CHARACTERIZATION OF MYCOBACTERIAL ISOLATES FROM LYMPH NODES OF PATIENTS WITH TUBERCULOUS LYMPHADENITIS IN DERA WOREDA, NORTH SHOWA, ETHIOPIA

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List of Abbreviations

AFB	Acid fast bacilli
AHRI	Armauer Hansen Research Institute
AIDS	Acquired immuno deficiency syndrome
ALERT	All African Leprosy and Tuberculosis Rehabilitation Research and Training Center
ATCC	American Tissue Culture Collection
Bp	Base pair
CTAB	Cetyltrimethyl ammonium bromide
DNA	Deoxyribonucleic acid
DOTS	Directly observed treatment short – course
E.C	Ethiopian calendar
EPTB	Extra pulmonary tuberculosis
FNA	Fine needle aspirate
HIV	Human immunodeficiency virus
IS	Insertion sequence
LJ	Lowenstein - Jensen
MOH	Ministry of Health
PCR	Polymerase Chain Reaction
SDS	Sodium dodceyl sulfate
TB	Tuberculosis
TBLN	Tuberculous lymphadenitis
TLCP	Tuberculosis and Leprosy Control Programme
WHO	World Health Organization
ZN	Ziehl Neelsen

Abstract

Tuberculosis is a major health problem throughout the world causing a large number of deaths, more than that from any other single infectious disease. Extra pulmonary tuberculosis can occur in isolation or along with a pulmonary focus as in the case of patients with a disseminated form of tuberculosis. Tuberculous lymphadenitis (TBLN) is a common form of extra pulmonary tuberculosis. Lymph nodes (cervical, axillary and inguinal) are the most common sites of involvement. Diagnosis of TBLN is a formidable challenge in developing countries where there is high rate of human immunodeficiency virus infection. The aim of this study was to identify the etiological species of Mycobacteria responsible for TBLN in Derra, rural Ethiopia, where the status of TBLN was not known. A total of 153 patients who fulfilled the inclusion criteria were included in the present study during the study period between January 2004 and February 2005. Out of 153 study participants, fine needle aspirate specimens from 145 study participants were processed and analyzed using Ziehl Neelsen staining, culture, cytology and polymerase chain reaction. Out of 145 FNA samples, 66 (45.5%) of the isolates demonstrated growth on Lowenstein - Jensen medium. Among 145 clinically suspected TBLN patients, 115 (79.3%) were confirmed as TBLN cases by the combined results of AFB smear examination, culture, cytology and PCR. Two primer sets were used to identify the etiological agent at the complex (targeting the IS6110 insertion sequence) and species level (targeting the *pnc*A gene allelic variation). From a total of 145 clinically suspected TBLN cases, 108 (75%) were positive by PCR at the complex levels. Based on PCR for detection of allelic variation at position 169, 107/108 (99.1%) were positive for *M. tuberculosis* and only 1(0.9%) was

positive for *M. bovis.* The results indicated that *M. tuberculosis* is the causative species for tuberculous lymphadenitis in Dera.

CHAPTER I : Background and Justification

1.1 Tuberculosis

Tuberculosis (TB) is an infectious disease that is thought to have existed at least as long as mankind, but still remains a major global public health problem (Evans, 1998). TB is the most frequent cause of death from a single infectious disease in persons aged 15 - 49 years (Van Soolingen, 2001). It is the world's second commonest cause of death from infectious disease, after human immunodeficiency virus (HIV) / acquired immuno - deficiency syndrome (AIDS) (Frieden *et al.,* 2003)

1.2 Global epidemiology of tuberculosis

The recent report of World Health Organization (WHO) revealed that one third of the world population is thought to be infected with tubercle bacilli, eight to ten million people develop new active TB and two to three million deaths occur annually (WHO, 2005). Due to accelerating human immunodeficiency virus (HIV) pandemics and development of drug resistant strains, TB was declared as a global emergency in 1993 by WHO (WHO, 2004). Sub -Saharan Africa has the highest prevalence of TB. From sub - Saharan countries, Nigeria, Ethiopia, South Africa, Tanzania, Kenya, Democratic Republic of Congo, and Zimbabwe are among the 22 high TB burden countries worldwide (WHO, 2005). Twenty-two high TB burden countries in the developing world account for 6.9 million new TB cases, comprising over 80 % of the total global caseload. Of these, nine countries in sub - Saharan Africa account for 1.5 million of the cases with the incidence rates ranging between 305 and 525 per 100, 000 population (WHO, 2004).

A substantial rise in the number of TB cases reported from sub - Saharan Africa has been observed following an alarming increase in the HIV infection (Frieden *et al*., 2003). It is believed that the clinical pattern of TB has demonstrated a dramatic change due to co-

infection with HIV (Bekedam *et al.,* 1997). A significant number of extra pulmonary cases are reported among HIV infected patients.

However, the mechanisms with which HIV changes the clinical forms of TB are not well elucidated (Bem *et al*., 1997). It is also not clear as to what exactly causes the increase in extra pulmonary forms, whether it is related with changes in epidemiological trends or other factors (Bouakline *et al.,* 2003). Extra pulmonary tuberculosis (EPTB) is an important clinical problem where TB occurs at body sites other than the lungs (Razanamparany *et al.,* 2001). The commonest form of EPTB is tuberculous lymphadenitis (Singh *et al*., 2000). Historically, TBLN was referred to as "scrofula" meaning "glandular swelling'' (Latin) and "full necked sow'' (French) (Sharma and Mohan; 2004).

1.3 Epidemiology of tuberculous lymphadenitis (TBLN)

In developing countries where the incidence of tuberculosis is high, TBLN is a cause for 30 - 64% of lymphadenopathy, responsible for about 80% of all exclusively EPTB cases and more than 6% of all tuberculosis cases (Brainard, 2001). Approximately 75% of the infection is associated with cervical lymph nodes (Singh *et al*., 2000). A study conducted in Iran on 31 patients with TBLN depicted that cervical lymph nodes were the most frequently involved sites and comprised 50% of the cases (Velayati, *et al*., 1998). In another study done in Texas, USA, from a total of 1,878 TB patients, 538 (28.6%) were identified EPTB cases among African – Americans. Of these EPTB cases, the most common sites of infection were lymph nodes 43% (Gonzalez. *et al*., 2003).

Infection with human immuno-deficiency virus (HIV) has had a substantial impact on the global epidemiology of tuberculosis by causing a progressive and ultimately profound reduction in cell mediated immunity that increases the likelihood of developing active tuberculosis and lymph node involvement (Daley *et al*., 1995). Prior to the epidemic of HIV infection, approximately 85% of reported cases were limited to the lungs, with the remaining 15% involving extra pulmonary sites. However, due to the expansion of HIV infection, EPTB now accounts for more than 50 % of all cases of TB (Sharma and Mohan, 2004). Studies

conducted in developing and developed countries have substantiated the impact of HIV on the epidemiology of TB (Ilgazi *et al.*, 2004).

A study conducted in Dar as Salaam demonstrated that of 128 patients with peripheral lymphadenopathy, TBLN was proven in 89(69.5%) of the patients of whom 69(54%) were HIV sero- positive (Perenboom *et al.*, 1995). A retrospective study conducted in Taiwan on 118 adult AIDS patients who were hospitalized at National Taiwan University Hospital showed that 29/118 patients (24.6%) had TB. Among these TB patients, 22 (75.9%) had EPTB with the most common site being the lymph nodes (73%) (Hsieh *et al.*, 1996). Research findings reported that around 21% of extra pulmonary tuberculosis cases seen in the United States were associated with HIV infection (Brainard, 2001).

In China, tuberculosis kills more than 250,000 people each year. A study from Shanghai, China also showed that the proportion of EPTB among all TB was 9.7 - 11.8%. EPTB accounted for 14.1 - 17.6% of all deaths due to TB and among EPTB, TBLN ranked first with 38% (Huang, 2000). A study conducted in Lusaka, Zambia, on lymph node biopsy revealed an increased burden of TBLN in central Africa (Bem *et al.,* 1996). Another study done in Spain has also reported that tuberculous lymphadenitis was proven in 25/65 (39%) of the patients clinically suspected of tuberculous lymphadenitis (Singh *et a*l., 2000). A study from Germany indicated that TBLN is the most common form of extra pulmonary tuberculosis comprising about 7 to 8 % of all cases of tuberculosis (Rimek et *al*., 2002).

Even though the prevalence of TBLN is rising, only a few systematic studies have so far been conducted in sub –Saharan Africa (Gomez *et al.,* 2000). A few studies and hospital reports indicate that TBLN comprises a significant proportion of EPTB in Ethiopia, particularly in rural areas where there is limited access to health services (Kidane *et al*., 2002, Yassin *et al*., 2003). Our present study was initiated to investigate the situation of TBLN in Dera, rural Ethiopia. Dera was relatively isolated from urban centers until the construction of Jema bridge over the Jema River in 2002. We were interested to investigate whether the etiological agents and disease pattern of TBLN in the study locality differs from other rural parts of the country due to geographical variations, feeding habits, culture and other socio - economic factors.

1.4 Situation of tuberculosis in Ethiopia

In Ethiopia, tuberculosis is one of the most important public health problems affecting large segments of the population. Ethiopia is number seven out of 22 high TB burden countries that account together for 80% of all newly diagnosed TB cases in the world (WHO, 2004). The TB and Leprosy Control Programme of Ethiopia reported that new cases of all forms of TB were 105, 567 in 1994 and 109,346 in 1995 E. C (MOH, 1995 E.C). According to reports obtained from different health institutions found in the country, TB is the leading cause of morbidity, the most frequent cause of hospital admission, 9.4% of all cases admitted to hospitals and the leading cause of hospital deaths, accounting for 27% of all patients who die in hospitals (MOH, 1995 E.C). Table 1 depicts trends of TB case notifications in Ethiopia along with incidence rate, DOTS coverage and treatment success.

Table 1. Trend of TB case notifications in Ethiopia, 2002 - 2003. *(Global tuberculosis control, WHO report, 2005).*

Latest estimates		Trends	2002	2003
Population	70 678 002	DOTS geographical coverage (%)	95	95
Global rank by estimate number of cases	7th	Notification rate of all TB cases/ 100 000 population	160	166
Incidence (all cases / 100 000 Population / year)	356	Notification rate of new smear positive cases / 100 000 pop	53	56
New smear positive incidence cases / 100 000 pop/ year	155	Detection of all cases of TB (%)	47	47
Prevalence (all cases/ 100 000 pop/ year	533	Case detection rate (new smear positive, %)	36	36
TB mortality (all cases/ 100 000 pop/ year)	79	DOTS case detection rate (new smear positive, %)	38	38
TB / HIV co- infection (Adults aged 15 - 49, %)	21	DOTS treatment success from DOTS implemented areas (%)	76	No report

Nationally, 33% of all reported cases were extra pulmonary tuberculosis with the largest group being taken by TBLN. Reports from some areas of the country such as Tigray and Amhara regions depicted that EPTB is estimated to account for over 39% of all TB cases (MOH, 2002). The results of the evaluation made by the Annual Joint Review Mission of TB and Leprosy Control Programme of Ethiopia also noted that in a number of treatment units more than half of all new cases of registered TB cases were found to have extra pulmonary tuberculosis with the majority being diagnosed as TBLN that mostly involved cervical lymph nodes. The Review Mission finally recommended that studies should be undertaken to assess the magnitude of TB among patients coming to health institutions with enlarged lymph node (MOH, 2003).

According to previous findings about TB, one could expect that amongst 100 new TB cases, 15 of them would be expected to have EPTB, accounting for 30 - 40% of all TB cases. (Hopper, 1972, Stybol, 1991). The situation of Ethiopia seems in agreement with above reported epidemiological findings with over 39% reporting rates of EPTB from all forms of TB (MOH, 2002).

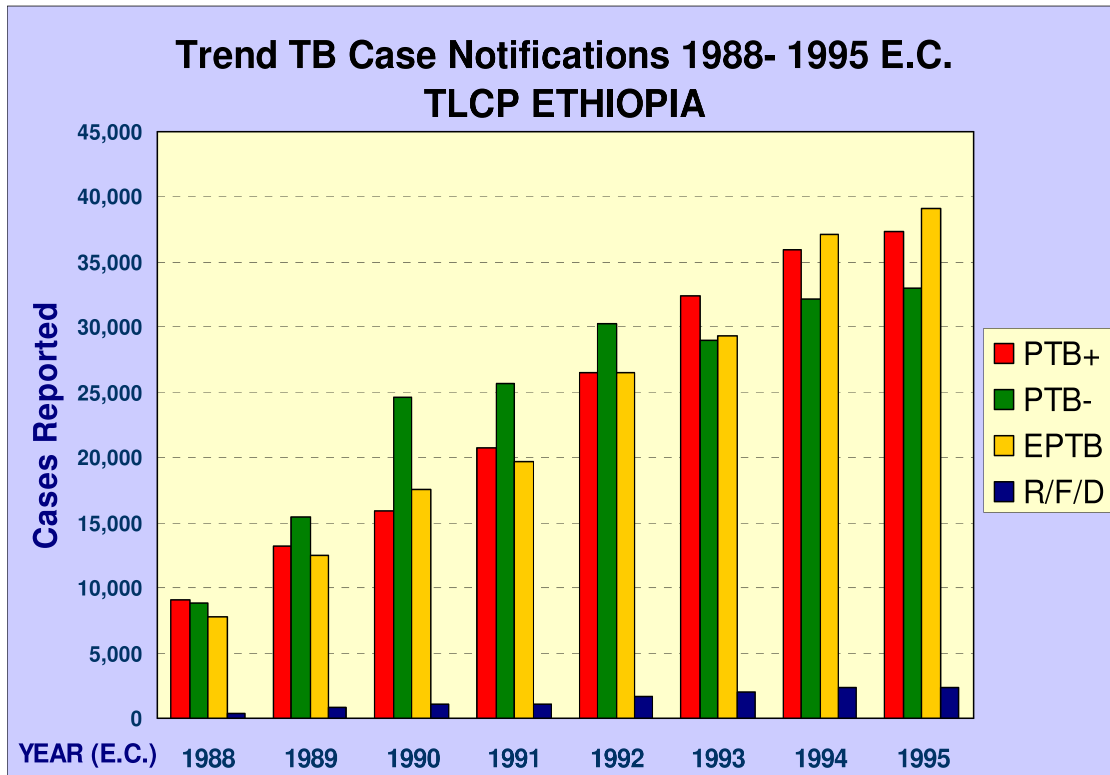

Figure1.**Trend of TB case notifications in Ethiopia, 1988-1995 E .C.**

(TLCP, Ministry of Health, Ethiopia, 2002*)*

A few studies conducted in the country also indicated increased reporting of TBLN in some localities. A study conducted in Butajira, rural Ethiopia by Kidane *et al* (2002) demonstrated that out of 72 patients with a clinical diagnosis of TBLN,40(55.6 %) were confirmed tuberculous lymphadenitis cases on the basis of combined clinical and cytological criteria. Of the 40 patients, 11(27.5%) were HIV sero- positive. Another study conducted in the same area by Yassin *et al* (2003) also showed that out of 147 patients, 107(72.8%) were confirmed as cases of TBLN by fine needle aspirate (FNA) cytology and acid-fast stain examination. The findings of the study also depicted that 24/107(2 2.4 %) TBLN cases were HIV sero - positive. Moreover, a study from Tikur Anbessa Hospital confirmed that from a total of 205 clinically suspected TBLN patients, TBLN was diagnosed in 194(94.6%) cases using microscopic examination of cytological and acid fast smears (Ergete and Bekele, 2000).

As shown in Figure 1, the proportion of EPTB is increasing from year to year. The possible reasons why EPTB, is rising in Ethiopia could be due to: increased TB burden, increase in HIV pandemic, under diagnosis of pulmonary tuberculosis, over diagnosis of cervical TBLN or transmission of other mycobacteria such as *M. bovis.* Therefore, due to high reporting rates in some areas of the country, it is important to study the situation of TBLN in different regions of the country. Hence, our present study was conducted in Dera; to assess the situation of TBLN in the locality.

Dera was a relatively isolated area for a long period of time due to lack of access to road until 2002. Therefore, due to socio-economic factors and geographical location, it was believed that the epidemiology and the etiology (*M. tuberculosis* versus other species) responsible for TBLN infection might be different from other rural areas of Ethiopia. As the risk factors for *M. bovis* and *M. tuberculosis* infection are likely to be different and because *M. bovis* is inherently resistant to pyrazinamide, proper identification of these organisms has epidemiologic as well as clinical implications and is therefore, required for effective TB control (Kurabachew *et al.,* 2004). The present study was designed to isolate and identify the etiological agents responsible for TBLN in the study area. Thus, this study would provide concrete evidence on the etiological agents and the general situation of tuberculous lymphadenitis in Dera.

1.5 Characteristics of Mycobacteria

The mycobacteria are classified under the class Actinomycetes, order Actinomycetales, family Mycobacteriaceae and genus Mycobacterium (Shinnick and Good, 1994). The mycobacteria are rod shaped, acid fast, aerobic or micro-aerophilic, non-spore forming, non-motile, non-capsulated and lipid rich bacteria (Frieden *et al.,* 2003). Most of them grow slowly on solid Loweinstein - Jensen (LJ) medium, are resistant to drying, disinfectants and remain viable in clinical samples for a long period of time (Evans, 1998).

The wall of mycobacteria is thick consisting of a plasma membrane surrounded by a complex wall structure harboring virulence factor such as peptidoglycan, arabinogalactans, mycolic

acids (long chain fatty acids), glycolipids, and lipoarabinomanans (Brennan and Draper, 1994, Alamelu, 2004). On the basis of experimental evidence, cell wall glycolipids interact with polymorphonuclear leukocytes and macrophage lysosomal membranes to prevent their fusion with phagosomes. It is also conceivable that wall glycolipids, lipoproteins, and / or lipid may play a role in the stimulation of delayed type of hypersensitivity and /or immunity (Flynn and Chan, 2001).

1.6 Etiological agent

The causative agent of tuberculosis was first detected in 1892 by the German scientist Robert Koch. The genus Mycobacterium includes members of the *Mycobacterium tuberculosis* complex, which contain medically important species (Shinnick and Good, 1994). The species found in the complex are *M. tuberculosis* (the most widely spread bacterium responsible for tuberculosis), *M.bovis* (world wide but limited by pasteurization of milk and it has the broadest host range of species in the complex), *M. africanum* (mainly found in Africa), *M. microti* and *M. canetti* (limited to small rodents) (Sales *et al.,* 2001). *M. tuberculosis*, *M. bovis*, *M. africanum* and non-tuberculous mycobacteria can cause lymphadenitis, but lymphadenitis caused by *M. tuberculosis* complex is more chronic as compared with lymphadenitis caused by other mycobacteria, which has a more rapid course (Singh *et al.,* 2000).

A study done on 1,817 bacteriological cultures that were received from 1,817 patients with tuberculous lymphadenitis in South east England, from 1981 to 1989, showed that 1,677 (92.3%) of the cultures were found to be positive for *M. tuberculosis*, 25(1.34%) were *M. bovis*, 21(1.16%) *M. africanum* and 94(5.17%) other environmental mycobacteria (Yates and Grange, 1992). In the United States, it was reported that 95 - 98% of mycobacterial lymphadenitis in adults is due to *M. tuberculosis* and 86-92% mycobacterial lymphadenitis in children is caused by atypical mycobacteria (*M. scrofulaceum*, *M. avium - intracellular a*nd *M. kansasii*) (Brainard, 2001).

In Ethiopia, the etiological agents for TBLN in rural area were recently reported by Kidane *et al* (2002) using PCR technique. The study demonstrated that among 35 PCR positive cases of tuberculous lymphadenitis in Butajira, rural Ethiopia, 29(82.9%) were caused by *M. tuberculosis* and 6(17.1%) were caused by *M. bovis.* However, this does not lead to a conclusion that *M. tuberculosis* is the major cause of TBLN everywhere at all times. This is because different studies demonstrated that the dominance of the different mycobacteria as a causative agent of TBLN vary depending on many factors such as geographical location, age, gender, race or ethnicity and cultural practices (Small *et al.,* 1994, dye *et al.,* 1999). A study conducted in Tanzania revealed that among 17 pastoralists suspected of suffering from TBLN, *M. bovis* was found in 4(23.5%) of the suspects who consumed raw milk (Kazwala *et al.,* 2001).

1. 7 Risk factors

Risk factors that accelerate tuberculous infection include: poverty, changing demographics with increasing crowding and changing age structure (children less than five years and the elderly greater than 65 years are more vulnerable) (Pena *et al.,* 2003). Other factors such as genetic disposition, inadequate health coverage, chronic infections (HIV/ AIDS, diabetes mellitus, renal disease, lung damage and various malignancies) enhance development of the disease (Preneboom *et al*., 1995). Neglect and under-funding of tuberculosis control programmes, previous exposure to mycobacterial infections, protein energy malnutrition, and cytotoxic therapy can also aggravate disease progression (Cantwell *et al.,* 1997). However, how much significant these risk factors are to isolated TBLN needs further investigations.

1.8 The disease transmission

Transmission of *M. tuberculosis* can occur from persons with active tuberculosis. Tuberculosis is spread through droplets, which are expelled when persons infected with the bacilli cough, sneeze or sing (Nachega *et al.,* 2003). Persons with prolonged or intense contact with infected individuals are at high risk of becoming infected. Others at risk include residents

and employees of high congregate settings, individuals living in TB endemic areas and health care workers who serve high risk clients (Raviglione *et al.,* 1995, Kazwala *et al.,* 2001). *M. bovis* can be transmitted to humans through ingestion of infected raw milk and close contact with infected animals (Skuce *et al.,*2003).

1.9 Pathogenesis

Extra pulmonary involvement can occur in isolation or along with a pulmonary focus as in the case of patients with disseminated TB. TBLN is considered to be the local manifestation of a systemic disease (Sharma and Mohan, 2004). Tuberculosis is spread by air borne droplet nuclei, which are particles of 1-5µm in diameter that contain *M. tuberculosis (*Frieden *et al.,* 2003). *M. tuberculosis* gets entry into the body via the respiratory tract and undergoes haematogenous and lymphatic dissemination (Raviligone *et al.,* 1995). 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 (Kumar, 2001). Hilar and mediastinal lymph nodes are initially involved. The infection then spreads through the lymphatic to the draining cervical lymph nodes (Alamelu, 2004).

Initially the lymph nodes are discrete and periadenitis results in matting and fixation of the lymph node. The lymph nodes coalesce and break down due to the formation of caseous pus which may perforate the deep fascia and present as a collar - stud abscess (Gomez *et al.,* 2003). Overlying skin becomes indurated and breaks down, resulting in sinus formation, which may remain, unhealed for several years and healing may occur with calcification and scarring (Abba *et al.,* 2001). In contrast, non-tuberculous mycobacteria gain entry into the lymph nodes directly via or-opharyngeal mucosa, salivary glands, tonsils, gingival or conjunctiva and lymph node involvement represents a localized disease (Sharma and Mohan, 2004).

1.10 Clinical presentation

Pulmonary TB patients usually have weight loss and productive cough for more than three weeks. Symptoms such as chest pain, dyspnea, fever, night sweating, anorexia and haemoptysis are common among TB patients (Nataraj *et al.,* 2002). Previous studies have reported that tuberculous lymphadenitis usually affects children and young adults with high female predilection (Velayati *et al.,* 1998). 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 (Sharma and Mohan, 2004).

Bem *et al* (1997) observed that among HIV negative as well as HIV positive patients, cervical lymph nodes were mostly affected as compared to axillary and inguinal lymph nodes. Patients with TBLN may manifest symptoms such as fever, weight loss, fatigue, cough and night sweating (Arora *et al.,* 1995). The enlarged lymph nodes may be of varying size, are usually firm, painless and may be discrete or matted. The lymph nodes are not tender unless secondary bacterial infection has occurred (Alamelu, 2004, Nataraj *et al.,* 2002).

1.11 Laboratory diagnosis of tuberculosis

The definitive diagnosis of tuberculosis depends on the isolation and identification of the etiological agents responsible for the infection. Identification and treatment of affected patients is the primary strategy for the control of tuberculosis (Frieden *et al*., 2003). The low sensitivity of conventional methods in detecting tubercle bacilli in clinical specimens makes the diagnosis of tuberculosis in general and extra pulmonary tuberculosis in particular, a major challenge in developing countries (Banavaliker, *et al.,* 1997). It is estimated that only 50- 60% of all patients with tuberculosis worldwide are actually diagnosed. Conventional methods (such as AFB staining of smears from FNA or sputum samples and mycobacterial culture) are still the methods of choice in most mycobacteriological laboratories (Daniel, 1989). For effective diagnosis of tuberculosis, the combination of conventional methods and molecular techniques has been recommended (Kurabachew *et al*., 2004).

1.11.1 Ziehl Neelsen (ZN) staining

The detection of acid-fast bacilli using Ziehl Neelsen staining is the primary method for the diagnosis of tuberculosis (Nataraj *et al.,* 2002). Although specific and rapid, the technique has low sensitivity in the detection of tubercle bacilli in various clinical specimens (Bouakline *et al.,* 2003). The specificity of direct smear examination is considered to be 98% in areas where the prevalence of non - tuberculosis bacteria is low (Yates and Grange, 1992). Before the advent of HIV, the sensitivity of the technique range from 30 - 40 % with a single sputum specimen and 65 -75% with repeated smear examinations (Daniel, 1989).

However, the rate of acid-fast bacilli (AFB) positivity in FNA smears of TBLN is in the ranges of 41- 56.4 % (Ergete and Bekele, 2000). Previous studies conducted on FNA revealed a wide variation in the frequency of AFB positivity. AFB positivity is low in epithelioid granuloma without necrosis (5.8 - 30%) but significantly higher in epithelioid granuloma with necrosis (32 - 65%) (Rajwanshi *et al.,* 1987). 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 sputum must be present to permit detection by acid fast staining (Daniel, 1989).
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1.11.2 Cytology

Fine needle aspirate (FNA) cytology is a good diagnostic approach in the diagnosis of tuberculous lymphadenitis or extra pulmonary tuberculosis (Dannapat, *et al.,* 199). FNA cytology is a useful diagnostic method in areas where it is difficult to perform molecular techniques.
Diagnostic criteria for tuberculous lymphadenitis include: Clinical signs such as enlarged, discrete or matted, usually unilateral non-tender lymph nodes with or without draining sinuses. Thick yellowish appearance of fine needle aspirates; cytological finding showing epitheloid granulomas with caseous necrosis (Singh *et al*., 2000; Ergete and Bekele, 2000). In addition to epithelioid cells, the smear may contain clumps of amorphous acellular debris or caseous necrotic materials. Lymphocytes, Langhan giant cells and neutrophils may be present

or; epithelioid granulomas without caseous necrosis. Groups of epithelioid cells found along with a variable number of lymphoid cells or; necrotic materials without epithelioid granuloma and clumps of amorphous acellular material (Sharma and Mohan, 2004).

1.11.3 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 (Hung and Sun, 2000). Culture is often used as a reference method due to its high sensitivity (as high as 89%) and specificity (greater than 98%). However, in significant proportion of clinical samples, low numbers and slow growth rate of tubercle bacilli limit the sensitivity of culture (Velayati, *et al.,* 1998).

Different media (egg based, agar based and liquid media) have been devised for cultivating of tubercle bacilli .The ideal medium for isolation of tubercle bacilli should be: economical, simple to prepare from readily available ingredients, inhibit the growth of contaminants, support luxuriant growth of small numbers of bacilli and permit preliminary differentiation of isolates on the basis of colony morphology. Among the aforementioned media, bacterial culture on egg - based Lowenstein Jensen (LJ) media are commonly used in most laboratories since they meet most of the above-mentioned requirements (Singh *et al.,* 2000).

LJ 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*. Both should be used in countries or regions where patients may be infected with either organism (Small *et al.,* 1994). However, bacterial culture takes at least 3 to 8 weeks to observe a positive growth after incubation at 35 -37 ^{o}C (Szewzyk, *et al.,* 1995).

1.11.4 Polymerase Chain Reaction (PCR)

In order to reduce the time needed for and improve the sensitivity of identification methods, a combination of the above cited techniques and PCR methods have been recommended (Gonzalez *et al.,* 2003). 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 extra pulmonary specimens. Nucleic acid methods are currently employed for rapid identification of mycobacteria from both cultured and uncultured clinical specimens (Espinoza *et al.,* 1998).

DNA amplification using PCR is considered as one of the most sensitive approaches for detection of mycobacterial DNA, which involves genomic DNA extraction, amplification and identification (Kurabachew *et al.,* 2004). It has allowed great progress to be made in the rapid and accurate diagnosis of infections due to organisms that are not cultivable by *in vitro* means, that require complex media, or cell cultures and prolonged incubation times or for which culture is too insensitive (Bouakline *et al*., 2003).

In contrast to pulmonary samples, PCR has less sensitivity in detection of mycobacterial DNA from extra pulmonary samples (Delportillo *et al.,* 1996). This might result from the use of very small sample volumes and the presence of inhibitors, which interfere with amplification based techniques. Inhibitory activity was often checked for discrepant results between culture and PCR results (Shah *et al.,* 2002). It is difficult to identify the substance (s) responsible for this inhibition, which might be blood, detergents or heparin. In order to minimize the inhibitory substance (s), simple dilution of the specimen is of paramount importance (Rimek *et al.,* 2002).

Molecular studies demonstrated that normal appearing human lung tissues rather than glaucomatous lesions harbor DNA; a finding strongly suggested that latent TB bacilli reside in apparently normal tissue, which can result in false positive PCR results (Singh *et al.,* 2000). PCR has a specificity of 100% and sensitivities ranged from 20 to 94% for extra pulmonary specimens. It is important that any PCR - based assay include positive and negative internal

control to allow proper evaluation of DNA preparation and amplification (Bouakline *et al.,* 2003).

1.12 Treatment

Anti - tuberculosis treatment is extremely important in the management of extra pulmonary tuberculosis. WHO recommends directly observed treatment, short - course (DOTS) approach for patients with EPTB (WHO, 2005). According to the WHO report, the DOTS strategy had been adopted by more than 150 countries and was available to over 60% of the world's inhabitants (WHO, 2004). Tuberculosis is treated with the first line drugs, which include isoniazid, rifampicin, streptomycin, ethambutol and pyrazinamide.

In order to avoid the occurrence of drug resistance, the drugs are provided in combination of two or more (Sharma and Mohan, 2004). In countries with high TB burden, special emphasis has to be given to the treatment of new TB cases by performing drug susceptibility tests that are relevant in the identification of resistant strains (Nachega, 2003). For management of enlarged lymph nodes, see Appendix I.

1.13 Significance of the study

There are reports of rising of TBLN infection from sub – Sahara African countries due to an alarming increase of HIV pandemic, TB/ HIV co- infection and multi drug resistance TB (WHO, 2005). In Ethiopia, TBLN is not well studied so far and little is known about the etiological agents of TBLN and patterns of the disease transmission among the population. Therefore, the present study aimed to isolate and identify Mycobacteria that are responsible for TBLN infection from patients clinically suspected for TBLN in Dera, rural Ethiopia. Hence, it is believed that this study would provide a general overview of the status of TBLN infection in the study locality and helps to fill the existing gap in our knowledge on the situation of TBLN in rural areas.

1.14 Hypothesis

Tuberculous lymphadenitis in Dera is caused by *M. tuberculosis* in over 80% of the cases.

1.15 Objectives

1.15.1 General objective

To characterize the species of Mycobacteria causing tuberculous lymphadenitis in Derra.

1.15.2 Specific objectives

1. To identify the etiological species responsible for tuberculous lymphadenitis.
2. To compare the relative frequency of mycobacterial isolates responsible for tuberculous lymphadenitis.
3. To evaluate the detection rate of different diagnostic methods in the diagnosis of TBLN from fine needle aspirates.

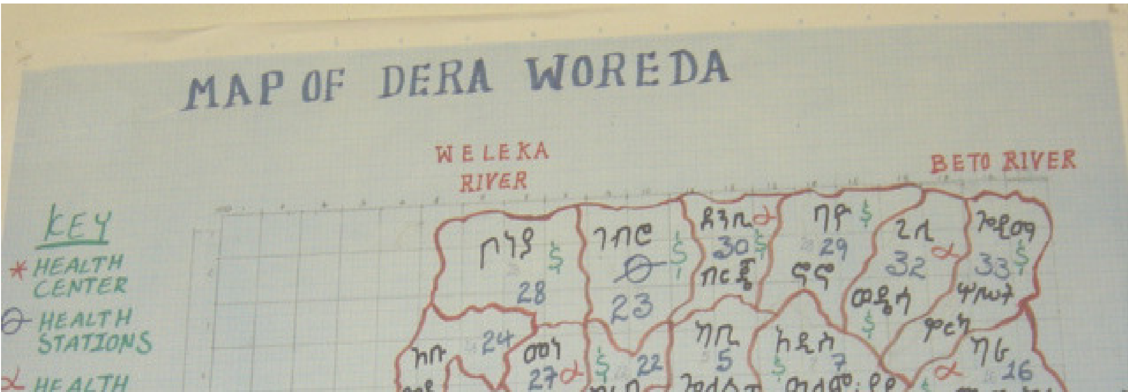
CHAPTER II : Materials and Methods

2.1 Study site

Dera is one of the 12 districts of North Showa Zone of Oromiya Regional State. The district is located about 225Km away from the capital city, Addis Ababa. The district has a common boundary with Warra Jarso and Hidhabu Abote districts in the south and south east, Amhara regional State in the north, north west, north east and south east of its land mass. The district of Dera has a total land area of about 1,528 square kilometers. Four major land use types are identified in the district. These are cultivated land consisting of 47.8% pastureland, forest and woodland and bush land covering 15.8% and non-productive land covering 27.9%. Land used for other purposes constitutes 8.5% of the total land area of the district.

According to the population and housing census of 1994, the population of the district was 139, 661. The population of the district was projected to reach 180, 925 in the year 2003. The ethnic composition of the district constitutes mainly of Oromo and Amhara.

Its town, Gundo - Mesqual is situated at a distance of about 110 Kms from the zonal capital, Fiche. The town serves as an administrative center for the district and has a total population of over 3,000. Two agro- ecological zones characterize Derra district, high land (Dega) covering about 50% and low land (Kola) sharing the same percentage of the total land area of the district. The mean annual rainfall of the district ranges between 800 mm to 1,500 mm mainly from June to August and the average annual temperature of the district ranges from 18°C to 25°C.



Map of Dera Woreda, North Showa, Ethiopia.

Source: Dera Woreda Health Office, Gundo- Meskel, 2004. (Photo: Dr. Howard Engers).

Regarding the health infrastructure, the district has one health center, two clinics and ten health posts. Currently, the health coverage in the district is 47%, which is below the regional average of 52.3%. As a result of lack of all weather roads, accessibility to basic services outside the main road is difficult for most of the rural population (Sera Project, 2004).

According to the new health policy of Ethiopia, there are four health care delivery structure i.e. health centers, district, regional and specialized hospitals.

The primary health care unit is the basic level of health care in the system and consists of a health center with five satellite health posts.

One health center provides health services for about 25,000 people. The five satellite health posts each give services to about 5,000 people living in the catchment area (WHO, 2005).

Based on the report obtained from the Dera health center, the most prevailing diseases in the district are malaria, rheumatic diseases, tuberculosis, upper respiratory tract infections, pneumonia and diarrhea. For TB services, patients are referred from the clinics and health posts to the Gundo Meskel health center for diagnosis and treatment. In the health center, there is one TB clinic, which registers and supervises anti TB treatment based on the DOTS strategy. The clinic also provides follow - up care of TB patients.

The laboratory service given for TB patients at the health center is the routine sputum smear examination. Patients whose smear examination is positive for acid-fast bacilli and patients negative for smear examination but clinically suspected for TB are put on anti - TB treatment. On the other hand, patients who are critically ill and negative for sputum smear examination are referred to Fiche Hospital for further evaluation and management.

2.2 Study design

This was a health center based cross - sectional study conducted in Dera Woreda, North Showa, Ethiopia, between August 2004 and February 2005.

2.3 Target population

The target population was patients aged 15 to 65 years who had complaints related to a presumptive clinical diagnosis of TBLN.

2.4 Inclusion criteria

Patients with enlarged, non-tender lymph nodes that did not respond to a two-week course of broad-spectrum antibiotics were included in the study.

2.5 Exclusion criteria

- Patients who were critically ill.
- Patients who were already taking anti-tuberculosis treatment.
- Those patients unwilling to undergo fine needle aspiration.
- Patients whose age was less than 15 and greater than 65 year of age.

2.6 Sample size

The minimum sample size was determined based on the prevalence of *M. bovis* (17.1%) in a previous study conducted in Butajira (Kidane *et al.,* 2002). With 17.1% prevalence, the required sample size was estimated at 216. However, due to shortage of time and unavoidable constraints of slow patient flow during the study period, the total number of samples collected was 153 (71% of the calculated sample size).

2.7 Specimen collection and transportation

A trained health officer working in Derra health center collected fine needle aspirates (FNA) after receiving signed informed consent from each study participant. The FNA was collected from the swollen superficial lymph nodes by using a 22-gauge needle with an attached 10-ml syringe, which was mounted on an aspiration cameco gun. During each pass, the needle was moved within the lesion a few times while aspirating.

From each study participant, a small amount of FNA (approximately 50-60 micro liters) was collected. Some portion of the specimen was used for the preparation of AFB and cytology smears on the site. The rest of the specimen was transferred into Nunc Cryo Tubes (Nalge Nunc International, Denmark) containing one ml of phosphate buffer saline (pH = 7.4) for culture and DNA extraction. Each Cryo Tube was initially labeled with a code number corresponding to each patient using a permanent marker in order to easily identify each specimen.

After collection, the specimens were put at $4^{o}C$ until transported to Armauer Hansen Research Institute (AHRI) laboratory. For transportation of the specimens from study site to AHRI

laboratory, a cold chain system with icebox was used. After the specimens were transported to AHRI laboratory, they were kept at $4^{o}C$ and immediately processed the following day.

2.8 Microscopic examination of smears

2. 8.1 Ziehl Neelsen staining technique

This method is recommended for the preliminary diagnosis of tubercle bacilli in various clinical samples. FNA samples obtained from each study participant were smeared on clean slides in the field. The slides were air-dried and heat fixed in Dera health center laboratory. By using a slide box, the heat fixed slides were transported to AHRI laboratory for further processing. The staining procedure was carried out at AHRI laboratory as per the staining protocol for sputum smear as described by Cheesbrough (2000) with certain modifications. In brief, the slides were covered with freshly filtered carbol - fuchsin and heated until steam came out. The heated slides were continuously stained by carbol - fuchsin for 10 minutes and washed with water. Carbol- fuchsin stained slides were further decolorized with 25% sulfuric acid and 96% alcohol for 5 minutes each. The decolorized slides were stained with methylene blue (counter stain) for 2 minutes, rinsed with clean tap water and air-dried.

Finally, the slides were examined for acid-fast bacilli under a 100x oil immersion objective by a medical laboratory technologist. Under microscopic examination, acid-fast bacilli appear red on a blue background while other organisms appear dark blue. Negative and positive control smears were stained concurrently to assess the quality of the staining procedures.

2. 8.2 Cytomorphological staining

FNA samples were smeared on clean slides on the spot of specimen collection. The slides were air dried and fixed with methanol in Dera health center laboratory. The slides were transported to AHRI laboratory using a slide box for staining. In the AHRI laboratory, the slides were flooded with freshly filtered Wright's stain and buffered with clean tap water. The buffered slides were continuously stained with Wright's stain for 10 minutes, washed with tap water and air dried. Finally, the slides were examined by a pathologist to evaluate whether the morphology was suggestive for tuberculous lymphadenitis or not.

Cytological examination of FNA smears was considered diagnostic of TBLN when they contained a thick, yellowish material showing either necrotic background associated with the presence of lymphohistiocytic and the presence of a significant polymorphonuclear cell population or the presence of a granulomatous inflammatory reaction consisting of giant cell, and/or epithelioid cell clusters and lymphohistiocytic cell population (Yassin et al., 2003).

2. 9 Mycobacterial Culture

One ml of FNA and phosphate buffer saline (pH = 7.4) mix was transferred into falcon tube of 15 ml capacity (BD, Lab. Ware, USA).The samples were decontaminated from non-mycobacterial organisms by sodium dodecyl sulfate (Groothuis and Yates,1991).
The decontaminated samples were centrifuged at 3,000 rpm for 15 minutes. An aliquot of 100 μl of the neutralized samples were cultured into four LJ tubes (two with pyruvate and two with glycerol) for primary isolation of the organisms.

The inoculated tubes were incubated at 35 to 37°C for 3 to 8 weeks and observed once a week for the growth of mycobacteria. Growth of the mycobacteria was confirmed by visual detection of colonial morphology and by microscopic examination of the colonies for acid-fast bacilli (AFB). Subcultures were also performed for further laboratory tests and storage of the samples.

To check for the quality of the LJ media, fast growing *M. kansasii* (ATCC 12478) strain was run in parallel with each batch of inoculated media. In addition to this, uninoculated LJ tubes were incubated at the same time to control for contamination.

2. 10 Molecular techniques

2.10.1 Genomic DNA Extraction

The DNA extraction of mycobacteria was performed as per the extraction protocol described by van Soolingen *et al* (1995). Briefly, bacterial strains grown on Lowenstein - Jensen media were resuspended in 400 µl of 1x TE buffer with pH 8.0. The mixture was incubated in a water bath at 80°C for one hour to kill the bacteria and cooled to room temperature. Fifty µl of lysozyme (10mg/ml) (Sigma, Saint Louis, USA) was added to lyse the bacteria and the mixture was incubated in a 37°C water bath for one hour. In the lysozyme treated samples, 75µl of 10% SDS / proteinase K mix (Sigma, Saint Louis, USA) was added and incubation was continued for 10 minutes at 65°C. To remove inhibitors, 100µl of 5M NaCl and 100 µl of cetyltrimethylammonium bromide (CTAB)/NaCl solution were added to the sample, vortexed and the mixture incubated for 10 minutes at 65°C .

DNA extraction was performed with 750 µl of chloroform - isoamyl alcohol in the 24:1 (volume / volume) ratio respectively. Four hundred fifty micro liter of isopropanol was added to the aqueous phase to precipitate and obtain the DNA pellet. After the addition of isopropanol, the pellet was placed in -20^{0}C freezer for 30 minutes. The DNA was recovered by centrifugation at 12,000 rpm for 15 minutes. The harvested DNA pellet was washed with 1ml cold 70% ethanol to remove CTAB and NaCl. The ethanol washed pellet was treated with 10 mg /ml DNase free RNaseA (Sigma, Saint Louis, USA) and incubated for one hour at 37° C. Finally, the pellet was dried at room temperature, re- dissolved in 1x TE buffer (pH 8.0) and stored at 4°C for immediate processing or at -20°C freezer until required for further analysis.

2. 10.2 Polymerase Chain Reaction

Polymerase chain reaction (PCR) was performed on 145 FNA samples to amplify the gene of interest from mycobacterial DNA. PCR amplification of the IS6110 (an insertion sequence element used to identify members of the *M. tuberculosis* complex) was carried out with complex specific primer pairs, IS5 (5'- CGGAGACGGTGCCTAAGTGG -3') and IS6 (5'- GATGGACCGCCAGGGCTTGC -3') to amplify a 980 bp of the gene (Del Portillo *et al*., 1996).

All reactions were performed in a total volume of 25µl containing 100 ng of purified DNA template and 2µl from each primer. The PCR mix was prepared in a final volume of 25µl using Ready To -Go - Beads (Amersham Pharmacia Biotechnology, Freiberg, Germany). The amplification programme for IS6110 genes was one cycle of 94°C for 1 minute for denaturation, followed by 35 cycles each for 60 seconds at 94°C and 71°C for 90 seconds for annealing and at 72°C for 2 minutes for final extension and a final incubation at 72°C for 10 minutes, with both pairs of primers added into the tubes using a thermal cycler (Gene Amplification PCR system 9700, PE Bio. system, Norwalk, CT).

In addition to Mycobacterium complex, allele specific PCR system was also applied for the amplification of the *pncA* gene to differentiate *M. tuberculosis* from *M. bovis* (Espinosa *et al.,* 1998).

For the amplification of *pncA* gene, *pncA*TB -1.2 (5'- ATGCGGGCGTTGATCATCGTC -3') was used as forward primer; p*ncA*MT-2 (5'- CGGTGTGCCGGAGAAGCGG -3') and *pnc*AMB-2 (5'- CGGTGTGCCGGAGAAGCCG -3') were used as reverse primer. A common forward primer, *pncA* TB -1.2 and two reverse allele specific primers, *pncA* MT-2 and *pncA* MB-2, were designed to hybridize with the published sequences of *M. tuberculosis* and *M. bovis pncA* genes to amplify 185 base pair fragments of the *pncA* genes.

The reverse primer *pncA*MT-2 was designed to anneal only to the *pncA* sequence from *M. tuberculosis* and *pncA* MB-2 to that from *M. bovis (*Espinosa *et al*, 1998). For the *pncA*

method, the cycling programme was 1 cycle of 95°C for 2 minutes and 30 cycles of 94°C for 1 minute, 67°C for 1 minute, and 72°C for 1 minute. All the steps of sample processing and PCR were carried out in a separate PCR mix room using dedicated pipettes and autoclaved tips to prevent cross contamination.

2.10.3 Optimization of PCR conditions

PCR conditions were optimized with respect to annealing temperature and number of amplification cycles. Using DNA extracted from H37Rv reference strain of *M. tuberculosis*; ten fold of serial dilutions were prepared. Using the serial dilutions, PCR was performed using IS5/IS6 complex specific primer pairs. The analytical sensitivity of the PCR assay was determined by calculating the smallest concentration of DNA that could be amplified and detected. For each FNA sample, original and 1:10 diluted DNA extracts were amplified by PCR to control false negative results due to the presence of amplification inhibitors. DNA extracted from cultures of *M.tuberculosis* (ATCC35836) and *M. bovis* (RIVM 12716) (Bilthoven, The Netherlands) were used as a positive controls. Moreover, sterile distilled water was used as negative control in each assay.

2.10. 4 Agarose gel electrophoresis

Agarose gel electrophoresis was performed to verify the amplification of the desired gene of interest. The PCR products were analyzed by electrophoresis through 1.5 - 2% agarose gels containing 0.5µg/ml ethidium bromide (Bio-Rad Laboratories, Germany) at 100 volts and 50 ampere for 30 to 45 minutes in 50x Tris acetate EDTA (Sigma, Chem. Corp. USA) buffer with pH 8.0.

The reaction was considered to be positive when a 980 base pair (bp) fragment was detected for genus complex PCR system and 185 bp fragment for *pncA* gene that was matched with the size of the PCR product of similarly amplified DNA extracted from control cultures of

M. tuberculosis (ATCC35836) and *M. bovis* (RIVM 12716) (Bilthoven, The Netherlands), which were used as a positive control. Sterile distilled water was used to control for contamination and run in parallel with each test. Finally, the gels were visualized under UV-transilluminator (Boehringer - Mannheim, Germany) and captured with Polaroid 667 film (Sigma, Chem. Corp. USA). The molecular weight marker we used was 1 Kilo base DNA ladder (Sigma, Chem. Corp. USA).

2.11 Statistical analysis

Demographic and clinical data were collected using Case Record Form (see Appendix II). The data were double entered using EPI- info version 6.04b by two data entry clerks on two different computers. The data were verified to obtain clean data before using them for further statistical analysis. Chi- square tests (Mantel Haenszel, Fisher's exact and Yates) were used to compare the differences. A p- value less than 0.05 was considered statistically significant.

2.12 Ethical considerations

The project has obtained ethical clearance from both institutional (Addis Ababa University, Medical Faculty Research and Publication Committee and AHRI / ALERT Ethical Review Committee) and National Ethical Review Committees before commencement of the actual activities. Informed consent was obtained from each study participant and guardians before collection of FNA specimens (Appendix III and IV). Informed consent signatures were obtained from each adult study participants and from parents or guardians for adolescents between 15 to 17 years of age.

CHAPTER III : Results

A total of 153 FNA specimens were collected from 28 August 2004 to 27 February 2005 from patients clinically suspected for TBLN. Out of these specimens, eight were excluded because of inadequate sample volume and contamination problems. Therefore, 145 FNA samples were processed and analyzed in our present study. The majority of the aspirates, 88 (60.7%) were from cervical, followed by 28 (19.3%) inguinal, 17 (11.7 %) axillary, 2 (1.4%) cervical and axillary, 3 (2.1%) cervical and inguinal (patients sampled twice from different lymph nodes) and the remainder 7 (4.8%) were from other lymph nodes. In this study, out of 145 patients with clinical diagnosis of TBLN, 115 (79.3%) were confirmed as TBLN cases based on combined results of AFB smear examination, cytology, culture and PCR assay.

3.1 Socio – demographic characteristics of the study population

Selected socio - demographic variables were statistically cross- tabulated with TBLN and non -TBLN cases to see the association among different variables and infection. The results are shown below in Table 1. As indicated, females 64.3% (n =74) were more frequent than males 35.7 % (n = 41). Farmers accounted for 66.1% (n =76) of TBLN cases. There were more TBLN Patients from low land 58 .2% (n = 67) than high land 41.8% (n = 48).

As to the ethnic group of the study participants, 80(55.2%) were Oromo and the rest 65 (44.8%) were Amhara. Among these, TBLN infection was detected in 62 Oromo (53.9%) and 53(46.1%) Amhara. Statistical analysis showed that demographic factors such as ethnic group, geographical location, occupation and gender had no significant association with TBLN infection. However, consumption of raw milk demonstrated significant association with TBLN infection (P = 0.04).

Table1. Socio - demographic characteristics of TBLN cases in Dera Woreda, North Showa, Ethiopia, August 2004 to February 2005.

Variable		**TBLN (n = 115)**	**Non – TBLN (n = 30)**	**p - value (X^2 - test)**
Sex	Male	41 (35.7)	13 (43.3)	
	Female	74 (64.3)	17 (56.7)	p = 0.43
Occupation	Farmer	76 (66.1)	24 (80)	
	House wife	20 (17.4)	3 (10)	
	Student	17(14.8)	1 (3.3)	p= 0.47
	Merchant	1(0.8)	None	
	Clergy	1(0.8)	None	
Ethnicity	Oromo	62 (53.9)	12 (40)	
	Amhara	53 (46.1)	18 (60)	p = 0.55
Geographical location	Low land	66 (57.4)	19 (63.3)	
	High land	49 (42. 6)	11 (36.7)	p = 0.56
Consumption of raw milk	Yes	89 (77.4)	28 (93.3)	
	No	26 (22.6)	2 (6.7)	p = 0.04

Data are shown as n (%)

The age and sex distribution of the 145 study participants is depicted in Table 2.

Table 2. Age and sex distribution of the 145 patients clinically suspected for TBLN in Dera Woreda, North Showa, Ethiopia, August 2004 to February 2005.

Sex	Patients and age groups (in years) (%)					Total
	15 – 24	**25 – 34**	**35- 44**	**45-54**	**55- 64**	
Male	24 (45.3)	16 (42.1)	5 (17.9)	6 (42. 9)	3 (25)	54 (37.2)
Female	29 (54.7)	22 (57.9)	23 (82.1)	8 (57.1)	9 (75)	91 (62.8)
Total	53 (37)	38 (26.1)	28 (19.2)	14 (9.6)	12 (8.1)	145 (100)

As shown in Table 2, there were 54 (37.2%) male and 91(62.8%) female study participants, making the male to female ratio 0.59: 1. The ages of the patients ranged from 15 to 63 years. The mean and median age was 31($\pm$ 8.8) and 30 years old respectively. The peak age for clinical TBLN was observed between 15 to 24 years, where 37% of clinical TBLN cases were found.

Table 3. Age and sex distribution of the 115 confirmed TBLN patients in Dera Woreda, North

Showa, Ethiopia, August 2004 to February 2005.

Sex	Patients and age groups (in years) (%)					Total
	15 - 24	**25 - 34**	**35 - 44**	**45 - 54**	**55 - 64**	
Male	19 (45.2)	11 (34.4)	4 (20)	4 (40)	3 (27.3)	41 (35.7)
Female	23 (54.8)	21 (65.6)	16 (80)	6 (60)	8 (72.7)	74 (64.3)
Total	42 (36.5)	32 (27.8)	20 (17.4)	10 (8.7)	11 (9.6)	115 (100)

As indicated in Table 3, there were 35.7 % (n =41) male and 64.3% (n =74) female, making the male to female ratio 0.55: 1. The peak age group for TBLN was found to be in the age group of 15 to 24 year.

Table 4. Clinical variables in the group confirmed as positive for TBLN with AFB smear examination, culture, cytology and PCR compared with non - TBLN cases.

Variable		**TBLN (n = 115)**	**Non – TBLN (n = 30)**	**p – value (X^2 - test)**
Lymph node scar	Yes	65 (56.5)	14 (46.7)	p = 0.03
	No	50 (43.5)	16 (53.3)	
Clinical manifestations	Cough	29 (25.2)	11 (10)	p = 0.21
	Night sweating	90 (78.3)	25 (83.3)	p = 0.54
	Weight loss	109 (94.5)	28 (93.3)	p = 0.29

Data are shown as n (%)

Table 4 depicted that the manifestation of cough (25.2%) and weight loss (94.5%) was relatively more common in TBLN cases as compared with non-TBLN cases. Night sweating was observed as frequently in non -TBLN patients (83.3%) as in TBLN patients (78.3%). Lymph node scars were also observed in 56.5% and in 46.7% of TBLN and non -TBLN patients, respectively.

Among the lymph nodes involved in TBLN cases, cervical lymph nodes 75/ 115 (65.2%) were the most affected followed by inguinal 22/115(19.1%), axillary 14/115 (12.2%) and other lymph nodes constitute 4 (3.5%).

The minimum duration of enlarged lymph node was one month for both TBLN and non - TBLN cases, while the maximum duration was 240 months and 120 months for non -TBLN and TBLN cases, respectively.

The mean and median duration of enlarged lymph nodes was 36 months and 20 months in TBLN cases respectively. The duration of enlarged lymph nodes was 22 months for 64 of 115

(55.7%) TBLN cases. The minimum lymph node size was one cm for both non - TBLN and TBLN cases, while the maximum size was 4cm and 10 cm for non - TBLN and TBLN cases respectively. The mean and median lymph node size was 2.9 cm (±1.2 cm) and 3.5 cm respectively for TBLN cases.

Table 5. Comparisons of AFB smear, cytology, culture and PCR in the diagnosis of TBLN in 145 clinically suspected TBLN cases in Dera Woreda, North Showa, Ethiopia, August 2004 to February 2005.

Diagnostic technique	Positive (%)	Negative (%)
AFB smear	16 (11)	129 (89)
Cytology	58 (40)	87 (60)
Culture	66 (45.5)	79 (54.5)
PCR	108 (75)	37 (25)
AFB + Cytology	63 (43)	82 (57)
Culture + AFB	66 (45.5)	79 (54.5)
AFB + PCR	108 (75)	37 (25)
Cytology + Culture	83 (57.2)	62 (42.8)
Cytology + PCR	115 (79.3)	30 (20.7)
Culture + PCR	108 (75)	37 (25)

As shown in Table 5, from the total of 145 patients who participated in this study, 58(40%) had cytology suggestive of TBLN and 16 (11%) were positive by AFB smear examination. Out of 16 smear positive cases, 11(68.8%) had a positive cytology and the diagnosis of the rest 5 (31.2%) was confirmed by a positive result of AFB smear examination alone. On the other hand, out of 58 cytological findings, 11(19%) of them were positive for AFB smear examination and the diagnosis of the remaining 47 (81%) cases was confirmed by cytology results alone.

The results of this study also indicated that 66 (45.5%) of the samples demonstrated growth on LJ media, whereas 79 (54.5%) of the samples were culture negative. Among the 66 culture positive samples, 42 (63.6%) grew on glycerol containing media, 20 (30.3 %) on both glycerol and pyruvate containing media and the remainder 4 (6.1 %) grew on pyruvate

containing media. Out of 66 culture positive cases, 41(62.1%) of them were suggestive for cytology and the diagnosis of 25 (37.9%) cases was confirmed by a culture result alone. In this study, all AFB smear positive samples were also culture positive.

Combined results of cytology and AFB smear examination revealed that from a total of 145 patients, 63 (43%) were confirmed as cases of TBLN. Our combined result of cytology and culture in the detection of TBLN cases was 83 (57.2%) which was greater than the combined results of cytology and AFB smear examination (43%).

Out of 145 patients, TBLN was detected by PCR in 108 (75%) of the cases. Among the 108 PCR detected TBLN patients, *M. tuberculosis* and *M. bovis* DNA were diagnosed in 107 (99.1%) and 1(0.9%) of the cases respectively. To run the PCR assay, each FNA sample was tested in duplicate with the original extract and a 1: 10 dilution in order to avoid false negative results due to the presence of inhibitors (blood, detergents).

As shown in Figure 1, PCR products of FNA samples containing *M. tuberculosis* complex DNA would be differentiated by PCR assay with IS5/ IS6 complex specific primers yielding a product of 980 base pairs.

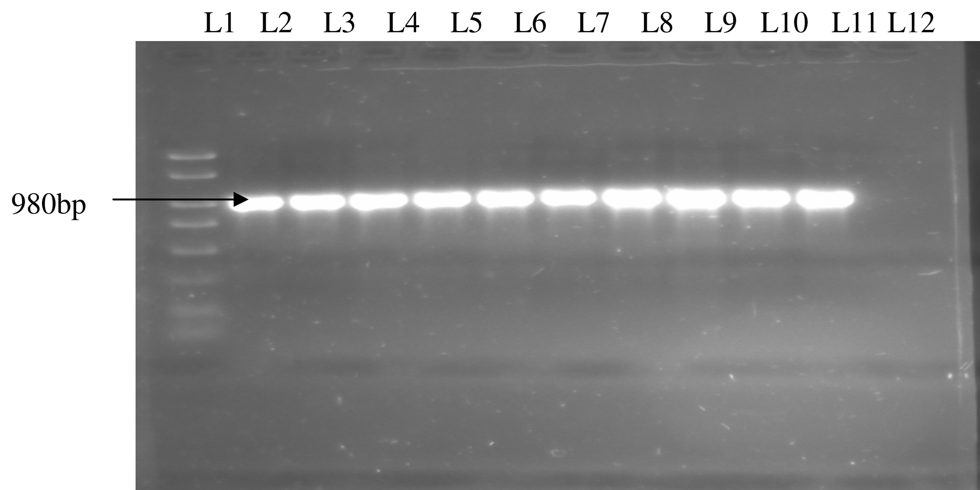


Figure 1. Electrophoretic pattern of the amplified IS6110 element PCR products from clinical samples in 1.5% agarosegel. Lane 1, 1.5Kb DNA ladder (molecular weight marker), lanes 2 to 10 clinical samples contained DNA from mycobacteria within *M. tuberculosis* complex. Lane 11and lane 12 are positive and negative controls, respectively.

As depicted in Figure 2, PCR products of fine needle aspirate samples containing *M. tuberculosis* and *M. bovis* would be differentiated by PCR assay with *pncA* allelic specific primers (variation at position 169) *pncA*MT-2 (specific for *M. tuberculosis) and pncA*MB -2 (specific for *M. bovis*).

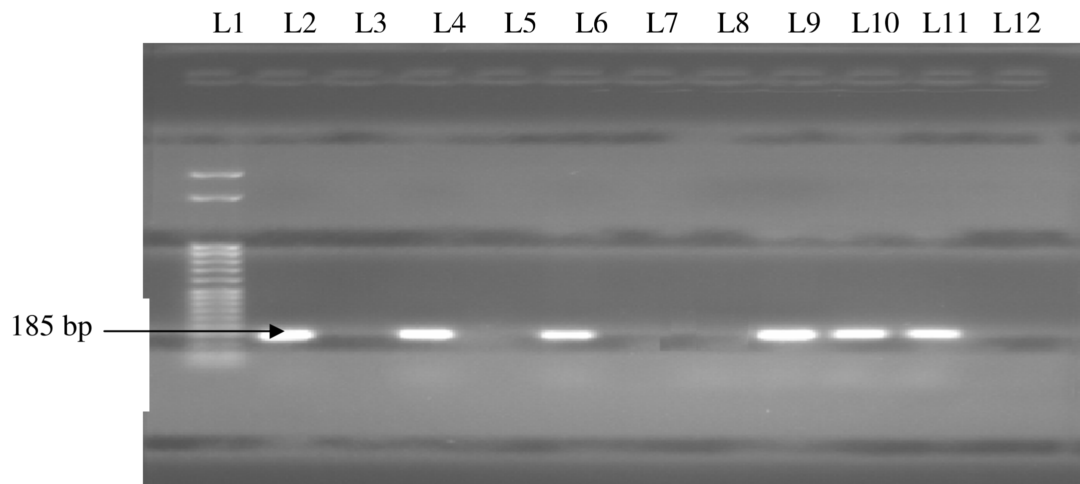


Figure 2. Electrophoretic pattern of the amplified *pnc*A genes (185 bp) PCR products from clinical samples in 2% agarose gel. Lane 1, molecular weight marker, lanes 2 to 7 clinical samples positive for *M. tuberculosis* and negative for *M. bovis,* L8 and L9 clinical sample positive for *M. bovis* and negative for *M. tuberculosis.* Lanes 10 and 11 are positive controls (*M. tuberculosis* and *M. bovis*) respectively. Lane 12 is a negative control with sterile distilled water alone.

The findings of the present study revealed that all culture and AFB smear positive samples were positive for PCR assay and 42/79 (53.2%) of culture negative samples gave a signal for PCR. Out of 58 FNA samples that were suggestive for FNA cytology, 51 (88%) of them were PCR positive and the rest 7 (12%) were diagnosed by cytology. In this study, clinical criteria and any specimen or patients positive for culture plus cytology were considered as true TBLN cases. In summary, the positivity of the various methods used in this study when applied separately to detect TBLN from FNA samples was 75% for PCR, 45.5% for culture, 40% for cytology and 11% for AFB smear examination.

Chapter IV : Discussion

Extra pulmonary tuberculosis is a very challenging clinical problem for diagnosis.
Tuberculous lymphadenitis comprises the majority of the manifestations and is relatively easier to suspect because of the visible clinical pictures. Nevertheless, the differential diagnosis of TBLN is multiple and laboratory confirmation is paramount importance. In a significant proportion of clinical samples, low numbers of the bacilli in the extra pulmonary specimens and their slow growth rate reduce the sensitivity of the conventional diagnostic methods (Kurabachew *et al*., 2004). The existing limitations of the conventional methods have contributed to the difficulties in managing patients with EPTB.

Researchers have demonstrated that a PCR method targeting the IS6110 insertion sequence is a highly sensitive and specific means of diagnosing TB (Bhattacharya et *al*., 2003). In classical human *M. tuberculosis* variants, IS6110 element is usually present in 8 to 20 copies, while in *M. bovis* BCG vaccine strains; this element is present in one copy (Van Soolingen *et al.,* 1991). *M. bovis* strains in general show low copy numbers, in the range of one to five copies (Szewzyk *et al.,* 1995). The 100% specificity of the PCR method, which amplifies specific nucleotides of the IS6110 genetic element, strongly justifies the use of selected IS6110 regions as highly specific targets for detecting TB. Hellyer *et al* (1996) reported that there was no cross reaction with 27 non-tuberculous strains using IS5/IS6 primers designed to amplify IS6110 elements.

In our present study, the peak age for TBLN was observed between 15 to 24 year age group and females were more affected than males. This result was in agreement with studies that reported increased TBLN infection in children and young adults with female predilection (Abba *et al.,* 2002; Sharma and Mohan, 2004). This is due to the fact that TBLN is a relatively early phenomenon following a primary TB infection and therefore usually seen in younger individuals than their elders because of weak immune response towards TB infection in younger individuals. In this study, the prevalence of TBLN is more frequent in women than their male counter parts which might possibly be related to the health care seeking behavior of women and / or because women are more sensitive to cosmetic problems than males.

In this study, cervical lymph nodes (65.2%) were the most affected in TBLN confirmed cases. Different studies have also reported comparable findings. A study reported from Kocaeli, Turkey, showed that out of 358 clinical TBLN cases, 92 (26%) had cervical lymph node tuberculosis (Ilgazi *et al.*, 2004). A retrospective study on FNA from San Francisco done on 47 TBLN patients confirmed cervical involvement in 29(62%) of the patients (Brainard, 2001). A study conducted by Yassin *et al* (2003) in Butajira, Ethiopia, revealed that 88 of 105 TBLN cases (82.2%) had cervical lymphadenopathy. In our present study, the rate of HIV co-infection among our study participants is not known. Considering the spread of HIV epidemic into rural areas, it is very crucial to study the association between TBLN and HIV infection in the study area.

In a majority of TBLN patients, weight loss (94.5%) and cough (25.2%) were more commonly observed as compared to non -TBLN patients. This might suggest that further studies should be conducted in Dera in order to assess the role of HIV in TBLN infection. Among the study participants diagnosed with TBLN using both clinical and laboratory parameters, 64 of 115 (55.7%) had enlarged lymph nodes of 22 months duration. The results of our present findings were higher than in previous reports (Yassin *et al.,* 2003, Bem, 1997). This might be linked to lack of access to health services, economical factors, and absence of cytology or histology services in the study area, which can create problem to implement the diagnostic algorithm of the National Tuberculosis Control Programme in the management of enlarged lymph nodes (Appendix I).

Statistical analysis of socio - demographic variables such as sex, geographical location, occupation and ethnicity showed absence of association with TBLN infection. The results of our present study was not far from the findings of similar studies done in developing countries (Bem *et al.,* 1996). It was indicated that 77.4% of TBLN cases had history of ingestion of raw milk, which was statistically associated with TBLN ($p = 0.04$). This study was consistent with previous studies that demonstrated an association between consumption of raw milk and risk of TBLN (Kazwala 2001; Skuce *et al.,* 2003). In our study, even though a large number of TBLN cases had a history of consumption of raw milk, the proportion of *M. bovis* detected in these patients was very small (0.9%) as compared to *M. tuberculosis* (99.1%).

This might be related to a possibly low prevalence of bovine tuberculosis in their cattle, which should be further explored. In addition to this, the laboratory technique of characterization and screening with primers targeting the deleted regions of *M. bovis* could be employed to confirm the PCR results.

Among 115 TBLN cases, almost half of them (56.5%) had lymph node scars and there was association between the presence of scar and TBLN infection (p = 0.03). Yassin *et al* (2003) reported a scar rate of 34.6% for Butajira patients with TBLN. This may suggest that among patients with lymhadenitis; those who have scars may probably be more likely to have TBLN. However, causes of scaring other than the natural course of the disease should be ruled out (example, herbal drugs and traditional practices).

In our study, two sets of primers were used to identify the etiological agents at the complex level targeting the IS6110 insertion sequence and at species level targeting the pncA gene and its allelic variation. Among 145 TBLN cases, 108 (75%) were positive by PCR at the complex levels. Our results were in agreement with a study which confirmed that 83% detection was achieved for lymph node aspirates from 23 patients for whom the cytological diagnosis was consistent with that of TBLN, based on the amplification of regions in the IS6110 insertion sequence which is present in most strains of *M. tuberculosis* (van Soolingen, 2001).

Another study conducted on TBLN patients demonstrated that 55% of the tested patients were PCR positive, based on the amplification of the devR gene (a single copy gene specific for organisms belonging to *M. tuberculosis* comple*x)* (Singh *et al*., 2000). A study conducted in Butajira, rural Ethiopia, by Kidane *et al* (2002) showed that *M. tuberculosis* complex was detected in FNAs from 35(87.5%) patients with clinical TBLN by PCR technique. Our present findings are in agreement with the aforementioned studies.

In recent years, several genes and insertion sequences have been targeted in an attempt to develop PCR assays to differentiate among mycobacteria in general and *M. tuberculosis* complex in particular (Bhattacharya *et al.,* 2003).

Single nucleotide changes in the *pnc*A gene (Espinosa *et al.,* 1998) have been exploited in the differentiation between *M. tuberculosis* and *M. bovis.*

Based on PCR for detection of allelic variation at position 169, 107 (99.1%) of the 108 were positive for *M. tuberculosis* and 1 (0.9%) was positive for *M. bovis.* The result of our findings was consistent with the results of a study conducted in India on 250 TBLN patients where it was reported that mycobacteria were isolated in 130 cases, of which 125 (96.6%) were identified as *M. tuberculosis* from FNA samples (Nataraj *et al*., 2002).

A study conducted in Butajira, rural Ethiopia, reported that among 35 PCR positive cases of tuberculous lymphadenitis, 29(82.9%) and 6 (17.1%) were positive for *M. tuberculosis* and *M. bovis* respectively (Kidane *et al*., 2002). The present study showed that PCR is a sensitive technique for the diagnosis of the etiological agents of TBLN at the complex and species level, provided that appropriate optimization conditions are made and if contamination and substances that can inhibit the amplification of PCR are avoided and/ or minimized. Our study revealed that *M. tuberculosis* was found to be the causative agent of TBLN in more than 99% of suspected patients in Dera.

In our present study, there was a relatively lower proportion of *M. bovis* in Dera compared to Butajira. This may be associated with differences in socio - economic factors, geographical location, culture, dietary and farming practices between the two communities, which need to be further, studied. Butajira farmers usually rely on enset for their livelihood whereas Dera farmers subsist on grains such as teff, maize and sorghum. Both communities rear cattle and small animals and consume raw milk as well as fresh milk products. The prevalence of *M. bovis* infection in their herds needs to be assessed to identify which of the above-mentioned factors are closely linked to the transmission of *M. bovis* into humans in these two different foci of tuberculous lymphadenitis.

Previous studies have suggested that PCR assays are sensitive for the diagnosis of smear and culture positive TBLN patients (Nataraj *et al.,* 2002). In our study, the positivity of PCR was

100% in specimens positive by both AFB smear and culture and 53.2% in culture negative specimens with an overall positivity of 75%. Our result was comparable with the study reported from India which indicated 100% PCR positivity in AFB smear and culture positive specimens and 51.8% in culture negative specimens with an over all PCR positivity of 69.4% (Banavaeliker, *et al*., 1998). Studies that have examined culture positivity have shown a range of 22 to 50% smear to culture correlation indicating low sensitivity of smears with respect to cultures (Daniel, 1987; Bekedam *et al*., 1997).

The over all positivity of our PCR for detecting TBLN (75%) greatly exceeded the combined positivity of culture and AFB smear (45.5%) and that of cytology (40%). In this study, we confirmed that PCR is a useful methodology for the diagnosis of TBLN. A meta analysis of papers on *M. tuberculosis* PCR has shown a sensitivity of less than 50% when PCR is performed on smear negative pulmonary specimens (Bouakline *et al*., 2003) correlating with a smaller number of tubercle bacilli. In specific studies of pulmonary and / or extra pulmonary specimens, the sensitivities ranged between 20 and 94% for PCR assays of extra pulmonary specimens (Bouakline *et al.,* 2003, Singh *et al.,* 2000). The discrepancies observed in these studies could be explained by the different degree of prevalence of TB in the study population, the number of smear positive samples, methods used in DNA extraction and the type of specimens used in the study.

In this study, cytology supported the diagnosis of TBLN in 58/145 (40%) of all confirmed cases and 16 (11%) were positive by AFB smear examination. Our result was comparable with pervious studies that reported a positivity of FNA cytology of 58% (Singh *et al.*, 2000), 8% positivity of AFB smear results (Bekedam *et al*., 1997) and 11% positivity of AFB smear was also reported in TBLN patients from Zambia (Bem *et al.,* 1996).

Another study depicted that AFB positivity is low in epithelioid granuloma without necrosis (5.8- 30%) but significantly higher in epithelioid granuloma with necrosis (32 - 65%) (Rajwanshi, *et al*., 1987). This showed that smear positivity could be affected by stage of the disease, nature of FNA sample and quality of smear.

Out of 66 culture positive, 41(62.1%) were suggestive in cytology. More importantly, in 25 cytology non - suggestive cases, culture was positive emphasizing the need for incorporating culture as a routine laboratory investigation in all clinically suspected TBLN cases. Our results indicated that the addition of a more sensitive technique like PCR would have helped strengthen the detection of TBLN in culture negative cases. From 66 culture positive isolates, 65 (98.5%) of the cultures were identified by PCR as harboring *M. tuberculosis* and 1(1.5%) *M. bovis*.

On the other hand, 42 (53.2%) culture negative and smear negative samples were also detected by PCR assays. This indicated that by using combined conventional methods, cytology and PCR; it is possible to enhance the detection rate and characterization of the etiological agents of TBLN infection. Possible explanation for the success of the PCR assay relative to the conventional methods is that the PCR assay is more sensitive than the conventional methods, particularly when low numbers of bacilli are present.

Conclusions and Recommendations

Extra pulmonary tuberculosis remains a significant fraction of the total TB cases in developing countries. Tuberculous lymphadenitis (TBLN) is a diagnostic challenge in sub Saharan Africa, including Ethiopia, where there is a high rate of HIV infection. Due to the increase in HIV infection and its impacts on the pattern of TB transmission, identification of the etiological agent(s) of TBLN in patients is of paramount importance for management of the disease. Co-infection with HIV may influence several clinical and laboratory findings among patients with TBLN infection. Understanding the changes in the epidemiology of EPTB / TBLN will help in making a prompt diagnosis, leading to appropriate therapy. Among the different diagnostic techniques used in the present study, PCR was the most powerful in the detection of TBLN cases. Among 108 PCR positive cases of TBLN from Dera, North Showa, Ethiopia, and 99.1% were caused by *M. tuberculosis* and 0.9% was caused by *M. bovis.* The burden of TBLN infection among the study participants appears to be high enough to warrant special attention.

On the basis of the above conclusions, the following recommendations were made:

1. Patients suspected for tuberculous lymphadenitis in rural areas should under go fine needle aspiration to rule out TBLN infection.
2. In regions with limited resources, introduction of easy, cost effective and time saving diagnostic methods is extremely important.
3. Due attention should be given to strengthening of district and / or rural laboratory services both in capacity building and through provision of the necessary laboratory supplies/ equipment.
4. Research issues such as the role of HIV in TBLN infection and typing of lung and lymph node strains are proposed.
5. It is believed that the results of the present study would help other researchers as base line data for further studies.

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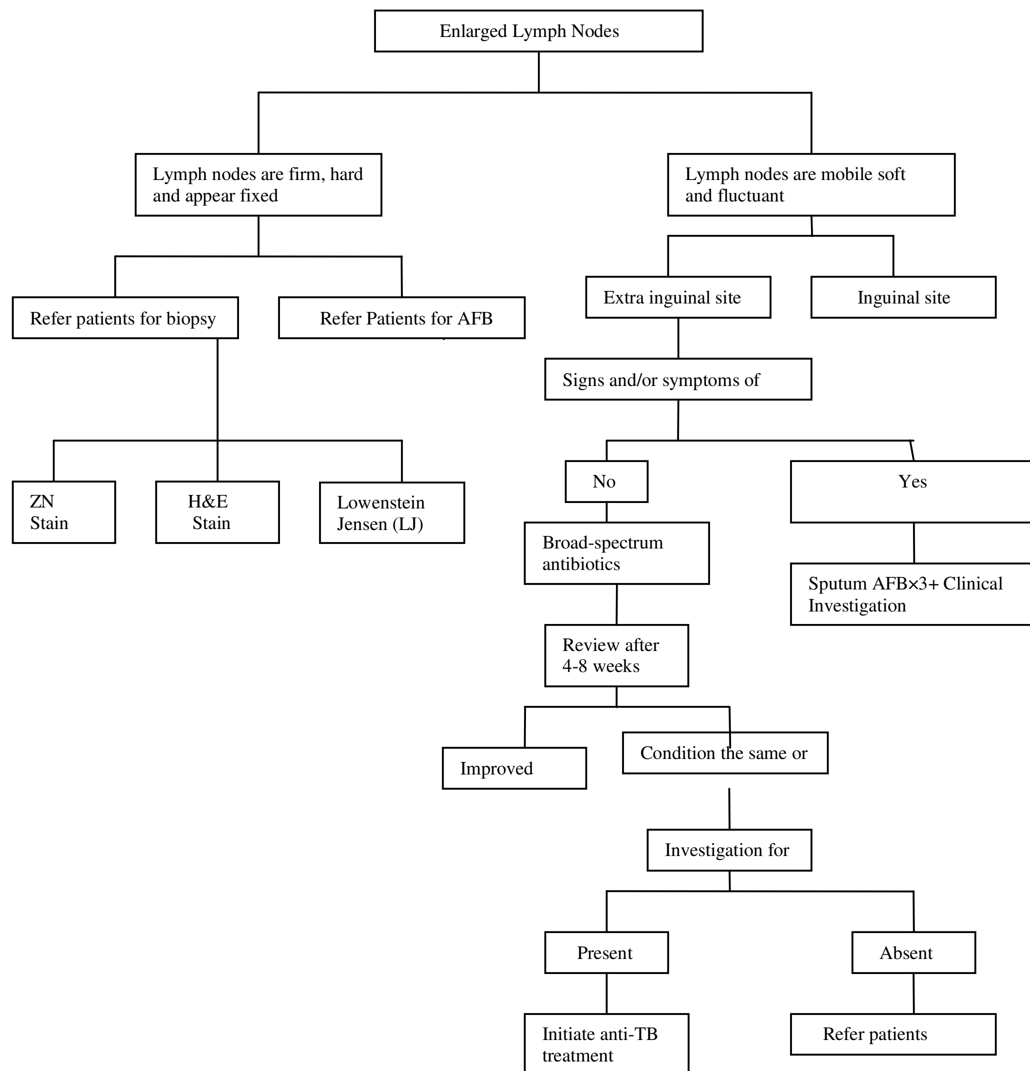
Appendix I. Management of enlarged lymph nodes (Algorithm)



Source: ***MOH, Manual of TLCP, 2002***

Appendix II. Case Record Form (CRF)

Case record form to be filled for investigation of tuberculous lymphadenitis in study participants clinically suspected for tuberculous lymphadenitis at Derra Woreda, North Showa, and Ethiopia. Put **x** where it is appropriate.

I. Patient identification

1. Patient identification number ________________
2. Full name ________________
3. Health Center card number ________________
4. Sex 1. Male ☐ 2. Female ☐
5. Age (Years) ________________
6. Address: (Woreda / Kebele) ________________
7. Occupation ________________
8. Ethnicity ________________
9. Geographical location 1. High land ☐ 2.Lowland ☐

II. Clinical data

1. Type of specimen ________________
   1.1 Who will collect the sample? ________________
   1.2 Is the sample obtained? 1. Yes ☐ 2. No ☐
   1.3 If no, why? ____________
2. Date of collection ____________
3. Time of collection ____________
4. Night sweating 1. Yes ☐ 2. No ☐
5. Weight loss: 1. Yes ☐ 2. No ☐
   Weight at the present moment __________
6. Cough 1. Yes ☐ 2. No ☐
   If yes, how long? _________
7. Lymph node

7.1 Site __________

7.2 Size (cm) __________

7.3 Duration (Months) __________

7.4 Scar 1. Present ☐ 2. Absent ☐

7.5 Other findings ____________

8. Have you ever had pulmonary tuberculosis before?

1. Yes ☐ 2. No ☐

9. Have you ever been treated for pulmonary tuberculosis before?

1. Yes ☐ 2. No ☐

If yes, for how long? _________

10. Did you take antibiotics within 2 weeks ago ?

1. Yes ☐ 2. No ☐

11. History of ingestion of raw milk

1. Yes ☐ 2. No ☐

Appendix III. Consent Form (English version)

Name ____________________

Serial number ____________________

Card number _____________________

The researcher has informed me well that I might have developed a form of tuberculosis, which is caused by bacteria known as *Mycobacterium tuberculosis* or *Mycobacterium bovis*. I have been appointed for an investigation that involves collection of fine needle aspirates (FNA) from my swelling in the neck or from other part of my body. The collected specimen will be processed in order to identify my disease problem. In connection to this, I have also been explained about the procedure of specimen collection, which is performed using needle and syringe. Moreover, I have been told that this procedure is practiced routinely and does not cause serious problems for me except minor bleeding which will be taken care of by the handling physician. I have also been requested to permit the use of the specimen for research so as to isolate, identify and type the etiological agents of the disease. Apart from this, I have been informed that the specimen is used only for this research purpose and I benefit from the free diagnosis and appropriate treatment will be provided to me for tuberculosis if it is confirmed through the diagnosis of the specimen. In addition to this, I have been informed that laboratory results and all information to be written on the case record form will be kept confidential and also the nature of the record form is private. The researcher has also explained to me that participation in the study is on voluntary basis and refusal to participate involves no penalty and I can withdraw my consent at any time without any prejudice to my case. I have been allowed to ask questions and have received clarification in a language I understand to my satisfaction. Finally, I confirm my agreement to participate in the study with my signature below.

Signature ---------------------------------

Date ----------------------------------

Appendix IV. Patient Information Sheet (English version)

Name of principal investigator Berhanu Seyoum
Name of the organization Armauer Hansen Research Institute (AHRI)
Name of the sponsor Armauer Hansen Research Institute

Title of the project:

Characterization of Mycobacterial Isolates from Lymph Nodes of Patients with Tuberculous Lymphadenitis in Derra Woreda, North Showa, Ethiopia.

1. Aim of the proposed study

Tuberculosis is an infectious disease that remains a major global public health problem. According to recent estimates of WHO (2004) about one third of the world is population is infected with tuberculosis. According to the report, there are more than 8 million new cases of tuberculosis and greater than 2 million people died of tuberculosis each year. Tuberculosis is a major cause of mortality in developing countries, where about 95% of tuberculosis cases occur. Ethiopia is among the 22 tuberculosis burden countries and ranks seventh (WHO, 2004). Therefore, the increase in tuberculosis favors for rising of extra pulmonary tuberculosis particularly lymph node tuberculosis. Hence, this study is aimed at isolating and identifying the species of mycobacteria that are responsible for tuberculous lymphadenitis from FNA specimens of patients clinically suspected for tuberculous lymphadenitis from Derra, rural Ethiopia.

2. Procedures

In order to achieve the designed objectives, fine needle aspirates will be taken from the study subjects using needle and syringe.

3. Risks and discomforts

Occasionally a small bleeding and some discomforts may occur during collection of specimen, which can be taken care of by the clinician.

4. Role of the study participants

Study participants who will fulfill the eligibility criteria are expected to give specimen (fine needle aspirates).

5. Rights of study participants

Participation in the study is on voluntary basis and participants have the right to refuse to participate in the study and with draw their consent at any time with out any prejudice to their case.

6. Benefits

- Patients will get diagnosis free of charge.
- Patients will get free treatment for other complications that will occur during the study period.
- continued interaction with tuberculosis control programme and with other units of Derra Woreda Health Team will be established to improve tuberculosis control programme.

7. Incentive

In order to take part in this study, you will not be provided any special incentive.

8. Consent

Participants will give their consent on voluntary basis.

9. Whom to contact

If you are interested to obtain further information, you are kindly requested to contact the following individuals.

About AHRI / ALERT ethical clearance, contact the chairperson Dr. Howard Engers, AHRI, Tel. No: 2113 34, Addis Ababa.

About the National Ethical Clearance Committee, contact Dr. Yemane Tekelai, secretary of the committee, Tel. No: 514447, Addis Ababa.

About the conduct of the study, contact the following individuals:

The principal investigator: Berhanu Seyoum Tel. No: (09) 677774
AHRI, Tel. No: 2113 34, Addis Ababa.

The project coordinator :

Dr. Abraham Aseffa, AHRI, Addis Ababa. Tel. No: 2113 34

Dr. Yimtubezenash WeldeAmanuel A.A.U, MF. Tel. No: 528726, Addis Ababa.