of culture proven TBLN cases. Making culture as a gold standard, sensitivity, specificity, PPV and NPV of the direct PCR were 94.6%, 33.8%, 55.2% and 88% respectively (**Table 4.7).**This indicated that 43/121(35.5%) of culture negative cases, positive with PCR. In similar way 3/121(2.5%) of the samples which negative for FNAC gave positive by molecular method. Of 104 ZN-AFB 63 were negative for acid fast bacilli, but PCR detected as positive 63/104 making detection rate (60.6%) over ZN staining.(**Table 4.6).**

Figure 7. *Electrophoretic pattern of the amplified element of PCR products from clinical samples in 1.5% agarosegel. Addis Ababa Ethiopia, February- October, 2013*

Lane1= 1kb ladder, Lane2= H37Rv (M.tuberculosis +ve control), Lane3=Qiagen H_2O(-ve control), Lane4= M. bovis (+ve control). Lanes 5 to 15 clinical samples from culture isolate contained DNA from mycobacteria within M. tuberculosis complex.

4.3.6. Spacer oligonucleotide typing (Spoligotyping) result

(MTC Strain identification)

Of total 74 clinical isolates 57/74(77%) were classified into one of 26 distinct spoligotype patterns shared international types (SIT) according to SpolDB4.0 (http://www.pasteur-guad-

eloupe.fr:8081/SITVITDemo/). The remaining 16/74(21.6%) isolates generate 13 different spoligotypes pattern that had not been previously reported to the SpolDB4.0. And one isolate was identified as *M.bovis* having the SB1176 spoligotype pattern according to web at www.Mbovis.org which accounted 1/74(1.4%).Among the distinct spoligotype pattern characterized, 8 patterns corresponding to cluster with 2-14 isolates per clusters were identified. The remaining 18 patterns represented by a unique (non-clustered) spoligotypes pattern were pseudo orphan which were represented as a single in the data base.

In addition to these patterns there were 16 isolates classified into 13 patterns previously unrecorded to the fourth international Spoligotyping database (spolDB4.0).Among these new spoligotyping pattern 10 were represented by a unique pattern which were true orphan according to spolDB4.0, whereas the remaining 3 pattern consisted of cluster with 2 isolates each per cluster were identified (T**able 4.9**).

One *M. bovis* isolated from human FNA sample in our study showed typical bovine tuberculosis having spoligotype profile lacking spacers 3-7, 9, 16, 24-26, 28-37 and 39-43. The largest cluster identified in our present study was SIT149 consisted 14 isolates,SIT53,SIT26, SIT37 and SIT523 were the commonest cluster identified consisted 7,5,4 and 3 isolates respectively.However,18/57(31.6%) isolates were represented by a unique(no-clustered) spoligotype patterns (**Table 4.8).**

Classification of the spoligotype pattern with web based SPOTCLUST data based showed different families “ill defined” (T), Central Asian (CAS), Family33, Family36, Haarlem (H) and Latino American-Mediterranean (LAM) were reported. Among these families the most predominant and prevalent family identified was T family consisted 41/74(55.4%) isolates with the following distribution: T3-ETH 14/41(34.1%), T1 20/41(48.8%), T3 6/41(14.6%) and T4 1/41(2.4%).The CAS families was the second largest dominant comprising 12/74(16.2%) of the total strains: the CAS1-DELHI clade accounted for 7/12(58.3%),CAS-KILI 2/12(16.7%) and CAS clade consisted 3/12(25%).Other families present were Family33,7/74(9.5%). Haarlem1, 3/74(4.1%), Haarlem3 1/74(1.4) and Haarlem4 contain 3/74(4.1%).The LAM7-TUR, LAM9, U,

X and Family36 were the least families in our study (**Table 4.8**).This web also classified strains into different lineage: modern lineage, Euro-American 49/74(66.2%), ancestor lineage, Indo-Oceanic 13/74(17.6%) and *M.bovis* lineage, 1/74(1.4%) and the rest 11/74(14.9%) identified were unknown lineages.

Table 4.8.Spoligotype patterns of *M.tuberculosis* and *M.bovis* strains, family and lineage assignment of TBLN from clinical isolates according to SpolDB4.0 data base and SPOTCLUST web based program in Addis Ababa, Ethiopia, February- October, 2013.

Binary Format	Octal Format	SIT	Family	Lineage	No(%) of strain in this study
1110000111101111111111000000000000111111111	703677740003771	954	CAS	Unknown	1(1.4)
1111111111111111111111111111111100001111	777777777760771	53	T1	Euro-American	7(9.5)
1111111111111111111111111111111111111111	777777777777771	523	Family33	Indo-Oceanic	3(4.1)
1111111111111111111111111111011100001111	777777777560771	462	T1	Euro-American	2(2.7)
1110000111111111111111000000000000110011	703777740003171	25	CAS1-DELHI	Unknown	1(1.4)
1111111111110111101111111111111100001111	777736777760771	217	T1	Euro-American	1(1.4)
1111111111111111111111111000000000001111	777777774000771	1952	X1	Indo-Oceanic	1(1.4)
1111111110000000000111111111111100001111	777000377760771	149	T3-ETH	Euro-American	14(18.9)
1111111111111111111000001001111100001111	777777404760771	41	LAM-TUR	Euro-American	1(1.4)
1110000000000000000000001110011111111111	700000007177771	910	U	Indo-Oceanic	1(1.4)
1110000111111111111111000000000000111111	703777740003771	26	CAS1-DELHI	Unknown	5(6.8)
1110000110111111111000000000000000011111	703377400001771	21	CAS1-KILI	Unknown	2(2.7)
1111111111111111111111111000000100001111	777777774020771	47	H1	Euro-American	1(1.4)
1111111111111111111111111111000100001110	777777777420731	817	H4	Euro-American	2(2.7)
1111111111110111111111111111111100001111	777737777760771	37	T3	Euro-American	4(5.4)
1110000111111111111111000000000000110011	703777700003171	2392	CAS	Unknown	1(1.4)
1101111111111111111111111111110100001011	677777777720571	699	H3	Euro-American	1(1.4)
1111111111111111110111111111111100001111	777777377760771	40	T4	Euro-American	1(1.4)
0000000000000000000000001111110100001110	000000007720731	830	Family36	Euro-American	1(1.4)
1111111111111111101111111111111100001110	777776777760731	336	X1	Euro-American	1(1.4)
1110000111111100000000000000000000001111	703760000000771	2102	CAS	Indo-Oceanic	1(1.4)
1111111111110001111111111111111100001111	777707777760771	2128	T1	Euro-American	1(1.4)
1110000111111111111111000000000000111011	703777740003571	289	CAS1-DELHI	Unknown	1(1.4)
1110000111111111111111000000000000111001	703777740003471	247	CAS	Unknown	1(1.4)
1100000101111110111111100010000000000100	602773761000200	SB1176	M.bovis	M.bovis	1(1.4)
1111111111111111111111111111000100001111	777777777420771	777	H4	Euro-American	1(1.4)
1111111110111111111111111111111100001111	777377777760771	1166	T1	Euro-American	1(1.4)
1111111110111110111111111111110100001111	777373777720771	Orphan	Orphan	Euro-American	2(2.7)
1111111111111111111111111111001000001111	777777776200771	Orphan	Orphan	Euro-American	1(1.4)
1111111111111111101111111111111100001101	777776777760661	Orphan	Orphan	Euro-American	1(1.4)
1111111110111011111111111111100100001111	777357777420771	Orphan	Orphan	Euro-American	1(1.4)
1111110101110111111000001111111100001111	772737407760771	Orphan	Orphan	Euro-American	1(1.4)
1101111111111101111111111111110100001011	677767777720571	Orphan	Orphan	Euro-American	1(1.4)
1011111010000000000001111111111100001101	575000077760671	Orphan	Orphan	Euro-American	1(1.4)
1111111110000000000011111111111100001111	777000177760771	Orphan	Orphan	Euro-American	1(1.4)
1111111110111001111111111111111101111101	777347777767671	Orphan	Orphan	Indo-Oceanic	1(1.4)
1111111110111001111111111111111100111111	777347777763771	Orphan	Orphan	Indo-Oceanic	1(1.4)
1111111101110111111101111111111100001111	776737677760771	Orphan	Orphan	Euro-American	1(1.4)
1111110111111111111111111110000000001111	773777776000771	Orphan	Orphan	Indo-Oceanic	2(2.7)
1111111110111001111111111111111100111101	777347777763671	Orphan	Orphan	Indo-Oceanic	2(2.7)

SpolDB4.0= fourth international spoligotyping database; SIT= Spoligo International Typing, CAS=Central Asian; LAM = Latin American-Mediterranean; H = Haaralem, T=Tuscany,U=Ural

Table 4.9.Spoligotype patterns of orphan *M.tuberculosis* strains, family and lineage assignment of TBLN from clinical isolates according to SpolDB4.0 data base and SPOTCLUST web based program in Addis Ababa, Ethiopia, February- October, 2013.

Binary Format	Octal Format	SIT	Family	Lineage	Probability	No(%) of strain in this study
1111111111110111110111111111110100001111	777737377720771	Orphan	T1/X1	Euro-American	0.3466/0.6533	2(2.7)
1111111111111111111111111001000000001111	777777776200771	Orphan	T1/H3	Euro-American	0.2267/0.7704	1(1.4)
1111111111111111011111111111111100001101	777776777760661	Orphan	T1/X1	Euro-American	0.3466/0.6533	1(1.4)
1111111110111011111111111111000100001111	777357777420771	Orphan	T1/H3	Euro-American	0.2267/0.7704	1(1.4)
1111110101110111111000001111111100001111	772737407760771	Orphan	LAM9	Euro-American	0.9984	1(1.4)
1101111111111101111111111111111010000101 1	677767777720571	Orphan	T1/H3	Euro-American	0.2273/0.7725	1(1.4)
1011111010000000000000111111111100001101	575000077760671	Orphan	T3	Euro-American	0.9999	1(1.4)
1111111110000000000001111111111100001111	777000177760771	Orphan	T3	Euro-American	0.9999	1(1.4)
1111111110111001111111111111111101111101	777347777767671	Orphan	Family33	Indo-Oceanic	0.9999	1(1.4)
1111111110111001111111111111111100111111	777347777763771	Orphan	Family33	Indo-Oceanic	0.9999	1(1.4)
1111111101110111111101111111111100001111	776737677760771	Orphan	T1	Euro-American	0.9999	1(1.4)
1111110111111111111111110000000000001111	773777776000771	Orphan	H1	Indo-Oceanic	0.9788	2(2.7)
1111111110111001111111111111111100111101	777347777763671	Orphan	Family33	Indo-Oceanic	0.9999	2(2.7)

SpolDB4.0= fourth international spoligotyping database; SIT= Spoligo International Typing; LAM = Latin American-Mediterranean; H = Haaralem, T=Tuscany

Figure 8. Autoradiograph of some of the spoligotypes, Addis Ababa, Ethiopia, February- October, 2013

4.3.7. Immunological study (Flowcytometric Analysis)

We analyzed 12(4 males and 8 females) TBLN confirmed patients by culture or/and cytology or/and direct PCR/ZN-AFB and 10(5males and 5 females) HIV negative healthy controls. The mean age ±SD of patients (cases) and controls were 24.4± 4.5 and 25.1±2.0 respectively. The age range of all the study participants was 18-30 years old.

Flowcytometric analysis showed that the mean number (±SD) of CD3^{+}T cells and CD3^{+}CD4^{+} helper T cells of patients with TBLN were 1414.8 cells/µL±424.1 and 752 cells/µL±270.9 respectively. These cells had relatively decreased mean percentage in TBLN cases when we compared with the healthy individuals (72.0%, 40.3%) versus (73.3%, 43.1%) respectively. Moreover the difference of mean percentage of T helper cells between cases and controls was statically significance(**P=0.05**).However the difference of mean percentage of total T lymphocytes was statically insignificance (**P=0.672**).In contrast the proportion of mean percentage of CD3^{+}CD8^{+} cytotoxic T cells in TBLN cases relatively increased versus healthy controls (29.2% **versus** 27.2%) (**p=0.560**)(**Table 4.10**)

Table.4.10. Distribution of mean number and percentage of T lymphocytes population among TBLN confirmed case and healthy control in Addis Ababa, Ethiopia, February-October, 2013

Mean number and percentage of T lymphocyte populations		Case (N=12)	Mean ±SD	Control (N=10)	Mean ±SD	P-value
CD3^{+}	Mean No(cells/µL)	1414.8	1414.8±424.1	1871.5	1871.5±817.5	0.107
	Mean %	72.0	72.0±6.2	73.3	73.3±8.0	0.672
CD3^{+}CD4^{+}	Mean No(cells/µL)	752.8	752±270.9	1004.8	1004.8±310.4	0.05
	Mean %	40.3	40.3±7.8	43.1	43.1±6.4	0.381
CD3^{+}CD8^{+}	Mean No(cells/µL)	578.3	578.3±280.5	543.1	543.1±165.5	0.731
	Mean %	29.2	29.2±8.8	27.2	27.2±6.3	0.560
CD3^{+}CD4^{+}/CD3^{+}CD8^{+}		1.3(1.38)		1.85(1.58)		

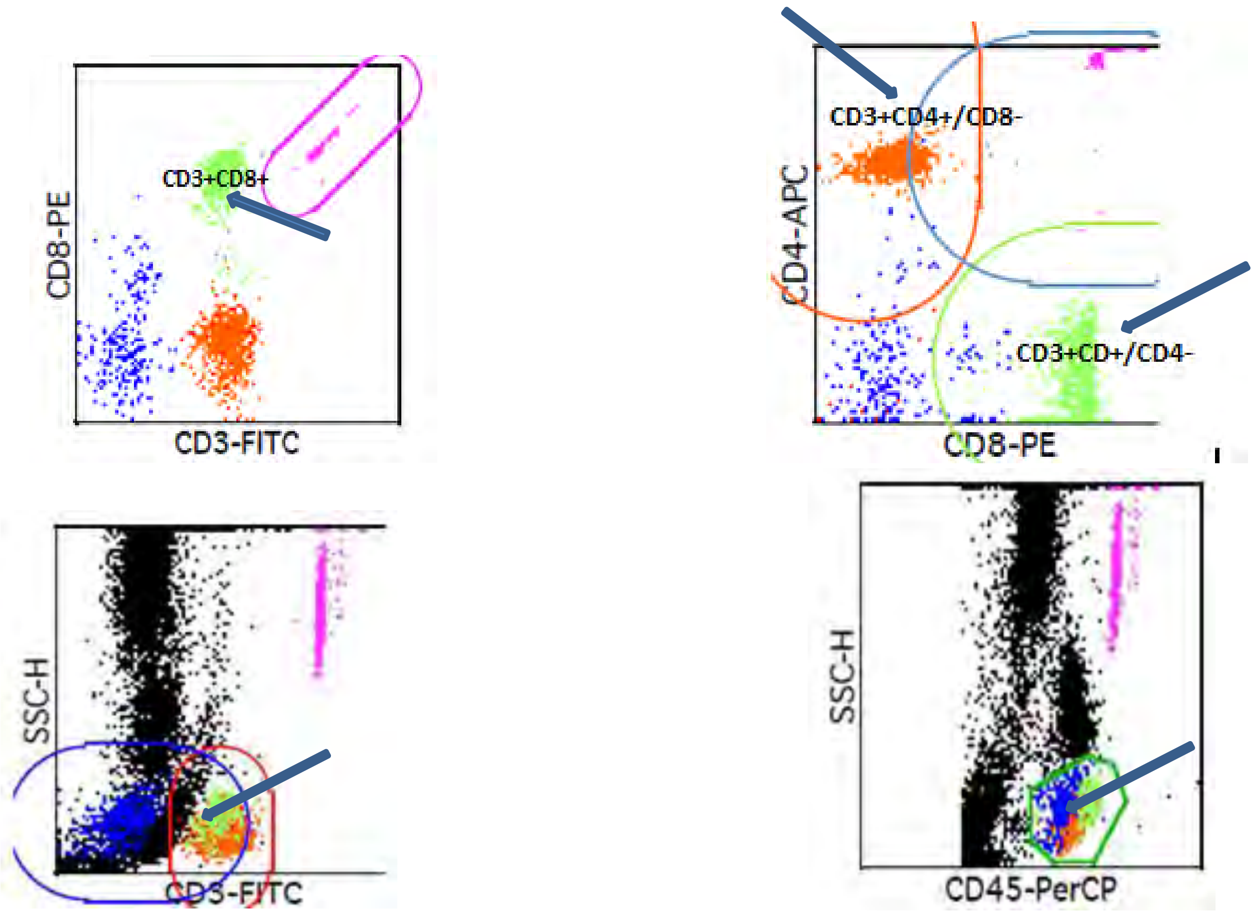


Figure.9 *Distribution of T lymphocytes populations among TBLN confirmed case in Addis Ababa, Ethiopia, February- October, 2013*

5. DISCUSSION

The proportion of extrapulmonary TB among all TB cases varies from country to country. Of the 22 high burden countries the highest proportion was reported from Cambodia (40%) and the lowest from China (<1%) [1]. Extrapulmonary TB is a significant health problem worldwide because of difficulties in its diagnosis and in monitoring its treatment, in which TBLN is accounting for 30-64% of cases in reported series [93]. Our present finding showed that high prevalence of TBLN in which it was reported 74/206(36%) by culture, 166/206(80.6%) by cyto-histology and 28.6% and 79.3% by ZN staining and PCR respectively. A few studies conducted in our country, Ethiopia, also indicated high prevalence of TBLN in different diagnostic modalities by different investigators [70, 71, 102, 103].

Our present study comprised the age range from 18-71 years old. More than half (54.1%) of TBLN cases found among the younger age group (18-27).This finding is supported by studies from different parts of Ethiopia [70,103-105],in which TBLN was reported high among the youngest age. This also similar with other finding somewhere in the world, in which high TBLN was observed among younger age [74, 79,106]. Our finding indicated also that, there was female dominance than males. This is in line with other studies elsewhere in the world [75,107-110]. Moreover, it has been suggested that in male dominated communities, where women experience poorer living conditions, young females generally notice differences in their appearance earlier than males[111].

Tuberculosis lymphadenitis culture positive cases were higher in farmers (27%) followed by students (18.9%).This might be due to fact that the farmers constituted the majority of the population in Ethiopia, with low nutritional and economic background, and possible also be due to occupational background. A considerable number of TBLN cases were found to be students. This might be due to, this category of patients are among young reproductive age and more vulnerable to HIV infection with high prevalence than other age group [72,103]. But the difference of this two occupations were not statically significant(p=0.513).Though we didn‟t assessed the HIV sero-satatus of individuals, the EPTB manifestation of TB is prevalent in 10-

34% of non HIV cases while it occurs in 50-70% of patients co-infected with HIV [99]. Still single consisted more than half TBLN confirmed cases (55.4%),if single get together with divorced and widowed they represented the high risk group, prone to HIV and TB infections.

The diagnosis of TBLN has been a true challenge solely by clinical evidence both in developed and developing countries,particularily in developing countries due to limited diagnostic facility on hand [112]. TBLN positivity was confirmed by FNAC and Histopathology in 94%, by culture in 75.5%, by Zeihl Nelson staining in 59.5% and by direct PCR in 86.8% of caseous material.We attempted to correlate different gross appearance aspirates with different diagnostic modalities.The highest positivity rate was recorded in caseous material in different diagnostic methods. Our finding is comparable with studies done by others[113,114].This might be due to the fact that the caseous aspirate believed to contains large numbers of bacilli in advanced stage of the disease[103]. Besides the high positivity of caseous aspirates among different diagnostic modalities were statically significant ($p \leq 0.05$).

In countries with high tuberculous infections and limited laboratory capacity and no sophisticated tools for diagnosis of TBLN, the use of FNAC features from lymph node aspirates as diagnostic tools has been shown to be higher efficient, safe procedure and strongly suggestive of TBLN [115,116].FNAC has assumed an important role in the evaluation of peripheral adenopathy as a possible noninvasive alternative to excisional biopsy. The cytological and histopathological criteria for diagnosis of possible TBLN have been clearly defined epitheloid cell granulomas with or without multinucleate giant cells, with or without necrosis and caseous necrosis without granuloma [108].Our present study revealed that 166/206 (80.6%) were confirmed as positive with cyto- histopathologically. Study from India and Tanzania consistent with our finding [61,106]. But others studies showed low number [70,104,105,117]. The reason might be the kind of patients seen at different geographical location and the stage of disease at the period of data collection.

Zeihl Nelson staining to investigate the AFB in FNA and biopsy is a simple and affordable method which can be done in determination of lymph node enlargement.Our study showed that 38/133(28.6%) was confirmed as TBLN from the FNA sample.This finding has low positive rate,as investigating bacilli in specimen by Zeihl Nelson staining requires more than 10,000 organisms/ml of sample ,thus in FNA sample the expected bacilli is very low.Other investigator also agreed with our present study [103], but different from other studies[118-120], which consisted 37%-42.7% of efficiency.Different reason might be arised:the method and the reagent used,the sample size and the distribution/homogensity of bacilli in aspirate can be make difference between our finding and their findings.Eventhough the sensetivity of this method was found to be relatively low (43.8%),the high specificity(85.5%) was reported. Acid fast bacilli alone give enough confident to the clinician in determination of initiation of anti-TB treatment.This in turn very important to eliminate antibiotic and anti-TB trials so that the patient to get the treatment early.

The presence of 10-100 live bacilli/ml of sample is enough for positive culture result. Seventy four (36%) cases of culture proven TBLN patients were identified from the total of our study participants. This finding is in agreement with 10-60% reported by different investigators [70,108,121-124].We observed as low as 36% of culture positive rate. Several reasons can support these low findings: Previous treatment by one or more course of anti-biotic before they visit the health institution could suppress and inhibit bacterial growth. Besides, scanty nature of the bacteria in the FNA sample and the decontamination process also had the contribution in suppressing the growth of bacilli [125,126]. Of our study participants, 44.2% were previous treated with one or more course of antibiotics. As it was seen in our study high culture positivity rate (75%) was recorded in caseous aspirates materials. This is might be high number of bacterial load in caseous aspirates with advanced stage of disease, as it was reported by others [110].

Nucleic acid amplifications techniques like PCR, has a considerable impact on disease diagnosis on account of their speed, specificity and enhanced sensitivity. Therefore the application of PCR to the diagnosis of TBLN has the potential to resolve one of the foremost challenges facing a

diagnostic laboratory. This method is also very important currently, for rapid identification and diagnosis of mycobacteria from both cultured and uncultured clinical specimens [88, 91].Our present direct PCR investigation of FNA sample showed that, there was 96/121(79.3%) positive for PCR all of them being *M.tuberculosis*. The different investigation on PCR from FNA samples to detect bacilli positivity rate of PCR varies from 61-94% [102,127,128-131]. The positive rate of our findings was relatively low when we compared with others [69, 102, 103,127,128]. The possible reason might be the method used and different inhibition factors during the extraction process. The sensitivity and specificity of PCR were 94.6% and 33.8% respectively, which in line with others [88,129].The high positive rate of PCR was seen in caseous material, 33/38(86.8%).Few dead and live bacilli is enough to detected by PCR, so that caseous material consists of more bacilli [108,128].

In our present study we used region of difference (RD) to identify or to characterize the members of MTC. Polymerase chain reaction of region of difference profile was employed for rapid, simple and reliable technique which detect MTC chromosomal region of difference deletion loci [132,133]. Of our studies participants, RD9 analysis showed that 73/74(98.6%) culture proven TBLN where RD9 present, which they showed 396bp in gel electrophoresis, and they were all *M.tuberculosis*. The remaining 1/74 (1.4%) was deleted for RD9 showed the band at 575bp (this may be *M.africanum* or *M. bovis*) but finally it was confirmed as *M. bovis* by RD4 deletion and spoligotyping. Our finding was supported by different investigator, in which they had reported majority of causative agent of TBLN were *M. tuberculosis* [69, 75,107, 103, 121].

Ethiopia has the largest cattle in Africa, practices such as the consumption of raw milk and meat, mean that the opportunity for zoonotic transmission of BTB is high [134]. Based on PCR, RD9 and RD4 commercial kit, of the total 74 culture isolates, we found 1.4% of bovine tuberculosis in our present study. As it was discussed before most of the ethologic agent of TBLN was *M. tuberculosis*, even though other MTC particularly *M. bovis* is not excluded. However, our finding was lower than the study done in Buta Jira, South Eastern, Ethiopia, which they reported 17.1 % [98]. And other study conducted in Tanzania showed 10.7% of *M. bovis* was reported

from human TBLN case [135]. The reason for this might influenced by sample size, geographical and ethnic composition, socio economic factors, geographical locations, culture, dietary and farming practice in the community.

Spoligotyping was used as a primary typing tool because of its ease of use, straight forward coding and international data base of global isolates for comparative analysis and is known to reproducibly reconstruct family tree [136,137]. In our present study, we used molecular finger printing techniques to have better understanding about mycobacterial strains and to clarifying the epidemiology of tuberculosis transmission in TBLN. We included 74 isolates for spoligotyping, of which 57 isolates were classified into 26 distinct spoligotypes pattern, whereas the remaining 16 isolates were classified into 13 different spoligotypes pattern, that had been new to the international data base and one isolate was identified as bovine tuberculosis according to analysis of international database [138,139].

The most dominant cluster in our present study were SIT149,SIT53,SIT26 and SIT37 respectively in decreasing prevalence which they constituted 52.6% of total identified according to the international data base, SpolDB4. Our finding was comparable with the study done in Ethiopia [140] in which the higher percent was with the common strain with ours. In our present study clade analysis showed that, SIT149 was highly dominant. In addition to this 62/206(30.1%) of our participants have had a history of tuberculosis and they had took the anti TB treatment at least once. Of which 12 isolates were identified in which three isolates were grouped under SIT149 and one isolate under SIT523, which had been reported previously as MDR-TB [141]. Therefore we strongly suggested that this strain might be MDR-TB strains circulate in this country.

Spoligotyping in this study identified 8 international families, according to the SPOTCLUST web based program [8]. Internationally „ill-defined"T, Central Asia(CAS),Haarlem(H),Latin America-Mediterranean(LAM),Ural(U),X,Family33 and Family36. According to this web based

program T and CAS families together comprised 53/74(71.6%) isolates. When combined with the Haarlem clade it increased to 81.1% isolates, in which T, CAS and Haarlem constituted 55.4%, 16.2% and 9.5% respectively. As previous study indicated the T family and CAS family were the dominant families [136,139,140,142] identified which in line with our study. The genotype assignment demonstrated that a major proportion of the strains analyzed belonged with T (55.4%) and CAS (16.1%) genotype which means they are clustered and having high probability to be evolved in the same chain of the recent TB transmission.

T family further grouped in sub families (T1-T4), which T1 families were the dominant in our finding. Of these sub families, T3-ETH predominant strains in Ethiopia and also described in the international data base as predominant genotype in Ethiopia [143,144].But in our study, only 14/74(19%) were from T3-ETH genotype. More over T3-ETH which had SIT149 was previously shown to be frequent in Ethiopia and in Denmark among Ethiopia immigrants. This genotype is believed to specific for Ethiopia and is rarely reported in other countries. In general the overall dominance of T family resembles the distribution pattern in Eastern Africa countries suggesting a similar transmission trend [139].

The second most prevalent genotype was Central Asian (CAS) family which accounted 16.2% from the total isolates: CAS1-DELHI, CAS1-KILI and CAS, comprised 58.3%, 16.7% and 25.0% respectively of the CAS strains.CAS1-DELHI commonly found in India, Indian sub content and Pakistan [139,144]. As it was stated, this genotype highly prevalent in Central Asian because of it might have biogeographic specificity to this parts of world and it has been suggested that this family might be transported by trade, tourism or migration from India to other part of world [145].Our finding of this strains also supported by different researchers [136,139].CAS1-KILI, unlike our finding it was prevalent among MDR-TB patients in Ethiopia [145]. This genotype also highly prevalent in Tanzania and Kenya [139], this might be due to, biogeographic specificity of the strains.

Haarlem also reported in 7/74(9.5%) of our cases, this is very similar with the study conducted in Ethiopia [136, 1349, 143].Haarlem genotype is common in Central America, Europe and the Caribbean, and also has been reported from Kenya, Malawi, Mozambique, Tanzania and West Africa [139]. The other families were LAM, X and U genotypes comprising 2, 1 and 2 isolates respectively. Among the LAM family, the LAM-TUR is believed to be restricted Turkey [146], whereas LAM9 has been reported from different parts of the world including Africa [136]. In our finding, it was one isolates identified as *M.bovis* which have spoligotypes pattern of SB1176 according to the web at **www.Mbovis.org**. This typical bovine TB was first identified in government farm from cattle in Holeta district near to Addis Ababa, Ethiopia [134]. Our data showed the present finding of typical *M.bovis* was isolated from human sample, FNA around **Sabeta, neighbor of Holeta**. Therefore this strain was highly dominant and circulated in central Ethiopia as zoonotic importance of the disease.

According to the SPOTCLUST web based program [8], currently we identified three principal lineages with Euro –American (modern lineage) which was the dominant (66.2%) lineage, followed by the Indo-Oceanic (ancestor) lineage accounted (17.6%), *M.bovis* (ancestor) lineage (1.4%) and the unknown lineage which constituted (14.8%).Although „modern" strains of *M. tuberculosis* are more prevalent worldwide, the „ancient" strains of *M. tuberculosis* strains are also responsible for the spread of tuberculosis [147].The most prevalent lineage in our current assessment was Euro-American lineage in which it accounted 66.2% of strains analyzed belonged. Our data further confirmed that the old ancestral lineages such as Indo-Oceanic and *M.bovis* lineage are circulating in study population even if in relatively low rate. About 11 isolates were have of unknown lineage and 21.6% of isolates were new to the data base, therefore other test with more discriminatory power have to implement to have full information about these strain, which might indicated geographical localization of the strains to Ethiopia.

Understanding the components of host immune response at a basic level is likely to lead to a better understanding of the pathogenesis of tuberculosis in humans and to result in better and novel approaches to prevent and therapy of this disease[23]. *M.tuberculosis* is a classic

example of a pathogen for which the protective response relies on cell mediated immunity. Both CD4+ and CD8+ T-cells are important for successful immunity to tuberculosis. They have many effector functions such as cytolysis and release of potent antimycobacterial cytokines like IFN- γ and TNF- α [148,149].

Therefore, an evaluation of change of lymphocyte percentage can reflect the state of a disease pathogenesis. Our present Immunological study by flowcytometry analysis conducted on 12 TBLN patient cases and 10 currently health individuals" volunteers to evaluate the change of T-lymphocytes populations in TBLN confirmed patients. Different investigators reported different outcomes regarding the proportion of different lymphocytes populations. Our finding showed, there were a change of T lymphocytes populations in TBLN cases when we compared with the healthy controls. According to the flowcytometry analysis there were a decreased mean percentage of CD3+ T cells and CD3+CD4+ T cells, when we compared with controls in which(**72.0% Vs. 73.3%)** and (**40.3% Vs. 43.1%)** respectively. In contrast the mean percentage of CD8+T lymphocytes were slightly increase in TBLN confirmed case in compared with controls (**29.2% Vs 27.2 %.**).Many investigators also reported in similar way with ours [149,150].Moreover, the difference of mean percentage of helper T cells between cases and controls was slightly statistically significant (**p=0.05**), but the difference of the mean percentage of total T lymphocytes and the cytotoxic T cells between the two groups were not significant (p>0.05).

This finding was supported by one study done in USA between active TB and negative cases in which patients with active TB had lowest numbers of CD4+ and their naïve, effector and late differentiation memory subset, and this was in contrast with TB negative subjects who had the highest CD4+ counts. These observations may reflect a higher cell differentiation disease caused by *M.tuberculosis* [151]. Alternatively, T-lymphocyte depletion may be explained by impaired thymus function or increased apoptosis levels [149,152]. In contrast, remarkably a higher number and mean percentage of T cells was observed in healthy control group, suggesting that the infection by *M. tuberculosis* by itself may exert a significant impact in the immune

response, resulting in changes in peripheral T-lymphocyte numbers, possibly reflecting cell homing and differentiation [151,153,154].

In addition, this group of investigator also supported our idea in assessing of CD8+ T cells. CD8+ T-lymphocyte activation was evaluated by the surface expression of CD38 and HLADR. In comparison with the negative group, the mean percentage of this cytotoxic T cells were higher, in the group of patients with active pulmonary disease, suggesting an overall increase in cellular activation in response to ongoing *M. tuberculosis* disease [151,153],our finding also in line with this particular study in which they reported the increment of cytotoxic T cells in TBLN case than healthy volunteers. This transitory state of activation of CD8+ T lymphocytes during active infectious diseases, including tuberculosis, possibly mirrors of the cytotoxic activity against intracellular pathogens [151,155].

CONCLUSION AND RECOMMENDATIONS

CONCLUSION

Although conventional diagnostic technique remains the methods of choice in regions with low-resource setting, PCR may be employed in case with strong clinical suspicion and equivocal result, especially at early stage of disease for better diagnosis, management and treatment. From this our finding we conclude that the current prevalence of TBLN with the gold standard technique tool was 36%. The numbers of patient for repeated cases were high in numbers, from which some isolates were identified which requires the drug susceptibility test. Genetically MTC are highly related species causative agent for tuberculosis, particularly *M tuberculosis* and *M.bovis*. In our experience majority of the causative agent of TBLN was *M.tuberculosis*, though the contribution of *M.bovis* is not excluded.

Molecular epidemiology is important in molecular typing of MTC and in assessing the global dissemination of strain and in understanding of the transmission as well. This finding indicated that the shared international spoligotyping of SIT 149, SIT53 SIT26 and SIT37 were the most dominant and prevalent strains identified which comprised 36.4% total clustered strains of MTB isolates belonged clusters suggesting that the extent of recent transmission and majority (66.2%) of them were modern TB lineages. In fact the true orphan (21.6%) strain identified also considered to insight and suggestive geographical location in Ethiopia.

Based on flowcytometric study there were a changes in T lymphocytes population distribution in patients suffering from TBLN compared to healthy controls. This study highlights the importance of cell-mediated immunity, particularly T lymphocytes populations in the patients suffering from TBLN. Although decrease in mean percentage helper T cells, main deference against TB and total T lymphocytes cells which significant impact in the immune response TBLN cases, there is increase in mean percentage of cytotoxic T cells due it is a mirrors of cytotoxic activity against intracellular pathogen, particularly in TBLN.

RECOMMENDATIONS

Based on our findings we forwarded the following recommendations.

1. Because of a true diagnostic challenge nature of TBLN, introduction of easy, cost effective, rapid, more efficient and time saving methods should be applicable.
2. The prevalence of TBLN still high in our study area, so early diagnosis and treatment should be implemented.
3. Though *M.tuberculosis* is a major causative agent of TBLN, the contribution of *M.bovis* must be considered. Therefore, community based health education should be launched in zoonotic transmission of TB and community based research should be conducted to get true prevalence of Zoonotic aspect of bovine TB.
4. The number of isolates from the repeated cases of TBLN was high; therefore further study on drug susceptibility test to screen the MDR-TB of TBLN should be employed.
5. To understand the insight of epidemiology and genetic diversity of *M.tuberculosis* spoligotyping is not the final option. Therefore for better understanding and confirmation of genetic diversity of *MTB* the most discriminative technique should be employed.
6. This study was conducted in the capital city only in which very few hospital and higher clinic such not representative to the national wide, thus we strongly recommend that further study on genetic diversity of *M.tuberculosis*, representative with better and more discriminative power of molecular technique should be implemented.
7. The genetic diversity on *M.tuberculosis* transmission, the different strain which might elicit different immune response still under questions. Therefore it is better if future researcher need to have further study on the strains and immune response of TB in humans and their immunogenicity and virulence.

6. REFERENCES

1. TB prevalence surveys: a handbook. Geneva, World Health Organization, 2013 (WHO/HTM/TB/2013 report).
2. WHO report 2006.WHO/HTM/TB, 362.Geneva; World Health Organization, 2006.
3. Shinnick TM. and Good RC. Mycobacterial taxonomy. *Eur J Clin Microbiol Infect Dis* 1994; 13:884-901.
4. Brosch R., Gordon SV., Marmiesse M., Brodin P., Buchrieser C., Eiglmeier K., Garnier T., Gutierrez C., Hewinson G., Kremer K, Parsons L M, Pym AS, Samper S., van Soolingen D.,and Cole ST. A new evolutionary scenario for the *M. tuberculosis* complex. *Proc.Natl.Acad.Sci.U.S.A* 2002;99:3684-3689
5. Smith N H., Kremer K., Inwald J., Dale J., Driscoll J. R., Gordon S. V., van Soolingen D., Glyn H. R., and Maynard S. J. Ecotypes of the *M. tuberculosis* complex. *J.Theor.Bio*2005.
6. Van Soolingen D., Hoogenboezem T., de Haas PE. Hermans PW., Koedam MA., Teppema KS., Brennan PJ., Besra GS., Portaels F., Top J., Schouls LM., and van Embden JD.A novel pathogenic taxon of the *M. tuberculosis* complex, Canetti: characterization of an exceptional isolate from Africa. *Int J Syst Bacteriol* 1997; 47:1236-45.
7. Spuy GD., Kremer K., Ndabambi SL., Beyers N., Dunbar R., Marais BJ., van Helden PD., Warren RM. Changing *M. tuberculosis* population highlights clade-specific pathogenic characteristics. *Tuberculosis* 2009; 89: 120–125
8. Run SPOTCLUST: http://tbinsight.cs.rpi.edu/run_spotclust.html (accessed Nov, 2013).
9. Gutierrez C., Brisse S., Brosch R., Fabre M., Omais B.,Marmiesse M.,Supply P. & Vincent V. *PLoS Pathogens* 2005;1: 1-7
10. McDonough K.A. and Kress Y. Cytotoxicity for lung epithelial cells is a virulence-associated phenotype of *M. tuberculosis*. *Infect. Immun* 1995; 63:4802–11
11. Grange JM. Mycobacterium bovis infection in human beings. *Tuberculosis* 2001; 81:71-77.
12. Dannenberg A.M. Roles of cytotoxic delayed-type hypersensitivity and macrophage-activating cell-mediated immunity in the pathogenesis of tuberculosis. *Immunobiology* 1994; 191:461–473.
13. Israel H., Hetherington H., and Ord J. A study of tuberculosis among students of nursing. *J.A.M.A* 1941; 117:461–473.

14. Kumar A. Lymph node tuberculosis. New Delhi. *Jaypee Brothers Medical publisher* 2001; 34:273– 84
15. Jayasankar K., Ramanathan VD. Biochemical and histochemical changes relating to fibrosis following infection with *M. tuberculosis* in the guinea pig. *Indian J Med Res* 1999;110:91-7
16. Chen M., Gan H., and Remold HG. "A mechanism of virulence: virulent *M. tuberculosis* strain H37Rv, but not attenuated H37Ra, causes significant mitochondrial inner membrane disruption in macrophages leading to necrosis." *J Immunol* 2006; 176(6):3707–3716.
17. Bodnar KA., Serbina NV. and Flynn JL. "Fate of *Mycobacterium tuberculosis* within murine dendritic cells," *Inf and Immun*; 69(2): 800–809.
18. Gonzalez-Juarrero M, Turner OC., Turner J., Marietta P., Brooks J.V., and. Orme I.M., "Temporal and spatial arrangement of lymphocytes within lung granulomas induced by aerosol infection with *M. tuberculosis*," *Inf Immun*2001; 69(3): 1722–1728.
19. Wolf A.J., Desvignes L., Linas B. et al., "Initiation of the adaptive immune response to *Mycobacterium tuberculosis* depends on antigen production in the local lymph node, not the lungs," *J Exp. Medicine* 2008;205(1):105–115.
20. Korf J., Stoltz A., Verschoor J., DeBaetselier P., Grooten J. The *M. tuberculosis* cell wall component mycolic acid elicits pathogen associated host innate immune responses. *Eur J Immunol* 2005; 35:890–900.
21. Kaufmann S.H., "Fact and fiction in tuberculosis vaccine research: 10 years later," *The Lan Inf Diseases* 2011; 11(8): 633–640.
22. Miranda MS., Breiman A., Allain S., Deknuydt F., and Altare F. The Tuberculous Granuloma: An Unsuccessful Host Defense Mechanism Providing a Safety Shelter for the Bacteria. *Clin Dev Immunol* 2012:1-14.
23. Schluger N W. and Rom W.The Host Immune Response to Tuberculosis. *Am J Respir Crit Care Med* 1998; 157: 679–691.
24. Mortaz E., Varahram M., Farnia P., Bahadori M., and Masjedi MR. New Aspects in Immunopathology of *M. tuberculosis*. *ISRN Immunology* 2012: 1- 11.
25. Ahmad S.,"Pathogenesis, immunology, and diagnosis of latent *Mycobacterium tuberculosis* infection," *Clin Dev Immunol* 2011; 1- 17.

26. Kaufmann S. H. E, "The macrophage in tuberculosis: sinner of saint? The T cell decides," *Pathobiology* 1991; 59(3):153–155.

27. Philips J. A. and Ernst J. D., "Tuberculosis pathogenesis and immunity," *Ann Rev of Path.* 2012; 7: 353–384.

28. Talreja J., Bhatnagar A., Jindal SK., Ganguly NK. Influence of *Mycobacterium tuberculosis* on differential activation of helper T-cells. *Clin and Exper Immunol* 2003; 131(2):292–298.

29. Boom WH., Canaday DH., Fulton SA., Gehring AJ., Rojas RE., Torres M. Human immunity to *M. tuberculosis*: T cell subsets and antigen processing. *Tuberculosis (Edinb)* 2003; 83:98- 106.

30. Rueda CM., Marín ND., García LF, Rojas M. Characterization of CD4 and CD8 T cells producing IFN-γ in human latent and active tuberculosis. *Tuberculosis (Edinb)* 2010; 90: 346-353.

31. Kaufmann SH. How can immunology contribute to the control of tuberculosis? *Nat Rev Immunol* 2001; 1(1): 20-30.

32. Bretsche PA., Ismail N., Menon JN., Power CA. Uzonna J.,Wei G. Vaccination against and treatment of tuberculosis, the Leishmaniasis and AIDS: Perspectives from basic immunology and immunity to chronic intracellular infections. *Cell Mol life sci* 2001; 58(12-13): 1879-96.

33. Kaufmann SHE., Parida SK. Tuberculosis in Africa: learning from pathogenesis for biomarker identification Cell Host. *Microbe* 2008; 4: 219–28.

34. Dorhoi A, Kaufmann SHE. Fine-tuning of T cell responses during infection. *Curr. Opin. Immunol* 2009; 21:367–77.

35. Trinchieri G. Regulatory role of T cells producing both interferon gamma and interleukin 10 in persistent infection. *J. Exp.Med* 2001;194: F53–7.

36. Scott-Browne JP., Shafiani S., Tucker-Heard G et al. Expansion and function of Foxp3 expressing T regulatory cells during tuberculosis. *J. Exp. Med* 2007; 204: 2159–69.

37. Kaufmann S H. Protection against tuberculosis: cytokines, T cells, and macrophages. *Ann Rheum Dis* 2002; (61):54–58.

38. Chan J. and Flynn J. "The immunological aspects of latency in tuberculosis," *Clin Immunol* 2004; 110(1): 2–12.

39. Beetz S., Wesch D., Marischen L., Welte S., Oberg H.-H, and Kabelitz D., “Innate immune functions of human γδ T cells,”*Immun* 2008; 213(3-4):173–182.
40. Tufariello J M., Chan J., and Flynn J L., “Latent tuberculosis: mechanisms of host and bacillus that contribute to persistent infection.” *The Lancet Infect Dis* 2003; 3(9):578–590.
41. Caruso A.M., Serbina N., Klein E., Triebold K., R.Bloom B., and Flynn J.L., “Mice deficient in CD4 T cells have only transiently diminished levels of IFN- γ , yet succumb to tuberculosis.” *J Immun* 1999; 162(9):5407–5416.
42. Cooper A.M., “Cell-mediated immune responses in tuberculosis,” *Ann Rev of Immunol* 2009; 27:393–422
43. Grotzke J.E. and Lewinsohn D.M, “Role of Ca8+ T lymphocytes in control of *Mycobacterium tuberculosis* infection, “*Microbe and Inf* 2005; 7(4): 776–788.
44. Jones, B. E., Young S.M., Antoniskis D., Davidson P.T., Kramer F., and Barnes PF. Relationship of the manifestations of tuberculosis to CD4 cell counts in patients with human immunodeficiency virus infection. *Am Rev Respir Dis* 1993; 148:1292-1297.
45. Guyot-Revol V., Innes J.A, Hackforth S., Hinks T., and Lalvani A. Regulatory T cells are expanded in blood and disease sites in patients with tuberculosis. *Am J Respir Crit Care Med* 2006; 173:803-810.
46. Klucar P., Barnes P. F., Kong Y., Samten B., Tvinnereim A., Spallek R., Nepom G.T, Singh M., and Shams H. Characterization of effector functions of human peptide-specific CD4+ T-cell clones for an intracellular pathogen. *Hum Immunol* 2008; 69:475-483.
47. Rodrigues D., Salomão R., and Kallas E G. Profound Peripheral T-Lymphocyte depletion and activation in disseminated Tuberculosis. *Bra J Infec Dis* 2006; 10(1):59-61.
48. Pessaran Z., Fosul FS. Oreizi F., Ghavaminejad A.,Kiani A., and Siadat ZD. Immunophenotypic Characterization of Peripheral Blood T-Lymphocytes and their Subpopulations in Tuberculosis Patients before and after Treatments. *Iranian J allergy, asthma Immunol* 2005; 4:23-26.
49. Antas P R.Z., Ding L., Hackman J., Hammock L R., Shintani A K., Schiffer J., Holland S M., Sterling TR. Decreased CD4+ lymphocytes and innate immune responses in adults with previous extrapulmonary tuberculosis 2005:1-32.
50. Jing X., Lin S., Xi-rong W., Qing M., Wei-wei J., Chen S., Dan S., Wei-xing F., Fang L., A-dong SH. Association of immunophenotypic characterization of peripheral lymphocytes

with different clinical phenotypes of tuberculosis in Chinese Han children. *Chinese Med J* 2012; 125 (2):1-10.

51. Deveci F., Akbulut HH., Celik I., Muz M H., and ˙Ilhan F. Lymphocyte Subpopulations in Pulmonary Tuberculosis Patients. *Mediators Inflamm* 2006:1–6
52. Wanchu A.,Bhatnagar A.,Talreja J.,Sapra S.,Suryanarayana BS.and Suresh P. Immunophenotypic and Intracellular Cytokine Profile of Indian Patients with Tuberculosis with and without Human Immunodeficiency Virus Co-infection. *The Indian J of Chest Dis Allied Sci* 2009: 208-211.
53. Wu Y., Zhang SW., Peng WG., Li KS., Li K.,Jiang JK., Lin JH. AND Cai YM. Changes in Lymphocyte Subsets in the Peripheral Blood of Patients with Active Pulmonary Tuberculosis. *The J of Inter Med Res* 2009; 37: 1742 – 1749.
54. Almeida A S., Fiske C T, Sterling T R., Kalams S A.Increased frequency of regulatory T cells and T lymphocyte activation in persons with previously treated extrapulmonary tuberculosis. *Clin Vaccine Immunol*. 2011: 45-52.
55. International statistical classification of diseases and related health problems, 10th revision (ICD-10), 2nd ed. Geneva, World Health Organization, 2007.
56. Small PM., Schecter GF., Goodman PC., Sande MA., Chaisson RE.,Hopewell PC. Treatment of tuberculosis in patients with advanced human immunodeficiency virus infection. *N Engl J Med* 1991; 324(5):289-294.
57. Clark RA, Blakley SL, Greer D, Smith MH, Brandon W, Wisniewski TL: Haematogenous dissemination of *Mycobacterium tuberculosis* in patients with AIDS. *Rev Infect Dis* 1991,13(6):1089-1092.
58. Seibert AF, Haynes J Jr., Middleton R, Bass JB Jr.: Tuberculous pleural effusion. Twenty year experience. *Chest* 1991, 99(4):883-886.
59. Maurya AK., Kant S., Nag VL.,Kushwaha RAS., Dhole TN. Detection of 123 bp fragment of insertion element IS6110 *Mycobacterium tuberculosis* for diagnosis of extrapulmonary tuberculosis 2012;30 (2) :182-186.
60. Aggarwal P., Wali JP., Singh S.,Handa R.,Wig N, Biswas A. A clinico-bacteriological study of peripheral TBLN. *J Assoc Physcians India* 2001; 49: 808-12.
61. Cowie RL. and Sharpe JW. Extra-pulmonary tuberculosis: a high frequency in the absence of HIV infection. *Int J Tuberc Lung Dis* 1997; 1:159-162.

62. Kale M.,Khatavkar P.,Satpute M.,Telang N., Wagh S. Chest, Microbiology,K.E.M.Hospital,Pune,Maharashtra,India, Mycobacterial characteristics of tuberculous lymphadenitis in a tertiary care hospital in India. *Thematic Poster Session, Hall*; 40: 1-5.
63. Peto HM., Pratt RH., Harrington TA., .LoBue PA., and Armstrong LR. Epidemiology of Extrapulmonary Tuberculosis in the United States, 1993–2006. *Clin Infect Dis* 2009; 49:1350–7.
64. Samaiala MA.and Oluwole OP.EPTB: Fine needle aspiration cytology diagnosis. *J Clinical Pract* 2011; 14(3):297-299.
65. Perenboom RM., Richter C., Swai AB., Kitinya J., Mtoni I., Chande H., Kazema RR., Mwakyusa DH., Maselle SY. Diagnosis of tuberculous lymphadenitis in an area of HIV infection and limited diagnostic facilities.*Trop Geogr Med.* 1994; 46(5): 288-92.
66. Richter C.,Kox LF., Van Leeuwen JV., Mtoni I., Kolk AH. PCR detection of mycobacteraemia in Tanzanian patients with extrapulmonary tuberculosis. *Eur J Clin Microbial Infect Dis* 1996; 15(10): 813-7.
67. Sharafeldin GS., Khalil EA., El Hag IA., Elsiddig KE., Elsafi ME., Aijafari AS., Elnour AA.,Hussein AM.,Elkhidir IM.,El-Hassan AM. Haematogenous dissemination of tuberculous lymphadenitis. *East Afr Med J.* 2007; 84(1):3-7.
68. Ministry of Health (2005/2006) National Tuberculosis and Leprosy Control (NTLC) Ethiopia, Ministry of Health Ethiopia.
69. Beyene D., Franken KL., Yamuah L., Aseffa A.,Wiker H G., Kolk A., Engers H., Klatser P. and Sviland L. Serodiagnosis of tuberculous lymphadenitis using a combination of antigens. *J Infect Dev Ctries* 2010; 4(2): 096-102.
70. Derese Y., Hailu E., Assefa T., Bekele Y., Mihret A., Aseffa A., Hussien J., Ali I., Abebe M. Comparison of PCR with standard culture of fine needle aspiration samples in the diagnosis of tuberculosis lymphadenitis. *J Infect Dev Ctries* 2012; 6(1):53-57.
71. Belay M., Bjune G.,Ameni G.,Abebe Diagnostic and treatment delay among Tuberculosis patients in Afar Region,Ethiopia:Across-sectional study. *BMC Public Health* 2012; 12(369): 1-15.

72. Deribew A, Negussu N, Melaku Z, Deribe K. Investigation Outcomes of Tuberculosis Suspects in the Health Centers of Addis Ababa, Ethiopia. *PLoS ONE | www.plosone.org.* 2011:1-5.
73. Velayati AA., Masjedi MR., Salehi M., Baghai N. Tuberculous Adenitis: Report of 31 patients with tuberculous adenitis. *Iranian J. Infec and Trop. Dis* 1998; 5:47- 51.
74. Sharma SK and Mohan A. Extrapulmonary tuberculosis. *Indian J. Med. Res* 2004; 120: 4316 -53.
75. Nataraj G., Kurup S., Pandit A., Mehta P. Correlation of fine needle aspiration cytology, smear and culture in tuberculous lymphadenitis: a prospective study. A prospective study. *J Postgrad Med* 2002; 48:113–116.
76. Bayazit YA., Bayazit N., Namiduru M. Mycobacterial cervical lymphadenitis. *J Oto Rhino-Laryngology* 2004; 66:275-280.
77. Bouakline SH., Vincensini JP., Giacuzzo V., Lagrange PH., Herrmann JL. Rapid diagnosis of extra pulmonary tuberculosis by PCR: Impact of sample collection and DNA extraction. *J. Clin. Microbiol* 2003; 41:2323 -29.
78. Yates and Grange JM. Bacteriologic survey of tuberculous lymphadenitis in southeast England, 1981- 1989. *J. Epidemiol Community Health* 1992; 46:332 - 35.
79. Ergete W. and Bekele A. Acid fast bacilli in fine needle aspiration smear from tuberculous patients. *Ethiop. J. Heal. Devep* 2000; 14: 99 - 104.
80. Daniel TM. Rapid diagnosis of tuberculosis: Laboratory techniques and application in developing countries. Review. *Infec Dis Supply* 1989; S471 – 8.
81. Bezabih M., Mariam DW., Selassie SG. Fine Needle aspiration cytology of suspected tuberculous lymphadenitis. *Cytopathology* 2002; 13:284-290.
82. Chao SS., Loh KS., Tan KK., Chong SM. Tuberculous and non-tuberculous cervical lymphadenitis: a clinical review. *Otolaryngol Head Neck Surg* 2002; 126:176-179.
83. Manolidis S., Frenkiel S., Yoskovitch A., Black M. Mycobacterial infections of the head and neck. *Otolaryngol Head Neck Surg* 1993; 109:427-433.
84. Artenstein AW.,Kim JH., Williams WJ., Chung RC., Mirza S., Restrepo BI., Mccormick JB., Fisher-Hoch SP. Diagnosis of tuberculosis lymphadenitis using a Polymerase Chain Reaction of peripheral blood mononuclear cells. *Am J Trop Med Hyg* 2003; 69:461-465

85. Bekedam HJ.,Boeree M.,Kamenya A.,Liomba G., Ngwira B.,Subramanyam VR., Harries AD. Tuberculosis lymphadenitis, a diagnostic problem in areas of high prevalence of HIV and tuberculosis. *Trans R Soc Trop Med Hyg* 1997; 91:294-297.
86. Hung J., Shen M., Sun Y. Epidemiological analysis of extra pulmonary tuberculosis in Shanghai. *Artic in Chinese* 2000; 23: 606 – 8.
87. American Thoracic Society (ATS). Diagnostic standards and classification of tuberculosis in adults and children. *Am J Respir Crit Care Med* 2000; 161:pp.1376-95.
88. Singh KK., Muralidhar M., Kumar A., Chattopadhyaya TK., Kapila K., Singh MK., Aharma SK., Jain NK., Tyagi JS. Comparison of in house polymerase chain reaction with conventional techniques for the detection of Mycobacterium tuberculosis DNA in granulomatous lymphadenopathy. *J Clin Pathol* 2000; 53:355-61.
89. Gonzalez OY.,Adam G., Teeter LD., Musser JM.,Graviss EA. Extra pulmonary manifestations in a large metropolitan area with a low incidence of tuberculosis. *Int. J.Tuberc. Lung. Dis.* 2003; 7: 1178 – 85.
90. Espinoza LE. , Monteros DL. , Galan JC., Gutierrez M., Samper F., Marine JG., Martin C., Dominguez L. , Rafel L. , Baquero F. Allele specific PCR method based on pncA and oxyR sequences for distinguishing M. bovis from M. tuberculosis: Intraspecific M. bovis pncA sequence polymorphism. *J. Clin. Microbiol*. 1998; 36: 239 - 42.
91. Li JYW., Lo STH., Ng CS. Molecular detection of M. tuberculosis in tissues showing granulomatous inflammation without demonstrable acid-fast bacilli. *Diagn Mol Pathol* 2000; 9:67-74.
92. Eisenach KD.,Cave MD., Bates JH.,Crawford JT.Polymerase Chain Reaction amplification of a repetitive DNA sequence specific for M. tuberculosis. *J Infect Dis* 1990; 161:977-981.
93. Kamerbeek J., Schouls L., Kolk A., Van Agterveld M., Van Soolingen D., Kuijper Simultaneous detection and strain differentiation of Mycobacterium tuberculosis for diagnosis and epidemiology. *J.Clin. Microbiol* 1997; 35: 907–914.
94. Parsons LM., Brosch R., Cole ST., Somoskovi A., Loder A., Bretzel G., van Soolingen D., Hale YM., Salfinger M. Rapid and simple approach for identification of M. tuberculosis complex isolates by PCR-based genomic deletion analysis. *J Clin Microbiol* 2002; 40:.2339-2345.

95. Ryan K.J.C., George Ray. Sherris Medical Microbiology; An introduction to infectious diseases 2004. MCGRAW-HILL, Medical Publishing Division.
96. American Thoracic Society, Centers for Disease Control, Diagnostic standards and classification of tuberculosis in adults and children. *Am J Respir Crit Care Med* 2000; 161 (4): 1376-1395
97. Mohammad A., Hossein S. Current trends in molecular epidemiology studies of Mycobacterium tuberculosis. *Biotech and Molec Biology Rev* 2007; 2 (5): 108-115.
98. Eyob G, Gebeyehu M, Gashu S, Girma M, Lemma E. Increase in tuberculosis incidence among the staff working at the tuberculosis demonstration and training center in Addis Ababa, Ethiopia. A retrospective cohort study (1989–1998). *Int J of Tuberc Lung Dis* 2002; 6: 85-88.
99. Triccas JA., Winter N,. Feng CG., and West NP. Immunity to M. tuberculosis. *Clin and Dev Immunol* 2011:1-2.
100. Tuberculosis 2007: From basic science to patient care. Available from *www.TuberculosisTextbook.com*
101. Wilton S., Cousins D. Detection and identification of multiple mycobacterial pathogens by DNA amplification in a single tube PCR. *Meth App* 1992; 1: 269–273.
102. Kidane D., Olobo JO., Habte A., Negesse Y., Aseffa A., Abate G., Yassin MA., Bereda K., Harboe M. Identification of the causative organism of tuberculous lymphadenitis in. Ethiopia by PCR. *J Clin Microbiol* 2002;11(40):4230-4234
103. Iwnetu R., van den Hombergh J, Woldeamanuel Y, Asfaw M, Gebrekirstos C, Nugusie Y, Bekele T, Ashenafi S, Seyoum B, Melaku K, Asefa A. Is tuberculous lymphadenitis over-diagnosed in Ethiopia? Comparative performance of diagnostic tests for mycobacterial lymphadenitis in a high burden country. *Scand J Infect Dis* 2009; 41:462–468.
104. Biadglegne F., Anagaw B., Anagaw B. Tesfaye W., Tessema B, Ulrich S., Arne C.R. Tuberculosis Lymphadenitis in Northwest Ethiopia: Implications for Public Health. Open *Med Micro* 2013; 3: 18-24
105. Abebe G., Deribew A., Ludwig A., Abdissa A., Deribie F., Woldemichael K., Shiffa J., Tesfaye M., Jira Ch. Bezabih M., Aseffa A., Bekele A., Robert C. Tuberculosis

lymphadenitis in Southwest Ethiopia: a community based cross-sectional study. *BMC Public Health* 2012; 12:504

106. Jha BC., Dass A., Nagarkar NM., Gupta R., Singhal S. Cervical tuberculous lymphadenopathy: changing clinical pattern and concepts in management. *Postgrad Med J* 2001; 77:185-187.
107. Seyoum B., Wolde Y, Aseffa A, Howard E, Lawrence KY. Characterization of mycobacterial isolates from lymph nodes of patients with tuberculous lymph adenitis in Dera Woreda, north showa, Ethiopia
108. Pahwa R., Hedau S., Jain, Neeraj Sh., Vivek J. Arora M., Kumar N. and Bhudev C. Assessment of possible tuberculous lymphadenopathy by PCR compared to nonmolecular methods. *Med Micro* 2005; 54: 873–878.
109. Fontanilla J-M., Barnes A., Reyn C.F.).Current Diagnosis and Management of peripheral Tuberculous Lymphadenitis 2011; *CID*.53.
110. Preeti M., Uma H., Harsh M., M.A.M.S., F.I.C.Path., F.U.I.C.C. Varsha G. Comparative Evaluation of Fine Needle Aspiration Cytology,Culture, and PCR in Diagnosis of Tuberculous Lymphadenitis. *Diagn. Cytopathol* 2011; 39:822–826.
111. Dandapat MC, Mishra BM, Dash SP, Kar PK: Peripheral lymph node tuberculosis: a review of 80 cases. *Br J Surg* 1990, 77:911–912
112. Kurabachew M., Enger O., Sandaa RA., Skuce R., Bjorvatn B. A. Multiplex PCR assay for genus, group and species specific detection of Mycobacteria. *Diagnostic Microbiol Infec Dis* 2004; 49: 99 – 104.
113. Swati P.,Hiten P.,Sweta P. ,Ritika P).Comparison of conventional diagnostic modalities(fine needle aspiration cytology & Ziehl-Nelson Staining) with culture(Bactect) for the diagnosis of tuberculous lymphadenitis: A prospective study. *Int J Res Med* 2013; 2:91-96.
114. Yogesh M.,Govind L.N.,Kalpesh M.,Rakesh R.,Rosy P.,Aksher GEfficacy of fine needle aspiration cytology, Ziehl-Nelson and Culture (Bactet) in diagnosis of tuberculosis lymphadenitis. *Nat J Med Res* 2012; 2:2249-4995.
115. Abedi AJ.,Khan Z.,Khalique N.,Fatima U.,Afroz N.(Utility of acid fast staining in tubercular lymphadenopathy compared to cytopathology. *Iranian J Publ Health* 2008; 37:98-100.

116. Suleiman A., Afshan S., Tazeen, et al., "A Comparison of Fine Needle Aspiration Cytology with Ziehl Nelson Staining Technique in Diagnosis of Tuberculosis Lymphadenitis," *A Comprehensive Cytopathology*2010;707-709.
117. Ramos J. M., Reyes F.,Tesfamariam A. "Childhood and Adult Tuberculosis in Rural Hospital in Southeast Ethiopia: A Ten Year Retrospective Study," *BMC Public Health* 2010; 10: 215.
118. Narcisse B., Jean CG., Zandanga G., Yango F.,Alexandre M., Leen R., Catherine PA., Brigitte G. and Gustave B. Fine-needle aspiration for diagnosis of tuberculous lymphadenitis in children in Bangui,Central African Republic Minime-Lingoupou Fanny *BMC Pediatrics* 2012, 12:19.
119. Ali.K.Ageep.Diagnosis of Tubeculous Lymphadenitis in Red Sea State, Sudan. *Inter J Trop Med* 2012, 7(1):53-56
120. Lau S.K,Wei W.I,Hsu C.,Engzell U.C.G.Efficacy of fine needle aspiration cytology in the diagnosis of tuberculous lymphadenopathy. *J of Laryng Oto*1990;104:24-27.
121. Garedew L., Mihret A., Abebe T., Ameni G. Molecular typing of mycobacteria isolated from extrapulmonary tuberculosis patients at Debre Birhan Referral Hospital, centralEthiopia,2013:*http://informahealthcare.com.*
122. Kanlikama M., Mumbuc S., Bayazit Y., sirikci A. Management strategy of mycobacterial cervical lymphadenitis. *J Laryngol Otol* 2000; 114:274-278.
123. Kanlikama M., Ozsahinoglu C., Akan E., Ozcan K. Mycobacterial species causing cervico-facial infection in turkey. *Eur Arch Otorhinolaryngology* 1993; 250:237-239.
124. Gupta sK., Chugh tD., sheikh ZA., al-Rubah NA. Cytodiagnosis of tuberculous lymphadenitis. A correlative study with microbiologic examination. *Acta Cytol* 1993; 37:329-332.
125. Sterling TR. The WHO/IUATLD diagnostic algorithm for tuberculosis and empiric fluoroquinolones use: Potential pitfalls. *Int J Tuberc Lung Dis* 2004; 8:1396-140.
126. Pamra S. P. & Mathur G. P. A cooperative study of tuberculous cervical lymphadenitis. *Indian J Med Res* 1974; 62, 1631–1646.
127. Manitchotpisit B., Kunachak s., Kulapraditharom B., Sura t. Combined use of fine needle aspiration cytology and polymerase chain reaction in the diagnosis of cervical tuberculous lymphadenitis. *J Med Assoc Thai* 1999; 82:363-368.

128. Baek, C. H., Kim, S. I., Ko, Y. H. & Chu, K. Polymerase chain reaction detection of Mycobacterium tuberculosis from fine-needle aspirate for the diagnosis of cervical tuberculous lymphadenitis. *Laryngoscope* 2000; 110: 30–34.
129. Goel M. M., Ranjan V., Dhole T. N., Srivastava A. N., Mehrotra A.,Kushwaha M. R. & Jain A. Polymerase chain reaction vs.conventional diagnosis in fine needle aspirates of tuberculous lymph nodes. *Acta Cytol* 2001; 45: 333–340
130. Kim S. S., Chung S. M., Kim J. N., Lee M. A. & Ha, E. H. Application of PCR from the fine needle aspirates for the diagnosis of cervical tuberculous lymphadenitis. *J Korean Med Sci* 1996; 11: 127–132.
131. Ersoz, C., Polat, A., Serin, M. S., Soylu, L. & Demircan, O. Fineneedle aspiration (FNA) cytology in tuberculous lymphadenitis. *Cyto-pathology* 1998; 9: 201–207
132. Szewzyk, R., et al, Molecular epidemiological studies of Mycobacterium bovis infectious in human and animals in Sweden. *J Clin. Microbiol* 1995; 33(12):3183-5.
133. Huard R.C.L.C, deOliveira Lazzarini W.R.,Butler D.,van Soolingen,J.L.Ho,.PCR based method to differentiate the subspecies of the Mycobacterium tuberculosis complex on the basis of genomic deletions. *J Clin Microbiol* 2003; 41:1637-50.
134. Ameni G., Aseffa A., Engers H., Young D., Gordon S., Hewinson G., Vordermeier M. High Prevalence and Increased Severity of Pathology of Bovine Tuberculosis in Holsteins Compared to Zebu Breeds Under Field Cattle Husbandry in Central Ethiopia. *Clin Vacc Immun* 2010; 14 (10): 1356–1361.
135. Cleavelanda S., Darren J., Shawa B., Sayoki G., Finangac M., Shirimad G., Rudovick R., Kazwalae Eblatee E., Sharpf M. *M. bovis* in rural Tanzania: Risk factors for infection in human and cattle populations. *Tuberculosis* 2007; 87: 30–43.
136. Mihret A, Bekele Y, Andre G. Loxton, Annemie M. Jordan, Lawrence Y, Aseffa A, 1 Rawleigh H, Gerhard W. Diversity of *M. tuberculosis* Isolates from New Pulmonary Tuberculosis Cases in Addis Ababa, Ethiopia. *Tuberc Res and Treat* 2012:1-7.
137. Gagneux, S. and Small, P.M. Global phytogeography of *M. tuberculosis* and implications for tuberculosis product development. *Lancet Infect Dis* 2007;7:328-337
138. Filliol, I., Driscoll, J. R., van, S. D., Kreiswirth, B. N., Kremer, K., Valetudie, G., Anh,D. D., Barlow, R., Banerjee, D., Bifani, P. J., Brudey, K., Cataldi, A., Cooksey, R. C.,Cousins, D. V.,Dale, J. W., Dellagostin, O. A., Drobniewski, F., Engelmann,

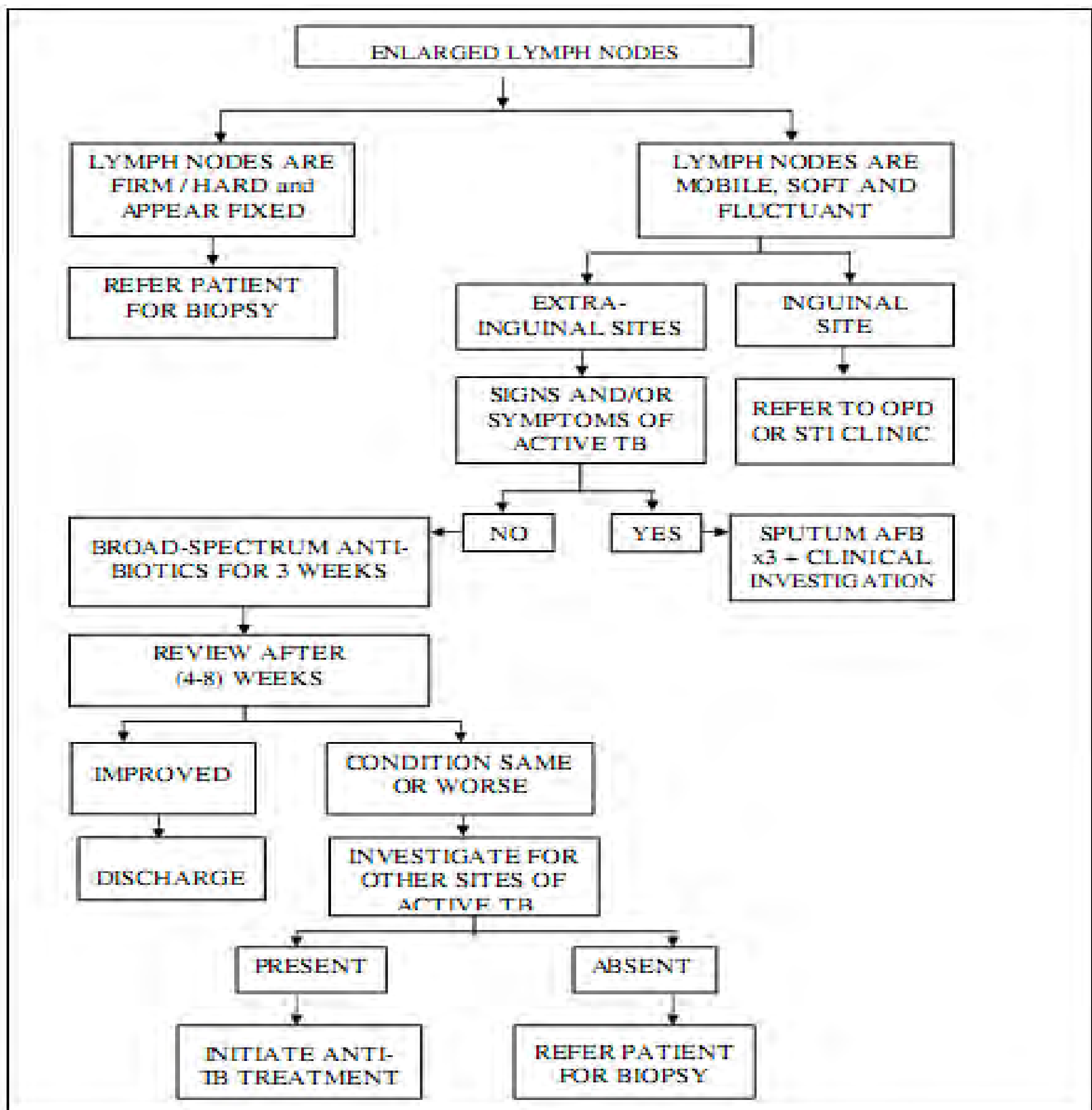
G.,Ferdinand, S., Gascoyne-Binzi, D., Gordon, M., Gutierrez, M. C., Haas, W. H.,Heersma, H., Kallenius, G., Kassa-Kelembho, E., Koivula, T., Ly, H. M.,Makristathis, A., Mammina, C., Martin, G., Mostrom, P., Mokrousov, I., Narbonne,V., Narvskaya, O., Nastasi, A., Niobe-Eyangoh, S. N., Pape, J. W., Rasolofo-Razanamparany, V., Ridell, M., Rossetti, M. L., Stauffer, F., Suffys, P. N., Takiff, H.,Texier-Maugein, J., Vincent, V., De Waard, J. H., Sola, C., and Rastogi, N. Global distribution of Mycobacterium tuberculosis spoligotypes. *Emerg.Infec Dis* 2002; 8:1347-1349.

139. Brudey K., J. R. Driscoll, L. Rigouts et al.,"*M. tuberculosis* complex genetic diversity: mining the fourth inter-national spoligotyping database (SpolDB4) for classification, population genetics and epidemiology."*BMC Microbiology* 2006; 23.
140. Ameni G., Tadesse K., Hailu E., Deresse Y., Medhin G., Aseffa A., Glyn H., Martin V., Stefan B. Transmission of Mycobacterium tuberculosis between Farmers and Cattle in Central Ethiopia,2013. *PLOS ONE | www.plosone.org:1-10*
141. Diriba B., Berkessa T., Mamo G.,Tedla Y., Ameni G. Spoligotyping of multidrug-resistant Mycobacterium tuberculosis isolates in Ethiopia. *Int J Tuberc Lung dis* 2013; 17(2):246–250.
142. Bruchfeld J, Aderaye G, Palme I B, et al. Molecular epidemiology and drug resistance of Mycobacterium tuberculosis isolates from Ethiopian pulmonary tuberculosis patients with and without human immunodeficiency virus infection. *J Clin Microbiol* 2002; 1636–1643.
143. Agonafir M, Lemma E, Wolde-Meskel D,Goshu S, Santhanam A, Girmachew F, Phenotypic and genotypic analysis of multidrug-resistant tuberculosis in Ethiopia. *Int J Tuberc Lung Dis* 2010; 14:1259 – 65.
144. Haiyan D., Zhiguang L.,Bing L,Yuanyuan Z., Jie L, Xiuqin Z.,,Jinghua L.,, Kanglin W. Spoligotypes of Mycobacterium tuberculosis from Different Provinces of China.*Clin Micro* 2010; 4102–4106.
145. Sreevatsan,S., Pan X., Stockbauer,K. E., Connell,N. D., Kreiswirth B. N., Whittam T. S. & Musser J. M.Proc. *Natl. Acad. Sci. U S A* 1997; 94: 9869-74.
146. Zozio,C. Allix, S. Gunal et al., "Genotyping of Mycobac-terium tuberculosis clinical isolates in two cities of Turkey:description of a new family of genotypes that is phylogeo-graphically specific for Asia Minor,"*BMC Microbiology* 2005.

147. Baker L., Brown Silent nucleotide polymorphisms and phylogeny for *M. tuberculosis*. *Emerg Infect Dis* 2004; 10(9): 1568-1577.
148. World Health Organization. The world Health Report: *Making a difference* 1999; 25.
149. al-aska a. I., al-anazi a. R., al-subaei s. s., al-Hedaithy M. a., barry M. a., somily a. M., buba F., Yusuf u., al anazi n. a. CD4+ T-lymphopenia in HIV negative tuberculous patients at King Khalid university hospital in Riyadh, Saudi Arabia. *Eur J Med Res* 2011,16: 285-288
150. Kony sJ, Hane aa, Larouze b et al. Tuberculosis-associated severe CD4+ T-lymphocytopenia in HIV – seronegative patient from Dakar. *J Infect* 2000; 41:167-71.
151. Rodrigues DSS., Medeiros E.A.S., Weckx L.Y., Bonnez W., Salomão R. Kallas E. Immunophenotypic characterization of peripheral T lymphocytes in Mycobacterium tuberculosis infection and disease, NY, USA. *Clin Exp Immunol* 2002;128:149–154
152. Das G., Vohra H., Saha B., Agrewala JN., Mishra GC. Apoptosis of Th1-like cells in experimental tuberculosis (TB). *Clin Exp Immunol* 1999; 115:324–8.
153. Ghasempour ShS ., Amiri MV. , Boloursaz MR., Dizaji MK., Mohammadi F., Mansoori D., Velayat AA. Lymphocyte Sub-populations and Lymphocyte Activation Markers in Pulmonary TB Patient. *Tanaffos* 2002; 1(4):37-44.
154. Jing X.,.Lin S., Xi-rong W., Qing M., Wei-wei J., Chen S., Dan S., Wei-xing F., Fang L., A-dong SH. Association of immunophenotypic characterization of peripheral lymphocytes with different clinical phenotypes of tuberculosis in Chinese Han children. *Chinese Med J* 2012; 125 (2):1-10
155. Silva CL, Silva MF, Pietro RC, Lowrie DB. Protection against tuberculosis by passive transfer with T-cell clones recognizing mycobacterial heat-shock protein 65. *Immunology* 1994; 83:341–6.

ANNEXES

Annex I. The national algorithm for management of lymph node enlargement



Addis Ababa University

College of Health Sciences

School of Medicine, Department of Microbiology, Immunology and Parasitology

Annex II. Case Record Form

1. General information

Name of the hospital/Clinic ________________ Code No.___________Card No. ____________

1. Age (in years)_______________________
2. Sex 1.Male 2.Female
3. Address 1.Urban Kef. Ketema _______ Keb. ______ House No.________ 2. Rural
4. Occupation: 1. Merchant 2. Student 3. Housewife 4. Gov't employee 5. Farmer 6. Others ____________
5. Educational Level: 1. Illiterate 2. Primary School 3. Secondary School 4. Diploma 5. Degree 6. Others _______________
6. Marital status: 1. Single 2. Married 3. Divorced 4. Widowed 5. Living with Partner

2. Clinical data

7. Fever: 1. Yes 2. No If yes, 1.High 2. Moderate 3.Low
8. Weight loss 1. Yes 2. No If yes, 1. Slight 2. Moderate 3.Significant
9. Night sweat 1.Yes 2. No
10. Poor appetite 1.Yes 2. No
11. Weakness 1.Yes 2. No
12. The duration of the neck swelling (in weeks) ___________________
13. Rate of increase of the neck swelling 1. Slow 2. Moderate 3.Fast
14. Presently felt pain at the swelling site 1. Present 2. Absent
15. Previous treatment for the swelling 1. Yes 2. No If yes, 1. Traditional (herbal) 2. Modern
16. Intake of antibiotic for the swelling 1. Yes 2. No If yes, what kind (if they know the name or describe the color, size, shape _____________________
17. History of anti-tuberculosis treatment previously 1. Yes 2. No If yes, had they 1. Finished the course 2. discontinued

18. History of contact with tuberculous patients 1. Yes 2. No If yes, Contact for how long
    1. Days 2. Weeks 3. Months
19. History of intake of raw milk/meat 1. Yes 2. No
20. History of BCG vaccination 1. Yes 2. No

3. Physical examination

21. Number of nodes 1. Single node 2. Few nodes (2-4) 3. Multiple nodes (>5)
22. Size (approximation) _________ cm
23. Mobility 1. Mobile 2. Non-mobile
24. Condition of the nodes 1. Discrete 2. Soft 3. Matted 4. Hard 5. Firm 6. Fluctuant
    7. Draining sinus 8.Other descriptions____________
25. Other pertinent findings ____________________________

7.2.የቃለ መጠይቅ መሙያ ቀፅ

1.ጠቅላላ መረጃ

የሆስፒታሉ ስም…………… መለያ ቁጥር………….የካርድ ቁጥር……………….

1. እድሜ (በአመት)…………….
2. ጾታ 1. ወ 2. ሴ
3. አድራሻ 1.ከተማ ክ/ከተማ………….. ቀበሌ……………….የቤት ቁጥር………… ገጠር ……………………..
4. ስራ 1. ነጋዴ 2. ተማሪ 3. የቤት እመቤ 4. የመንግስት ሰራተኛ 5. ገበሬ 6. ሌላ ይገለጽ……………
5. የትምህርት ደረጃ 1. ያልተማረ/ች 2. አንደኛ ደረጃ 3. ሁለትኛ ደረጃ 4. ዲፐሎማ 5. የመጀመሪያ ድግሪ
   6. ሌላ ይገለጽ…………
6. የጋብቻ ሁኔታ 1. ያላገባ 2. ያገባ 3. ፈት 4. የሞተበት 5. ከጓደኛ ጋር አብሮ የሚኖር

2.የጤና ሁኔታ መረጃ

7. ትኩሳት 1. አለ 2. የለም መልሱ አለ ከሆነ 1. ከፍተኛ 2. መካከለኛ 3. ዝቅተኛ
8. የክብደት በቀነስ 1. አለ 2. የለም መልሱ አለ ከሆነ 1. .መጠነኛ 2. መካከለኛ 3. ከፍተኛ
9. ለሊት ማላብ 1. አለ 2. የለም
10. የምግብ ፍላጎት መቀነስ 1. አለ 2. የለም

11. የድካም ስሜት 1. አለ 2. የለም

12. የአንገት እብጠቱ ምን ያህል ጊዜ ሆኖታል (በሳምንት)……………………………………

13. እብጠቱ የሚጨምርበት ፍጥነት 1. ዝቅተኛ 2. መካከለኛ 3. ከፍተኛ

14. በአሁኑ ወቅት እብጠቱ ላይ የሚሰማ ህመም አለ? 1. አለ 2. የለም

15. ለእብጠቱ ከዚህ በፊት ታክመዋል? 1. አዎ 2. የለም አዎ ከሆነ፡ 1. ባህላዊ 2. ዘመናዊ

16. የወሰዱት ፀረ-ባክቴሪያ አለ 1. አዎ 2. የለም አዎ ከሆነ መድሃኒቱነ ምንድነው?(ስሙን ካወቁ ወይም መልኩን፤ መጠኑን፤ ቅርፁን)………………………

17. ከዚህ በፊት የቲቢ ህከመና ወስደው ያውቃሉ?1. አዎ 2. የለም አዎ ከሆነ ህክምናውን ጨርሰዋል?1. አዎ 2. የለም

18. ከዚህ በፊት በቲቢ ህመም ከታመመ ሰው ጋር የቀረበ ግንኙነት ነበረዎት? 1. አዎ 2. የለም አዎ ከሆነ ለምን ያህል ጊዜ? 1. ለቀናት 2. ለሳምንታት 3. ለወራት

19. ጥሬ ስጋ/ወተት ተመግበው ያውቃሉ? 1. አዎ 2. የለም

20. ከዚህ ቀደም የፀረ ቲቢ ክትባት ወስደው ያወቃሉ? (ለህፃናት) 1. አዎ 2. የለም

3.የአካለ ምርመራ

21. የእብጠት ብዛት 1. አንድ 2. ጥቂት (2-4) 3. ብዙ (>5)

22. የእብጠት መጠን (በግምት)…………………..ሴ.ሜ

23. እብጠቱ ከቦታው ይንቀሳቀሳል? 1. አዎ 2. የለም

24. የእብጠቱ ሁኔታ 1. የተለያዩ 2. የተያያዙ 3. ጠንካራ 4. ለስላሳ

5. መግል ያለው 6. ሌላ ይገለፅ___________________________________

25. ሌላ በታማሚው ላይ የሚታዩ ምልክቶች ካሉ ይገለፅ______________________________

Annex III: Information sheet for study subjects **Date -----------**

You are kindly invited to participate in this study. The aim of this study is to identify the dominant species and strain of *Mycobacterium* from FNA and biopsy and to characterize different phenotype of peripheral T lymphocytes in Lymphadenitis Tuberculosis (LNTB) cases among individuals clinically suspected for tuberculous lymphadenitis at TASH and ALHC, Addis Ababa, Ethiopia and to compare the current algorithm with the routine one for the diagnosis of TBLN.

Purpose: the purpose of this research was to identify the dominant species and strain of *Mycobacterium* responsible for TBLN, to compare diagnostic performance of different laboratory method*s* in diagnosis of TBLN and to evaluate some phenotype of peripheral T lymphocytes in Lymphadenitis Tuberculosis (LNTB) cases among individuals clinically suspected for tuberculous lymphadenitis.

1. **Duration**: the duration of this study was about one year.
2. **Procedures to be carried on:** the procedure of sample collection is easy and straight forward. Small amount of fluid from the swelling by a needle and syringe was collected by pathologist. Under local anesthesia the same swelling was removed. Venous blood was collected by nurse.
3. **Risk and discomfort:** almost there was not risk associated during sample collection except little discomfort and pain feels to them while collecting the samples.
4. **Expected benefits:** They benefited from the study; because it was one part of their diagnosis and a key to their current and/or future problem if it was come up with positive result.
5. **Confidentiality:** All your personal information collected for the purpose of the present study was kept confidential.
6. **Compensation:** No compensation was provided by participating in this study.
7. **Termination of the study:** Participation in the study is voluntary, and refusal to participate involves no penalty or loss of benefits to which they were otherwise entitled. The study participants have a right to:

- Keep hold information
- Decline to cooperate in the study
- To refuse provision of specimens

I informed them that this study was approved by Department Ethical and Review Committee of the department of Microbiology, Immunology and Parasitology, School of Medicine, Addis Ababa University. For further information they used the following address.

1. **Olifan Zewdie(PI)** Address: Addis Ababa University, College of Health Science, department of Microbiology, Immunology and Parasitology, E-mail olifangdr@gmail.com
2. **Dr.Tamrat Abebe(advisor),** Address: Addis Ababa University, College of Health Science, department of Microbiology, Immunology and Parasitology.E-mail tabebezeleke@gmail.com
3. **Dr. Adane Mihret (advisor)**, Address: Addis Ababa University, College of Health Science, department of Microbiology, Immunology and Parasitology-mail: adane.mihret@aau.edu.et.
4. **Dr. Gobena Ameni (Co-advisor)** Address: Addis Ababa University, Aklilu Lemma Institute of Pathobiology, Department of Microbiology, Immunology & Parasitology E-mail: gobenachimdi2009@yahoo.co.uk.
5. **Dr. Tufa Gemechu** (Co-advisor) Address: Addis Ababa University, College of Health Sciences, School of Medicine, Department of Pathology. E-mail: tufa.gemechu@yahoo.com
6. **Dr.Bakure Tsageye (Co-advisor)** Address: Alem Tena Higher Clinic, Department of Pathology. E-mail:bekure_tsegaye@yahoo.com

7. **Addis Ababa University (College of Health Science),** address: Office of Associate Dean, Postgraduate Programs and Research. Tel. + 251-011-551-28-765, P.O. Box 9086, Addis Ababa, Ethiopia

7.3.የጥናቱ ተሳታፊዎች የመረጃ ቅጽ

1. **የጥናቱ ዓላማ፡-** የዚህ ጥናት ዓላማ በሊነፍ ቲቢ ህመም የተያዘ ሰው የሰውነት በሽታ የመከላከል አቅሙን ማወቅ ሆናል፡፡
2. **የሚፈጀው ጊዜ፡-** ይህ ጥናት እስከ አራት ወር ሊፈጅ ይችላል፡፡
3. **የናሙና አወሳሰድ፡** ፡-የዚህ ጥናት ተሳታፊዎች የደምና ከእብጠቱ ናሙና በመስጠ ከላይ የተጠቀሰውን የሰውነት የበሽታ የመከላከል አቅምን ማወቅ ይቻላል፡፡
4. **ሊደርስ የሚችል አደጋ፡-** በዚህ ጥናት ውስጥ ናሙና በሚወሰድበት ወቅት ከሚኖረው መጠነኛ ህመም በስተቀር አደጋ የሚያደርስ ድርጊት የለም፡፡
5. **የሚገኝበት ጥቅም፡-** የዚህ ጥናት ተሳታፊዎች የተሻለ የምርመራ ዘዴ በመጠቀም ለቲቢ ባክቴሪያ ምርመራ ይደረግላቸዋል፡፡

6. **<u>ሚስጥራዊነት</u>፡-** የማንኛውም የጥናቱ ተሳታፊ መረጃ በሚስጥር ይያዛል፡፡ የእያንዳንዱን ግለሰብ መረጃ ከዋናው ተመራማሪና አማካሪው በስተቀር ማንም ሊያገኝ አይችልም፡፡
7. **ክፍያ፡** ናሙናዉን እንድጠቀም ከፈቀዱልኝ ምንም ዓይነት ክፍያ የለዉም፡፡
8. **<u>ፈቃደኝነትን ስለማቋረጥ</u>፡-** የጥናቱ ተሳታፊዎች፤መረጃ ያለመስጠት፣በጥናቱ ለመሳተፍ ፈቃደኝነት ያለማሳየት እንዲሁም ናሙና ያለመስጠት መብታቸው የተጠበቀ ነው፡፡

አድራሻ ማወቅ ካስፈለግዎን የሚከተሉትን ይጠቀሙ፡-

1. **1.አሊፈን ዘውዴ፡** የማይክሮ ባዮሎጂ፤ኢምኖሎጂ እና ፓረሳይቶሎጂ ትምህርት ክፍል ፡ ህክምና ፋክሊቲ፤አዲስ አበባ ዩኒቨርሲቲ
2. **ታምራት አበበ፡** የማይክሮ ባዮሎጂ፤ኢምኖሎጂ እና ፓረሳይቶሎጂ ትምህርት ክፍል ፡ ህክምና ፋክሊቲ፤አዲስ አበባ ዩኒቨርሲቲ
3. **ዶ/ር አዳነ ምህረት፡** የማይክሮ ባዮሎጂ፤ኢምኖሎጂ እና ፓረሳይቶሎጂ ትምህርት ክፍል ፡ ህክምና ፋክሊቲ፤አዲስ አበባ ዩኒቨርሲቲ
4. **ህክምና ፋክሊቲ**፤ አዲስ አበባ ዩኒቨርሲቲ የድህር ምረቃ ፕሮግራምና ምርምር የተባባሪ ዲን ቢሮ

   የመ.ሳ.ቁ. 9086 አዲስ አበባ

   ስልክ.251-011-551-28-76

IV. Consent format

Name ________________________ Identification Code ________________________________

I have read the information sheet (or it has been read to me); I have understood that this study is about **"Molecular Epidemiology and Comparison of Diagnostic Methods of Tuberculous Lymphadenitis, Addis Ababa, Ethiopia"**. I have asked some questions and clarification has been given to me. I have given my consent freely to participate in the study, and I hereby to approve my agreement with my signature.

Participants signature _________________ Date____________________

Investigators signature _________________ Date____________________

Witness signature 1. _________________ Date______________

2 . ________________ Date________________

7.4.የፈቃደኝነት መጠየቂያ ቅጽ

ሙሉ ስም______________________መለያ ቁጥር _________________

የዚህ ጥናት ዋና ዓላማ "**Molecular Epidemiology and Comparison of Diagnostic Methods of Tuberculous Lymphadenitis, Addis Ababa, Ethiopia**." በዚህ ጥናት ውስጥ ምንም አይነት ጎጂ ድርጊት የለም፣እንዲሁም ማንኛውም መረጃ በሚስጥር ይያዛል፡፡ ስለዚህ በዚህ ጥናት በመሳተፍ አስፈላጊውን የደምና ከሊንፍ እብጠት የፈሳሽ ናሙና በመስጠት እንዲሁም ለአንድ አንድ ቃል መጠየቅ እንድትተባበሩኝ እጠይቃለሁ፡፡

እኔ_________________________ ከላይ የተጠቀሰውን አድምጬ ለእኔ ጠቃሚ መሆኑን ስለተረዳሁ በጥናቱ በመሳተፍ የሚፈለግብኝን ለማድረግ ተስማምቼለሁ፡፡

የጥናቱ ተሳታፊ ፊርማ ---------------------- ቀን---------------------

የተመራማሪው ፊርማ ------------------------- ቀን ---------------------

Declaration

I the undersigned, declare that this thesis is my original work, has not been presented for a degree in other university and that all source materials used for the thesis have been duly acknowledged.

Name of student

Olifan Zewdie Abil Signature ________________ Date_______________

Advisors' Name

1. Dr. Adane Mihret Signature __________________ Date______________
2. Dr. Tamrat Abebe Signature ___________________ Date______________

Examiners

1. ___________________________ Signature __________________ Date______________
2. ____________________________Signature __________________Date_______________