

**Dilemmas in the Diagnosis of Lymph Node Enlargement in
Ethiopia: A Study from Four Sites with High
Notification of Lymph Node Tuberculosis**

**By
Rahel Iwnetu**

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**Addis Ababa University
Graduate Program
Department of Medical Microbiology, Immunology and Parasitology**

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ABSTRACT

The proportion of extra-pulmonary tuberculosis has risen to over a third of the cases of tuberculosis in Ethiopia. The majority of this form of tuberculosis is reported as lymph node tuberculosis (LNTB). We evaluated the national algorithm currently in use for the diagnosis of lymph node enlargement in Ethiopia. One hundred and fifty suspected cases of lymph node tuberculosis, aged 5-65 years, with extra-inguinal lymphadenopathy were recruited following the national algorithm, from October 2004 to August 2005. Out of these individuals, 117 (78%) were diagnosed as LNTB on positive culture of fine needle (FNA) and/or biopsy specimens. FNA cytology (FNAC) and histopathology detected 88 (75%) and 105 (97%) of the culture proven LNTB patients, respectively. Eighty percent of DNA extracted from biopsy tissues gave signal for *M. tuberculosis*. All of the strains isolated from culture were identified as *M. tuberculosis*. Ziehl-Neelsen (ZN) staining of FNA and biopsy smears detected acid fast bacilli (AFB) in 28% and 25% of the patients, respectively. Macroscopic caseation in excised lymph node and aspirated material was found to be comparable with 79 (68%) of the nodes and 78 (67%) of the fine needle aspirates demonstrating caseation. Statistically significant association was found between the presence of caseation and the diagnosis of LNTB in both types of specimens ($p=0.002$). Combination of FNAC, ZN staining of FNA materials and macroscopic examination of aspirates detected 112 (96%) of the culture proven LNTB patients. The proportion of HIV seropositive individuals among the culture proven LNTB patients were 24%. There was statistically significant association between urban residence ($p=0.002$), female gender ($p=0.006$) and HIV in LNTB patients. There was no statistically significant association between LNTB and HIV. We found that the currently used algorithm detects a significant amount of culture proven LNTB patients even though it suffers from shortcomings. It is hoped that this will encourage wider consultation to review and revise the existing algorithm and improve LNTB diagnosis and reporting nationwide.

Part I

1. Background and rationale for the proposed study

1.1 Introduction

Tuberculosis (TB) is a leading cause of death worldwide, with about 98% of the 3 million TB deaths annually occurring in the poorer developing countries. With accelerating HIV pandemics and development of drug-resistance, TB was declared as a global emergency by the WHO in 1993 (WHO report, 1994). Sub-Saharan Africa has the highest prevalence of tuberculosis, and case-notification rates and treatment outcomes have not been satisfactory. Recently national TB control programs are expanding Directly Observed Treatment Short course (DOTS) coverage but still not to the levels needed to effectively control the disease (WHO report, 1999). Ethiopia ranks seventh among the 22 high burden countries which also includes Nigeria, South Africa, Mozambique, Kenya, Uganda and Zimbabwe in sub-Saharan Africa (WHO report, 2005). The 22 high burden countries account for approximately 80% of the estimated number of new TB cases (all forms) arising worldwide each year (WHO report, 2005). The largest number of cases occurs in the South-East Asia Region, which accounts for 33% of incident cases globally (WHO report, 2003). However, the estimated incidence per capita in sub-Saharan Africa is nearly twice that of South-East Asia, at 350 cases per 100,000 population.

A substantial rise in the number of TB cases reported from sub-Saharan Africa has been observed following the expanding HIV epidemic. The clinical pattern of TB has shown a remarkable change due to co-infection with HIV. More cases of extra-pulmonary TB (EPTB) are reported among HIV infected individuals but the exact mechanisms whereby HIV changes the clinical forms of TB are not known. It is also not clear as to what exactly causes the increase in extra-pulmonary forms; i.e., whether that is related to problems pertaining to sputum examination or true changes in epidemiological trends. Nonetheless, TB control programs still concentrate their efforts on smear positive pulmonary TB cases due to operational reasons. It has become increasingly obvious that this can no longer be the only approach to reduce the infection and death toll from TB.

The rate of EPTB cases is increasing, the commonest being lymphadenitis. However, studies on lymphadenitis are scanty worldwide. A clear mapping of the TB situation in sub-Saharan Africa, and improving the effectiveness of intervention strategies are urgently needed. The proportion of EPTB is growing progressively in sub-Saharan Africa relative to pulmonary TB cases and the increase appears to be associated with HIV infection (Bem *et al.*, 1993; Perenboom *et al.*, 1995). The etiology of lymphadenitis varies between geographic areas and time within a geographic area (Lai *et al.*, 1984).

The clinical presentation of lymphadenitis (site of lymph nodes affected and progression of disease) differs between tuberculosis and non-tuberculous mycobacteria. In developing countries, tuberculous lymphadenitis (LNTB) is believed to account for about 30-50% of all EPTB cases. Historically LNTB was caused mainly by *M. bovis* (Griffiths, 1937) but tuberculous lymphadenitis is now mostly due to *M. tuberculosis* (Cantrell *et al.*, 1975). However, literature suggests that there is a geographic and time dependent shift in the dominance of the different mycobacteria as causative agents of tuberculous lymphadenitis. There appears to be an increased risk of LNTB in certain ethnic groups, such as people of South Asian or Somali ethnic origin (van den Hombergh, 1999; Kempainen *et al.*, 2001).

1.2 The global picture of tuberculous lymphadenitis

Tuberculous lymphadenitis is the commonest form of extra-pulmonary tuberculosis (Jha *et al.*, 2001; Ilgazli *et al.*, 2004; Gonzalez *et al.*, 2003). Cervical lymph nodes are the most common lymph nodes affected by this disease – classically termed as “scrofula”. Tuberculosis that affects the cervical lymph nodes represents about 50% of extra-pulmonary tuberculosis (Fain *et al.*, 1999), though it could vary in different areas. In 1992, Khiery *et al.* drew attention to the fact that there were an increasing number of cases of lymph node tuberculosis in Khartoum. This finding was substantiated recently in a study that utilized molecular technique (PCR) for identification and characterization of the causative agent of tuberculous lymphadenitis in fine needle aspiration samples (Aljafari *et al.*, 2004). PCR allowed immediate characterization of *M. tuberculosis* in a vast majority (96%) of the cases in this study. Similar findings suggesting the presence of a

high percentage of glandular tuberculosis was indicated in a retrospective study conducted on all adult patients diagnosed in 1991 with tuberculosis in Djibouti (Rodier *et al*, 1993). In addition, a similar pattern of increment in tuberculous lymphadenitis cases has been documented especially in association with HIV infection in many African countries. A study conducted on 506 consecutive adult medical admissions to a hospital in Nairobi revealed that extra-pulmonary disease was more common in HIV seropositive than seronegative tuberculosis patients accounting for most of the excess cases of tuberculosis in seropositive patients (Gilks *et al*, 1990). Tuberculous lymphadenitis has been shown to be more common in HIV positive African patients in several studies conducted in Uganda (Nambuya *et al*, 1988), Tanzania (Perenboom *et al*, 1995), Zambia (Bem *et al*, 1997) and Malawi (Bekedam *et al*, 1997).

In the rest of the world the pattern of lymph node tuberculosis as the dominant contributor to extra-pulmonary tuberculosis has been evidenced in several publications. In eastern Australia a resurgence of extra-pulmonary tuberculosis has been documented of which lymphadenitis is the most common finding (Wark *et al*, 1998). *M. tuberculosis* amounted to 68% as a causative agent of lymph node tuberculosis in this 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 (Aggarwal *et al*, 2001). In contrast, a bacteriological survey of tuberculous lymphadenitis done in southeast England has shown a changing trend in the prevalence of pathogens over time (Yate and Grange, 1992). They concluded that the incidence of lymphadenitis due to *M. tuberculosis* is declining in contrast to environmental mycobacteria which are increasingly affecting more adults. In the assessment of lymph node tuberculosis in northern Germany, besides identifying *M. tuberculosis* as a major causative agent, the study stressed that this condition is still an important issue in developed countries and has to be considered in the differential diagnosis of lymph node enlargement (Geldmacher *et al*, 2002). In the Middle Eastern country of Saudi Arabia where the HIV seroprevalence of the population was considered to be low, this condition was also described with regard to its diagnosis and outcome of treatment (Memish *et al*, 2000). Tuberculous lymphadenitis was found to be predominantly

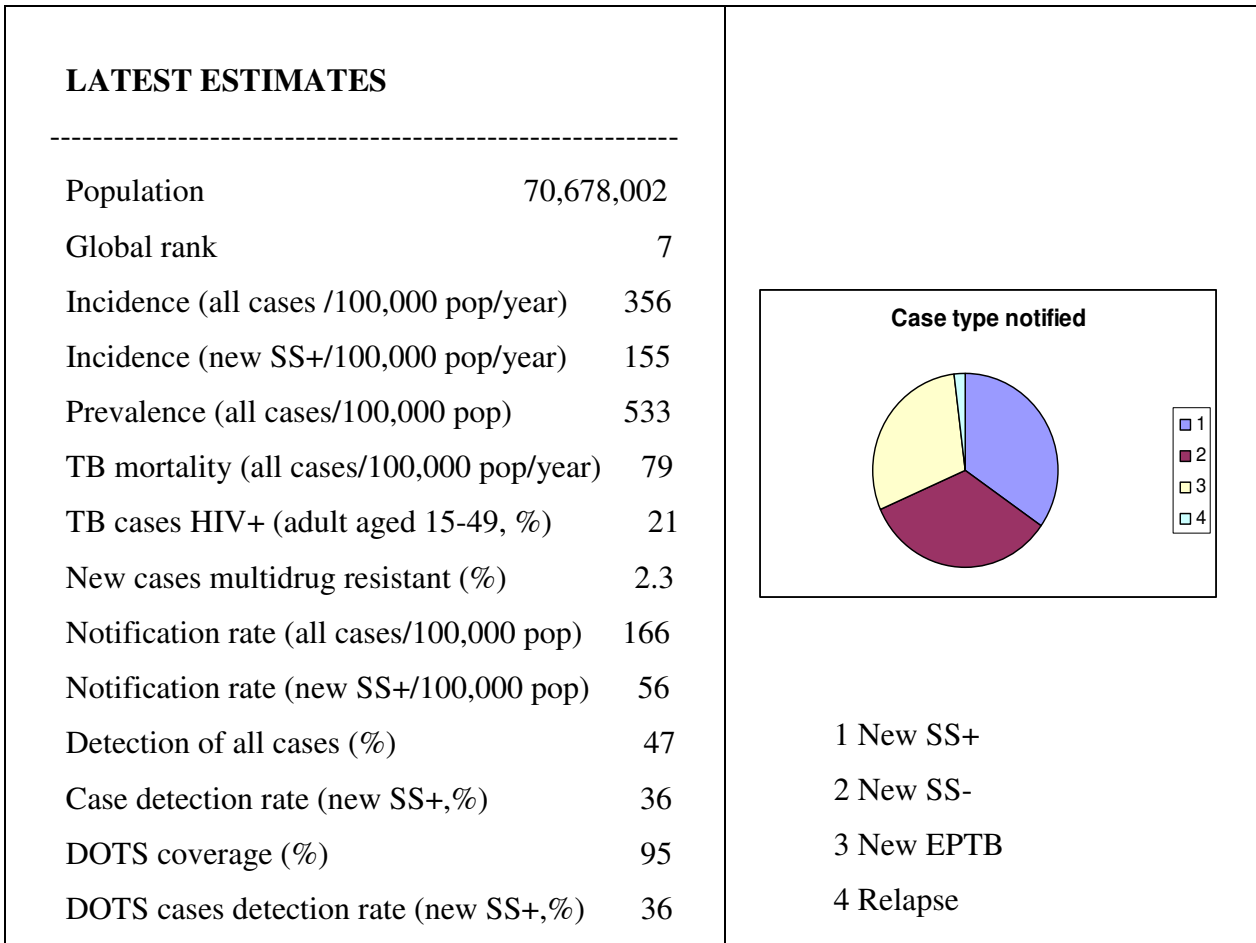
cervical in location and most patients responded well to chemotherapy. The relationship of HIV to extra-pulmonary tuberculosis has been clearly documented (Jones *et al*, 1996) but it is evident that extra-pulmonary tuberculosis especially lymph node tuberculosis does occur in the absence of HIV infection in young patients (Cowie and Sharpe, 1997) as well as in females (Gonzalez *et al*, 2003).

1.3 Tuberculous lymphadenitis in Ethiopia

Ethiopia ranks high in the top seven high burden countries in the world, and one of the top two in Africa, with regard to the number of tuberculosis patients (WHO, 2005). Over a third of the population has been exposed to tuberculosis and the annual risk of tuberculosis is estimated at 2.2%. An estimated 377,030 Ethiopians (0.6% of the population) have active TB of all kinds, with more than 120,000 new cases notified in the last year (2003/2004), nearly a third of which having smear positive TB (WHO, 2005). 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. Nearly a third of all estimated TB cases are fatal, killing over 42,000 people in Ethiopia this year, excluding those who had HIV/AIDS. The cumulative number of people living with HIV/AIDS is about 1.5 million out of which about 96,000 are children under 15 years of age (MOH 2004, AIDS in Ethiopia-Fifth Report). According to the Ministry of Health documents, of all TB case incidences in 2003, 54,000 (45%) tested seropositive for HIV. It was indicated that the proportion of TB patients with HIV in the Southern region of Ethiopia and Addis Ababa was considered to be 19% and 56% respectively. Even though the proportion of patients coinfecting often correlates with the prevalence of HIV, a recent study conducted in the Southern region has shown that there is an overall low HIV prevalence rate (11%) among patients with extra-pulmonary TB (Yassin *et al*, 2004).

Figure 1: Latest estimates of tuberculosis in Ethiopia, 2005

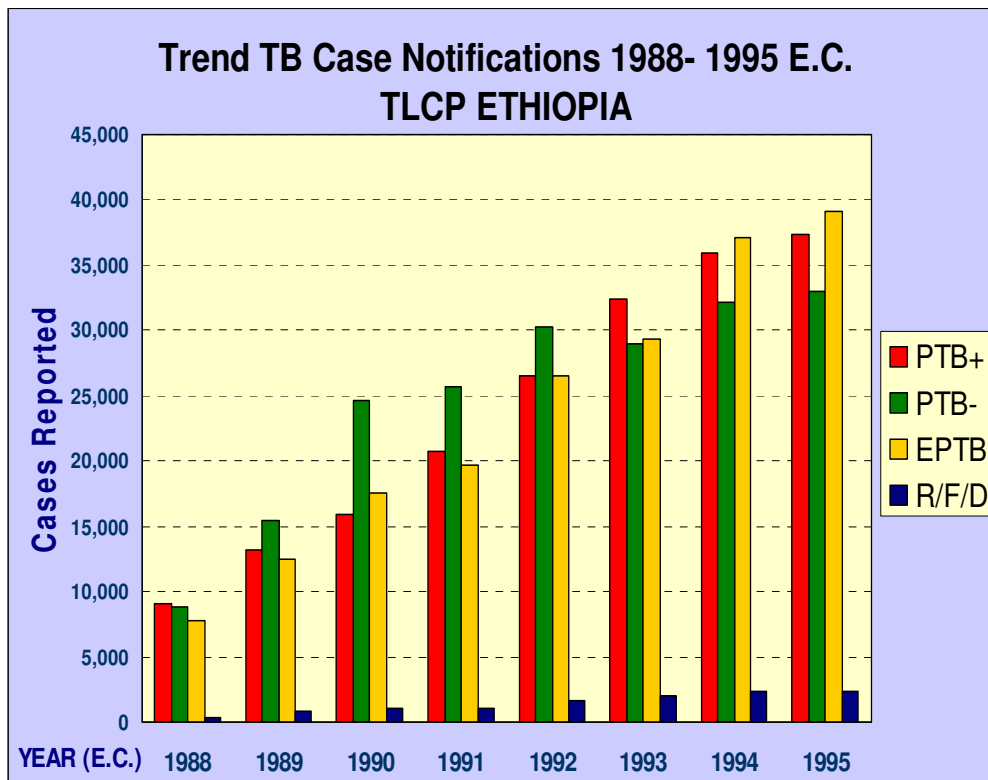
Source: WHO report (2005)



TB is one of the most important public health problems in Ethiopia, with over 121,026 new cases notified in 2004. Nationally 35% of all cases reported were extra-pulmonary (WHO report, 2005), with the largest group being LNTB. Since 2002, the proportion of EPTB has exceeded smear positive pulmonary tuberculosis for the first time. The trend of increase in EPTB cases over the years is shown in figure 1. This trend has continued similarly in 2003/04 report with a total of 41,430 (34%) smear positive cases reported in contrast to 42,477 (35%) new cases of EPTB. In some regions e.g. Tigray and Amhara, the figure rises to 42% and 39% respectively (Ministry of Health TLCP report, 2003/04). During the 2001 External Evaluation and the 2002 Joint Review of the TB and Leprosy Control Program (TLCP) of Ethiopia, it was observed that

in a number of treatment units, greater than 50% of all new cases registered were found to be EPTB cases, again with the vast majority being diagnosed as LNTB (Ministry of Health TLCP, 2001). In the majority of these cases, the cervical lymph nodes are predominantly involved. However “classic” TB epidemiology reports taught us that amongst 100 new cases, 15 will be EPTB cases, with lymph node the most common presentation of EPTB accounting for 30-40% of EPTB cases in reported series (Hooper, 1972; Styblo, 1991).

Figure 2. Trend of TB case notifications in Ethiopia, 1988-1995 E .C. (1994 - 2002 G.C.)
(TLCP, Ministry of Health, Ethiopia, 2002)



As cervical LNTB is the predominant form of EPTB reported in Ethiopia, this study proposed to concentrate on this form of EPTB only. The study made an attempt to address the following questions:

- Are the reported cervical LNTB cases true TB cases?
- If not, is there over-diagnosis of cervical LNTB cases (e.g. due to non-compliance of physicians with the diagnostic algorithm)?
- Are the physicians actually complying with the algorithm but still ending up with the diagnosis of LNTB disease? (In other words, is the algorithm sufficiently specific?)
- Could under-diagnosis of pulmonary TB play a contributing role in the apparently elevated proportion of EPTB reported in Ethiopia?
- Are the high notification rates of EPTB associated with the underlying HIV epidemic in Ethiopia?
- Are there other factors at play in Ethiopia (racial / genetic / transmission of *M. bovis*)?

Given that many health units lack appropriate diagnostic facilities, TB lymphadenitis may not be diagnosed appropriately in developing countries (Voetberg and Lucas, 1991). This has serious implications for TB control programs, since it may cause development of drug resistant strains, in addition to substantially increasing the cost of drug purchase. Over-diagnosis is certainly a possibility. A small study in Addis Ababa in the diagnostic accuracy of registered smear-negative PTB cases showed a significant proportion diagnosed after non-compliance with the diagnostic algorithm by the attending physicians (Ahmed *et al*, 1999). However, no studies have addressed this issue for LNTB cases. The HIV epidemic is associated with a marked increase in the total numbers of TB cases registered in programmes (Harries *et al*, 1995), and some reports have demonstrated a proportionally larger increase in the EPTB cases including LNTB (Drobniewski *et al*, 1995; Hopewell, 1995).

The technique of fine needle aspiration (FNA) of material from enlarged cervical lymph nodes, with examination for AFB (by ZN staining) and microscopic caseation (by H&E staining) has been advocated by some researchers as a simple, feasible field technique to enhance the accurate diagnosis of LNTB (Pithie and Chicksen, 1992; Bem *et al*, 1993). Other researchers have found that FNA had a high yield which was similar to histology and higher than that of culture on Lowenstein-Jensen medium (Bekedam *et al*, 1997). All in all, limitations in the traditional microbiological methods and the extensive potential differential diagnosis have made the detection and appropriate treatment of LNTB cases very challenging (Singh *et al*, 2000). This study has utilized all techniques reported by previous researchers and, based on its finding, has proposed a feasible diagnostic algorithm applicable for Ethiopia.

1.4 Etiologic agent

1.4.1 The Genus Mycobacterium

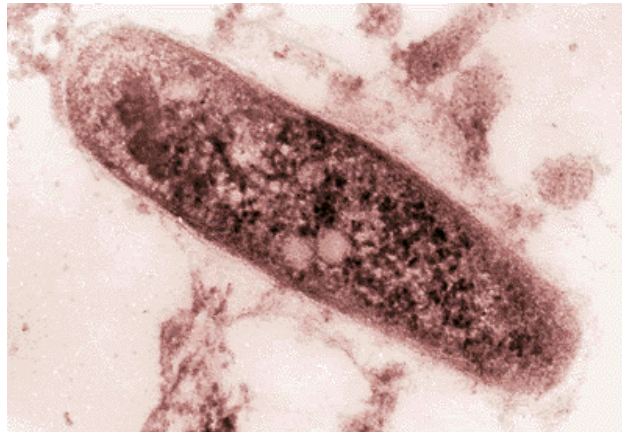


Figure 3: Electromicrograph of *Mycobacterium tuberculosis*

Source: file:///D:/Tuberculosis_files/image011.gif

Genus Mycobacterium has 95 well characterized species (Euzéby, 2003). Mycobacteria are slim, slow growing organisms that are 1-10 μ m long. The mycobacteria are rod shaped, acid fast, aerobic or micro-aerophilic, non-spore forming, non-motile, non-capsulated, lipid rich bacteria (Friden *et al.*, 2003). The Mycobacteria contain mycolic acids, and rare complex, long chain fatty acids. They have a cell envelope with a high lipid content. This accounts for the difficulty in staining them with conventional techniques.

1.4.2 The Mycobacterium Complex (MTC)

They are five closely related mycobacteria grouped in the *M. tuberculosis* complex: *M. tuberculosis*, *M. bovis*, *M. africanum*, *M. microti*, and *M. canetti* (van Soolingen *et al.*, 1997; van Soolingen *et al.*, 1998). The MTC are characteristically 99.9% similar at the nucleotide level, with identical 16S rRNA sequences (Brosch *et al.*, 2002). However, there are distinct phenotypic differences between the subspecies and not least their host range and pathogenicity (Collins *et*

al, 1983). The natural hosts of *M. tuberculosis* and *M. africanum* are humans, whereas *M. bovis* can cause disease in a wide range of domestic or wild animals like deer, badger, as well as in humans (Wayne and Kubic, 1986). According to their biochemical characteristics, two major subgroups of *M. africanum*, corresponding to their geographic origin in West (subtype I) or East (subtype II) Africa, have been described. Numerical analyses of biochemical characteristics have revealed that *M. africanum* subtype I is more closely related to *M. bovis*, whereas subtype II more closely resembles *M. tuberculosis* (David *et al*, 1978). *M. microti* has been reported to infect small rodents such as voles and more recently also humans (van Soolingen *et al*, 1998). *M. canetti* has been described as a novel pathogenic taxon of the MTC, and rare cases have been reported in patients living mainly in Africa (van Soolingen *et al*, 1997).

In a study conducted in the Sudan, *M. tuberculosis* was the causative agent in the vast majority of cases (96%) of mycobacterial lymphadenitis (Kheiry and Ahmed, 1992). While *M. tuberculosis* is the principal cause of mycobacterial lymphadenitis in Sudan, in Europe and the USA, the disease is frequently caused by *M. avium-intracellulare* in children (Falworth and Simpson, 1990). Information from Africa on *M. bovis* causing LNTB is scarce. In Tanzania *M. bovis* was isolated from four of 17 biopsies of clinically suspected LNTB (Daborn *et al*, 1996) and recently reports from other African countries indicate transmission of *M. bovis* causing clinical disease in humans (Cosivi *et al*, 1998). There is no sufficient data to draw upon to assess whether *M. bovis* is playing a role in Ethiopia today amongst LNTB cases. However, from veterinary sources in Ethiopia, bovine TB among cattle and dairy products appears to be prevalent in various settings (Ameni *et al*, 2002; Assegid *et al*, 2000). Recently Kidane *et al* identified 17% *M. bovis* from 40 sequential cases with clinically diagnosed LNTB using PCR techniques (Kidane *et al*, 2002; Yassin *et al*, 2003). However, this study was not supported with culture results.

1.5 Transmission

Mycobacterium tuberculosis is transmitted through the airborne route and there are no known animal reservoirs (American Thoracic Society, 2000). Droplet nuclei are produced when persons with pulmonary or laryngeal tuberculosis cough, sneeze, speak, or sing (American Thoracic Society, 2000). Only the droplet nuclei in the size range 1-5µm reach the terminal air spaces or alveoli; each contains only 1-3 bacilli (Long and Jessamine, 2000). Among patients with tuberculosis of the respiratory tract not all are equally efficient at transmission (Long and Jessamine, 2000). Patients whose sputum smears are positive for acid fast bacilli on smear have 5,000 or more organisms per millilitre of sputum (Yeager *et al*, 1967) and infect many of their close contacts, whereas those who are smear negative and culture positive infect far fewer contacts (Behr *et al*, 1999). Infection of human beings with *M. bovis* almost always occurs by inhalation of aerosols or consumption of milk containing the bacillus (Grange, 2001). In industrialized countries, the incidence of tuberculosis due to *M. bovis* in humans is almost at zero level as a result of pasteurisation of milk and milk products and eradication of bovine tuberculosis in cattle populations (Collins and Grange, 1983). However, in developing countries, bovine tuberculosis in animals can be widely distributed in regions where control measures are not applied or are conducted sporadically and pasteurization is rarely practised (Cosivi *et al*, 1998).

1.6 Pathogenesis

M. tuberculosis gains entry into the body via the respiratory tract (Sharma and Mohan, 2004). The organism is conveyed to regional lymph nodes at the hilum resulting in proliferation of bacteria in the lymph nodes (Vijayan, 2002). In primary *M. tuberculosis* infection of the lung, resulting bacteremia is thought to occur with dissemination throughout the body. Lymphatic involvement is thought to be an integral part of the tuberculous infection with generalized and haematogenous spread rather than a localized disease process. Immune response develops 4 to 6 weeks after inhalation and activated T cells recruit monocytes and mononuclear cells to the lung and lymph nodes, ultimately leading to granulomatous inflammation with giant cells, epithelioid cells and lymphocytes. In most of the patients, the primary infection resolves without becoming

clinically apparent and healing occurs by fibrosis and/or calcification (Vijayan, 2002). However disease may progress, and haematogenous dissemination may take place after primary infection, as well as months or years afterwards (post-primary tuberculosis), under conditions of reduced immunity (van Crevel *et al*, 2002). During the initial infection, regional, hilar, and mediastinal lymph nodes are always seeded with bacilli, and other lymph nodes may also be involved (American Thoracic Society, 2000). The infection in these nodes may progress directly to clinical disease, may become active after many years, or may never become apparent. However, some authors consider that cervical tuberculous lymphadenitis may be due to infection of the tonsils, adenoids and Waldeyer's ring resulting in cervical lymph node involvement (Dandapat *et al*, 1990).

1.7 Pathology

Once the bacteria are transported into the deeper tissues by macrophages and perhaps other phagocytic cells, additional macrophages gather at individual infected foci to form granulomas (Cosma *et al*, 2003). Granulomas likely begin as aggregates of mononuclear phagocytes that surround individual infected macrophages (Adams, 1976; Davis *et al*, 2002). The traditional view of a human granuloma is that of a central core of necrosis surrounded by epithelioid cells with a peripheral cuff of lymphocytes (Peters and Ernst, 2003). Mycobacteria are located within macrophages of granulomas and in large numbers within the central caseous region, when present (Bouley *et al*, 2001). Human *M. tuberculosis* granulomas can also be fibrotic and calcified, and such lesions only occasionally contain live bacteria, which suggest that the presence of these components may be related to healing or healed lesions (Cosma *et al*, 2003).

1.8 Clinical manifestations

Cervical lymphadenitis is the most common manifestation of mycobacterial infections encountered in the otolaryngologic practice (Bayazit *et al*, 2004). The lymph nodes in the neck are conveniently classified as being midline, anterior triangle and posterior triangle (including occipital and supraclavicular triangles) (Moore *et al*, 2001).

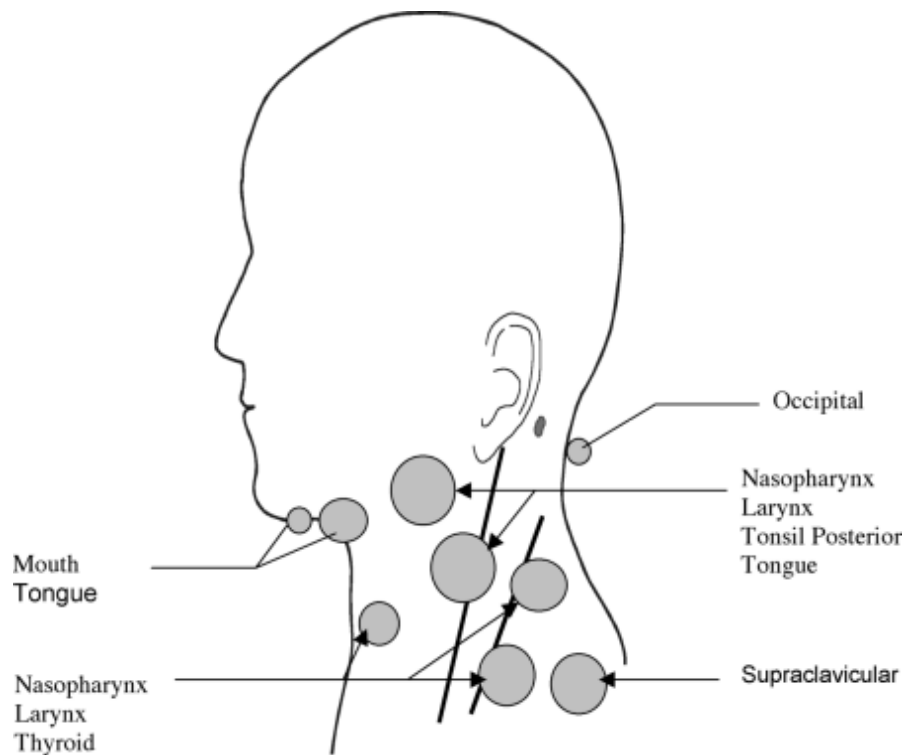


Figure 4: Cervical lymph nodes with their normal drainage areas

Source: The Medicine Journal, 2001; 43:1-2

Tuberculous lymphadenitis often affects children and young adults (Thompson *et al*, 1992; Subrahmanyam, 1993). Female predilection has been reported in a few studies (Thompson *et al*, 1992; Subrahmanyam, 1993; Chen *et al*, 1992; Fain *et al*, 1999). Some patients with lymph node tuberculosis may manifest systemic symptoms and these include fever, weight loss, fatigue and

occasional night sweats (Sharma and Mohan, 2004). Cough is a less prominent feature, seen in approximately 10 percent of patients (Dandapat *et al*, 1990; Lee *et al*, 1992).

Cervical lymphadenopathy is generally described as a painless, slowly growing neck mass or masses developing over weeks to months. Earlier investigators described a predilection to the posterior and anterior triangles of the neck (Cantwell *et al*, 1994; Alleva *et al*, 1988; Penfold and Revington, 1996). In a study from Khartoum by Khery and Ahmed, the most affected nodes were in the posterior triangle. The lymph nodes are usually multiple and matted but may be single, mobile, or fluctuant or with discharging sinuses (Al-Serhani *et al*, 2001). Peripheral tuberculous lymphadenopathy has been classified into five stages ranging from enlarged, discrete, firm and mobile nodes (stage I), to advancing nodal and perinodal involvement, to frank sinus tract formation (Jones and Campbell, 1962). Clinical presentations depend on the stage, nature and location of the disease with spectra of physical findings.

Chest X-ray may reveal findings consistent with tuberculosis in 14-20% of TB lymphadenitis cases (Kanlikama *et al*, 2000; Alleva *et al*, 1988; Ibekwe *et al*, 1997). The majority of patients showed a normal chest radiograph in Saudi Arabia though 15 cases revealed hilar lymphadenopathy (Memish *et al*, 2000). Similarly another study conducted in the same country showed that 83% of mycobacterial cervical lymphadenitis patients had normal chest X-ray with active and old lesions found in 7.3% and 9.1% of the patients, respectively (Jha *et al*, 2001).

1.9 Laboratory diagnosis

Mycobacterial cervical lymphadenitis remains a diagnostic challenge for many clinicians despite current advances in diagnostic laboratory techniques. It remains a diagnostic and therapeutic challenge because it mimics other pathologic processes and yields inconsistent physical and laboratory findings. A thorough history and physical examination, tuberculin test, staining for acid-fast bacilli, radiologic examination, fine-needle aspiration and PCR all would be instrumental in arriving at an early diagnosis and an early institution of treatment before diagnosis can be confirmed with biopsy and culture (Bayazit *et al*, 2004).

1.9.1 Fine Needle Aspiration Cytology (FNAC)

Fine-needle aspiration (FNA) has become a widely used diagnostic tool and it remains one of the most rapid and cost-effective methods of assessing a variety of pathologic conditions ((Leong and Stevens, 1996). It is being increasingly used as the main diagnostic procedure for establishing the diagnosis of tuberculous lymphadenitis (Sen *et al*, 1999). In areas where mycobacterial infections are prevalent, a diagnosis of tuberculosis can be made confidently when its cytomorphological features are met (Bezabih *et al*, 2002). History, physical examination, correct performance of the aspiration biopsy, and proper handling of the specimen are the four basic elements involved in this procedure. Microscopic evaluation includes assessment of overall cellularity, pattern of cell arrangement, identification of predominant cell type, and background elements (Frable and Kardos, 1988). The cytological criteria for diagnosis of tuberculous lymphadenitis have been clearly defined as being epithelioid cell granulomas with or without multinucleate giant cells and caseation necrosis (Lau *et al*, 1990).

In one study, the sensitivity and specificity of FNAC in the diagnosis of tuberculous lymphadenitis was stated as being 88% and 96% respectively (Chao *et al*, 2002) although other studies have shown that the sensitivity could range from 71% (Lau *et al*, 1988) to 90% (Patra *et al*, 1983). In contrast, a low diagnostic yield of 46% (Memish *et al*, 2000) has also been reported which might indicate some of the limitations of this technique. Sampling error that leads to

multiple aspirations, smearing and staining techniques, and reading of the slides are some of the issues that have to be considered and handled with well trained and experienced professionals.

ZN staining of FNA smears has shown results ranging from as low as 15% (Lau *et al*, 1990) to as high as 59% (Bezabih *et al*, 2002) depending on the nature of the lesion and immunological status of the individual. A much higher percentage of positivity with a higher density of acid-fast bacilli was found in HIV positive LNTB patients (Nayak *et al*, 2004). A distinct cytological pattern was also seen in HIV positive patients which makes this diagnostic tool very useful in the initial investigation of these patients presenting with lymphadenopathy (Nayak *et al*, 2003).

1.9.2 Histopathology

Histopathologic examination is one of the most important means for diagnosing mycobacterial cervical lymphadenitis (Manolidis *et al*, 1993; Artenstein *et al*, 1995). 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 (positive for lymph node tuberculosis) (Mirza *et al*, 2003). However, histology lacks specificity since granulomas with necrosis are seen in several infectious and non-infectious conditions. Despite this shortcoming, histological examination of extra-inguinal lymphadenopathy revealed a high yield of 82% in diagnosing lymph node tuberculosis in Malawi (Bekedam *et al*, 1997). In addition, macroscopic examination of excised lymph nodes for caseous necrosis also revealed similar results in the above study. It was found that examination of lymph nodes by the naked eye provides useful information for the diagnosis of lymph node tuberculosis in areas where such cases are endemic and laboratory facilities are not optimal (Bem, 1996).

1.9.3 Culture

In the isolation of mycobacteria by culture, the ideal medium should be able to support rapid and luxuriant growth, and allow the determination of its characteristic features, e.g. colony morphology, growth rate and pigment production (Ang *et al*, 2001). Most of mycobacterial culture media fall into egg-potato-base media and agar-base media (American Thoracic Society, 2000). The most popular egg-based media are the Lowenstein-Jensen buffered egg-potato medium and the American Trudeau Society egg yolk-potato flour medium. Among the agar-based media, Middlebrook 7H-10, Middlebrook 7H-11, and Dubose oleic-albumin agar are recommended. The advantages of egg-based media are the long shelf life (1 year when refrigerated) and the low cost of preparation. Egg media require heat for solidification, which, along with the presence of albumin, inactivates certain antituberculous drugs.

Lowenstein-Jensen medium which contains glycerol is used for the isolation of *M. tuberculosis* but glycerol is inhibitory for most strains of *M. bovis* (Grange *et al*, 2000). Media containing sodium pyruvate can be used for the isolation of *M. bovis*. Pyruvate improves the growth of *M. bovis* over that on glycerol medium.

Mycobacteria are slow growing and hence culture is not routinely done in all laboratories. The rapid radiometric culture system or BACTEC has been accepted for the culture isolation of mycobacteria using an enriched Middlebrook 7H12 containing ¹⁴C labelled palmitic acid (Mendoza *et al*, 1993). The use of the radiometric method has significantly improved the recovery rates and times of mycobacteria from respiratory secretions and other specimen sources.

Specimens collected from normally sterile body sites may be placed directly onto the culture media (American Thoracic Society, 2000) 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 (Singh *et al*, 2000). Mycobacterial cultures from such sources were positive in 35% to 65% of patients in

published studies (Gupta *et al*, 1993; Dandapat *et al*, 1990; Radhika *et al*, 1989). High culture positivity rates were found in materials which contained caseous and necrotic lesions. Though it involves an invasive procedure, homogenized and decontaminated biopsy materials can be used for culture. A high diagnostic yield of 88% had been reported from lymph node biopsy samples grown on LJ media in Tanzania (Perenboom *et al*, 1995).

Growing acid fast bacilli on solid media like LJ allows for identification of mycobacterial isolates by rate of growth, colonial morphology and biochemical tests. These traditional methods of identification of mycobacterial isolates are time consuming and laborious (American Thoracic Society, 2000). Although there are batteries of tests for biochemical identification, four simple *in vitro* cultural and biochemical tests have been selected after careful evaluation (Yates, 1984; Collins *et al*, 1982; Yates and Grange, 1988). These tests are nitrate reductase test, oxygen preference (aerobic or micro-aerophilic), susceptibility to the isoniazid analogue thiophen-2-carboxylic acid hydrazide and susceptibility to the anti-tuberculosis agent pyrazinamide or the pyrazinamidase test. *M. tuberculosis* reduces nitrate to nitrite with the production of red colour indicating the presence of the enzyme nitrate reductase. The same bacteria are resistant to TCH on slopes of 7H10 agar containing the drug whereas the reverse is true for *M. bovis*. The presence or production of the Pyrazinamidase enzyme differentiates *M. bovis* from *M. tuberculosis*. The former produces the enzyme and the production is indicated by a pink band which is produced in the upper part of the agar butt when ferrous ammonium sulphate is added.

1.9.4 Polymerase Chain Reaction (PCR)

With the advance of molecular diagnosis, various PCR methods in diverse clinical specimens have been introduced to identify *M. tuberculosis* more easily and quickly (Baek *et al*, 2000). Owing to the limitations of the traditional microbiological methods, paucibacillary nature of the specimen and the extensive differential diagnosis in extra-pulmonary tuberculosis, a rapid, sensitive and specific diagnosis is needed in developing countries (Singh *et al*, 2000). PCR has several advantages over culture, including confirmation of the presence of *M. tuberculosis*

within 1 to 3 days as compared to 6 weeks with conventional culture techniques (Kesarwani *et al*, 2004). Additional advantages of PCR over conventional methods include its high sensitivity, performance in few hours, and depending on the assay design, ability to differentiate between *M. tuberculosis* complex and mycobacterial species other than TB, and identification of gene mutations associated with drug resistance (Piatek *et al*, 1998; Richeldi *et al*, 1995).

Observing *M. tuberculosis* in tissues or smears using Ziehl-Neelsen staining or fluorescence method allows faster diagnosis (Li *et al*, 2000). Unfortunately, these methods are insensitive and non-specific. This called for development of a new and sensitive diagnostic technique like PCR. Key mycobacterial targets for PCR amplification are: the insertion sequence IS6110, 65KD heat shock protein, 38KD protein, and ribosomal RNA (Li *et al*, 2000). IS6110 is considered to be a good target for amplification as this insertion sequence is found in almost all members in high copy number in most strains of the *M. tuberculosis* complex (Eisenach *et al*, 1990). Amplification differences in RD regions reflecting variable deletions in this genomic region could also be used as a tool for differentiation of members of the mycobacterium tuberculosis (Parsons *et al*, 2002).

Fine needle aspirates from suspected cervical tuberculous lymphadenitis cases can be used for amplification of mycobacterial DNA extracted from such specimens. A sensitivity of 76% with specificity of 100% was reported by a study conducted on FNA obtained from 17 patients who were clinically diagnosed as having cervical tuberculous lymphadenitis (Baek *et al*, 2000). A slightly higher sensitivity rate of 78% was reported in another study which performed PCR on lymph node biopsy samples (Singh *et al*, 2000). Lymph node aspirates, in particular, pose a constraint and a challenge for the diagnostic laboratory because of the small volumes (a few microlitres to less than 2 ml) of specimens available for analysis and because of the low positivity with smear and culture techniques.

Yassin *et al* reported a high PCR positivity (65%) among clinical cases tested negative for tuberculous lymphadenitis by cytology, clearly marking a higher sensitivity of PCR. Since false

positive reactions are a major problem with PCR methods for the detection of *M. tuberculosis* (Kim *et al*, 1996; Eisenach *et al*, 1991), cautious interpretation of results is useful when using a very sensitive diagnostic tool like PCR. In a study conducted by Kim *et al*, it was shown that combining FNAC, culture and smear with PCR helped in establishing a definitive diagnosis of tuberculous lymphadenitis in 68% of cases. In this study, specimens showing typical suspected granuloma with caseation necrosis were over 60% positive, and even specimens with a few granuloma were 33% positive. However, in a similar work employing the above four diagnostic methods, a higher percentage (95%) was identified as being tuberculous lymphadenitis (Goel *et al*, 2001).

1.10 Treatment

Studies on extra-pulmonary tuberculosis have clearly established the efficacy of short course treatment in both children and adults (Balasubramanian and Ramachandran, 2000), with the overall favourable response varying from 87% to 99%. The WHO also recommends that DOTS be provided for effective management of patients with extra-pulmonary tuberculosis.

1.11 HYPOTHESIS

- *M. tuberculosis* is the etiologic agent in more than 80% of LNTB cases diagnosed according to the national algorithm for the diagnosis of tuberculous lymphadenitis.

1.12 OBJECTIVES

1.12.1 Main objective

- To evaluate the currently available diagnostic algorithm for LNTB in Ethiopia using culture as the gold standard and to propose a diagnostic guideline that has a maximum accuracy in field circumstances.

1.12.2 Specific objectives

- To assess the percentage of bacteriologically confirmed cases of *M. tuberculosis* amongst those cases clinically diagnosed as LNTB (having completed diagnostic algorithm as per the 2002 TLCP guideline).
- To determine whether transmission of *M. bovis* does indeed contribute to the incidence of LNTB in Ethiopia.
- To assess the contribution of other factors such as HIV to the reported increase of LNTB by comparing the proportion of LNTB in these different clinical conditions.

Part II

2. Materials and Methods

2.1 Study area



Figure.5: Map of Ethiopia showing the relative location of the study sites.

Source: www.countrywatch.com

2.1.1 Addis Ababa

Addis Ababa is the capital city of Ethiopia. The majority of its total population (73%) of about 2.9 million people is aged between 15 and 49 years (35% between 15 and 24 years) (Addis Ababa HIV/AIDS prevention and control office, 2004). The city is reported to have an annual growth rate of 2.8%, mostly due to the rural to urban migration. The distribution of health infrastructure includes 25 hospitals, 27 health centres and 136 health stations (Health Indicators 2003/04). In 2003/04, 2,805,000 people paid a visit to the out patient department of public health institutions out of which 4,957 deserved admission. In the same year, 3,931 (23%) smear

positive pulmonary cases were detected whereas smear negative and extra-pulmonary tuberculosis cases constituted 4,621 (34%) and 5,173 (38%) respectively. In Black Lion referral hospital, an average of 8-15 cases of tuberculous lymphadenitis patients were seen each month in the first half of 2005. Addis Ababa is reported to have one of the highest concentrations of HIV/AIDS cases in the country (Addis Ababa HIV/AIDS prevention and control office, 2004). According to recent surveillance reports of the Ministry of Health, the HIV prevalence rate in Addis Ababa amounts to 12.4% of the adult population.

2.1.2 Bahr Dar

Bahr Dar is 520 kilometres to the north-west of Addis Ababa. It is the capital city of the Amhara region. The region had a population size of 18,143,000 with females aged 15-49 constituting 23.4% (Population and Housing Census, 1994 GC). The region has 17 hospitals, 115 health centres and 1,128 health posts. In the region, 7,187 (29%) new smear positive cases, 8,012 (32%) pulmonary smear negative and 9,780 (39%) extra-pulmonary cases were detected in the year 2003 to 2004. In the regional referral hospital, on the average 8- 20 cases of LNTB were seen each month in the first half of 2005. The major ethnic group in Bahr Dar is Amhara. Farmers in the region subsist on small farms and keep cattle mostly for farming purposes.

2.1.3 Harar

Harar city is found 500 kilometres to the south-east of Addis Ababa. It has 5 hospitals, 2 health centres and 19 health stations. The population was estimated to be 185,000 (Population and Housing census, 1994 GC). New visits to the outpatient department were made by 128,682 patients and 12,600 were admitted for inpatient care (Health Indicators, 2003/04). Smear positive pulmonary cases were reported to be 261(30%) and smear negative and extra-pulmonary cases amounted to 126 (24%) and 136 (26%) respectively. In Harar TB centre, on the average 5 - 14 cases of lymph node tuberculosis were seen each month in the second half of 2005. It has a total of 8 health facilities that provide DOTS. In the city, Harari and Oromo constitute the major ethnic groups. Farmers around the city keep domestic animals such as goats and cattle and there is tradition of drinking raw milk.

2.1.4 Dire Dawa

It is a relatively large urban centre 515 kilometers to the south-east of Addis Ababa. Administratively, it is a self-administrative region with 370,000 people living in the locality (Population and Housing census, 1994 GC). It has 3 hospitals, 5 health centres and 13 health stations (Health Indicators, 2003/04). The health facilities were visited by 89,180 new patients of whom 4% and 2.4 % of the cases were given sputum and X-ray examinations respectively. Of the new patients who made a visit to the out patient department, 10.5% had been hospitalized. Seven hundred eighteen (33%) patients were found to be smear positive pulmonary cases. The proportion of smear negative pulmonary tuberculosis was found to be higher (34%) compared to extra-pulmonary cases (33%). In Dire Dawa's Dil Chora Hospital, on the average 5-10 cases of LNTB were seen each month in the second half of 2005. DOTS providing facilities include 1 hospital, 3 health centres and 1 health station. The ethnic composition is diverse with Amhara, Oromo, Somali and various other groups. The population in the urban area depend on trade for their day to day living.

2.2 Study design

The study is a cross sectional study conducted over a period of ten months between October 2004 and August 2005.

2.3 Sample size

The original study proposed 6 study sites with a calculated sample size of 246 based on the prevalence of *M. tuberculosis* (82%) in a previous study conducted in Butajira (Kidane *et al*, 2002). However, due to time constraints and unavoidable logistic problems the number of sites had to be reduced to four, resulting in a total enrolment of 150 patients.

2.4 Study population

The study population comprised of all consecutive patients between the ages of 5-65 years who presented themselves to the out patient department of the selected study sites and were diagnosed to have lymph node tuberculosis according to the national algorithm (TLCP 2002 manual).

Inclusion criteria:

- Patients from 5-65 years of age
- Domicile in the service area of the health facility
- Informed consent from subject or guardian
- Study subjects included with full conformity to the national algorithm
- Informed consent for HIV counselling and testing

Exclusion criteria:

- Patients who were critically ill
- Patients with medical contra-indications for biopsy (e.g. anticoagulant use)
- Proven pulmonary TB and on anti-tuberculosis treatment

2.5 Study period

Patients were recruited from October 2004 to August 2005.

2.6 Patient recruitment

Cases of LNTB diagnosed as per the national algorithm and who were willing to be HIV tested and gave informed consent were included in the study. Case record forms were filled by the examining physicians and depending on the condition of the patients investigations such as X-ray, sputum for AFB and routine haematological examinations were done. Patients were sent to VCT centre for counselling and HIV testing. Fine needle aspiration and excision biopsy was performed by surgeons in the minor theatre of the selected hospitals in Bahr Dar, Harar and Dire Dawa. In Addis Ababa, fine needle aspirates were done by a pathologist and subsequently patients were sent for excision biopsy.

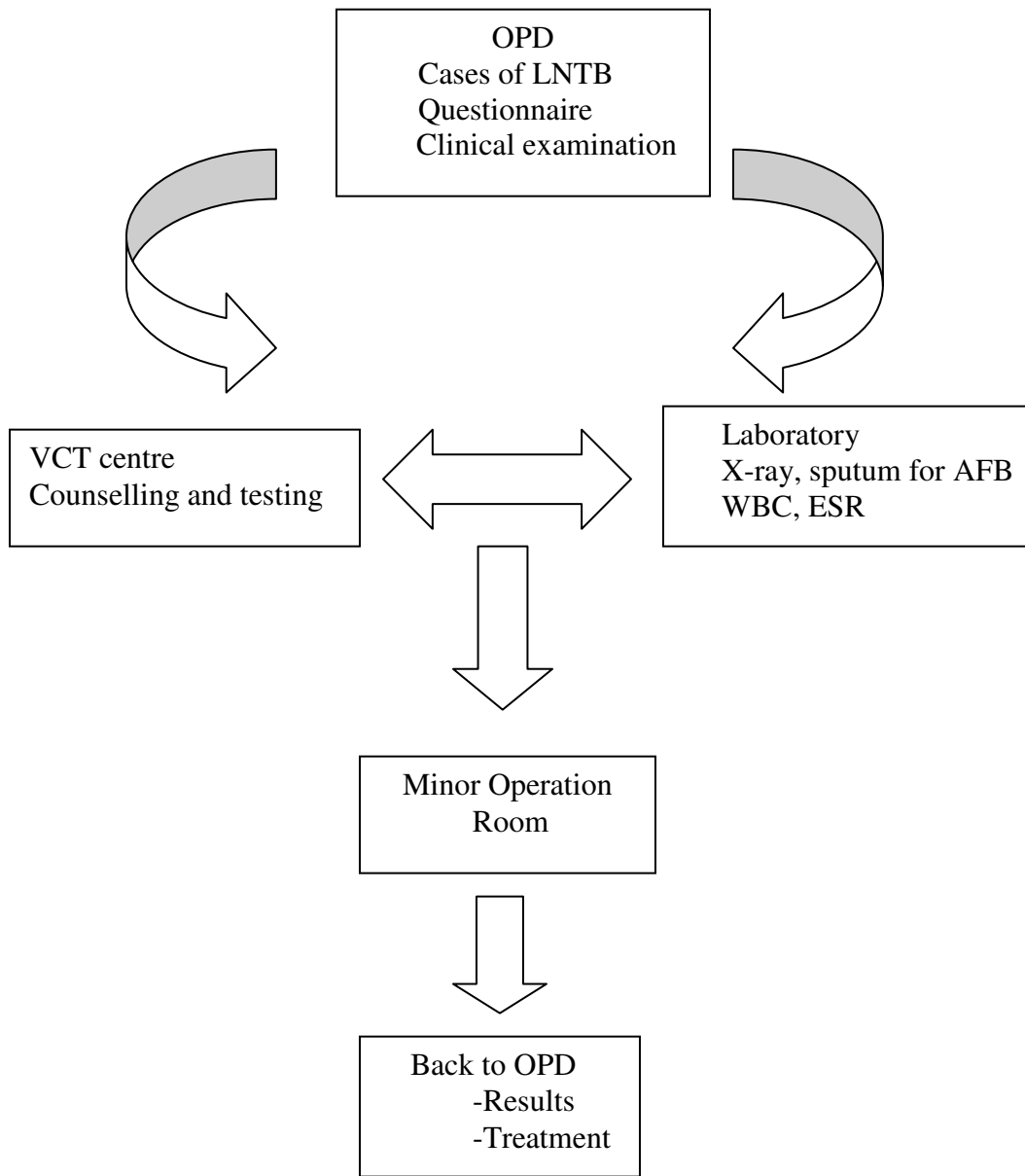


Figure.6: Flow chart showing patient recruitment procedures utilized in the four study sites during the study period (October 2004 – August 2005)

2.7 Sample collection

In Addis Ababa, a pathologist collected fine needle aspirates of the affected nodes whereas in the other study sites, a surgeon who had training in this technique performed the procedure. Excision biopsy on the other hand was taken in a minor operation theatre by certified surgeons in all of the sites.

2.7.1 Fine Needle Aspiration (FNA)

FNA was performed using 21 Gauge needle. A standard procedure was used. The overlying area was cleaned with 70% alcohol. The enlarged node was fixed and maintained in stable position by the left hand of the physician. Then the node was entered with a negative pressure applied to the syringe. Multiple (average six) in and out passes were made by the needle without exiting the node. After removing the needle a drop 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 AFB staining.

2.7.2 Biopsy

Following examination and selection of an appropriate lymph node for excision, a local anaesthesia (2% Lidocaine) was applied under aseptic conditions to the overlying skin. Maintaining homeostasis, a small incision was made with a surgical blade. The affected node was accurately dissected out from the surrounding tissue. The node is cut into two halves. One half is put in a bottle of formalin and the rest in physiological saline. The surgical incision was sutured with 2/0 catgut. The wound was dressed with a piece of gauze and the patient is appointed on the sixth day to the near by health institution for inspection of the wound. After a naked eye examination of the cut surfaces of the node, smears were made on a slide for AFB staining.

2.8 Sample storage

At the field sites samples in physiological saline were stored at 4⁰C until transportation. Storage period constituted a minimum of a day to a maximum of two weeks.

2.9 Sample transportation

The samples were transported to Addis Ababa by airplane in ice packed ice boxes. It took a minimum of 35 minutes and a maximum of 2 hours to reach the capital city. They were transferred from the airport to AHRI laboratory for sample processing.

2.10 Sample processing

The formalin bottles and FNAC slides were transferred to the Pathology Laboratory at AHRI whereas the samples in saline and the fine needle aspirates were prepared for culture under P3 safety conditions in the TB laboratory. Smears prepared from FNA and biopsy samples were stained by Ziehl-Neelsen staining for acid-fast bacilli

2.10.1 Acid fast staining

Standard Ziehl-Neelsen staining procedures were applied. Briefly, initially the slides were flamed to fix the samples. Carbol fuchsin was applied to cover the entire slide. Using a Bunsen burner, the slides were slowly heated until steaming. The steaming was maintained for 5 minutes by using low or intermittent heat (i.e. by occasionally passing the flame from the Bunsen burner over the slides). After this procedure, the slides were rinsed with water. Then they were flooded with 3% acid alcohol and allowed to decolorize for 5 minutes. After rinsing the slides thoroughly with water again, they were counter stained with methylene blue.

2.10.2 Fine Needle Aspiration Cytology (FNAC) and Histopathology

FNAC slides were air-dried. One ml of Wright stain was placed upon the smear for 1-3 minutes. After adding 2ml of distilled water, it was left to stand twice as long as in the initial step (2-6 minutes). Finally it was dried and examined under a light microscope.

The pathologist examined the biopsy materials macroscopically and initial slices were made. They were then paraffin embedded. The paraffin blocks were cut into 5µm pieces using a microtome blade. The slices were put on a slide, the tissues were deparaffinized in dry oven and subsequently stained by Haematoxylin and Eosin stain before further examination.

2.10.3 Culture

In the TB laboratory the biopsy specimen in saline was minced with a surgical blade. The bits were further homogenized with a mortar and pestle mixed with 3ml of PBS. Decontamination process begins by adding 3ml of SDS to 1ml of the homogenate (Groothuis and Yates, 1991). The sample is centrifuged for fifteen minutes at 3000 *rpm* after shaking for the same duration. After discarding the supernatant, the sediment was neutralized with bromocresol purple. The thoroughly mixed and decontaminated specimen was inoculated onto Lowenstein Jensen media (separate tubes of pyruvate and glycerol media) in a 100µl amount. The same method was applied for decontamination of FNA specimen prior to inoculation in the same amount and on the same media. Culture of the specimen was incubated at 37⁰C for 3-8 weeks. The media were checked for evidence of bacterial growth daily for the first week and weekly for the rest of the time until 8 weeks. Colony morphology, growth rate and preference of media were some of the parameters used for phenotypic characterization of the isolates.

2.10.4 Biochemical testing

2.10.4.1 Nitrate reduction test

Mycobacterial species may be differentiated on the basis of their ability to reduce nitrate to nitrite. The nitrate reduction test is based on the detection of a red color that is formed when a nitrite-sulfanilic complex reacts with N-naphthylenediamine. Initially, 4 drops of sterile distilled water was added to a screw capped tube. Subsequently, 2 loopful of growth from a 4 week old culture was transferred to the test tube. The tube was shaken by hand and incubated upright for 2 hours in a 37⁰C water bath after addition of 2ml of NaNO₃. Sequential addition of the following chemicals was performed to reach at the final result: 1 drop of Reagent 1(50:50 HCL and water), 2 drop of Reagent 2 (sulfanilamide), 2 drop of Reagent 3 (N-naphthylenediamine). A red colour

will be produced in the medium only when nitrite is present in the medium. Positive and negative controls constituting *M. tuberculosis* (ATCC 35836) and *M. bovis* (RIVM 12716) reference strains were included in each test.

2.10.4.2 Thiophen-2-Carboxylic Acid Hydrazide Susceptibility Test

The TCH test is used for distinguishing *M. bovis* from *M. tuberculosis* and other species. *M. bovis* is susceptible to low concentrations (2µg/µl) of this compound. Sensitivity to thiophen-2-carboxylic acid hydrazide was tested by inoculating a control and drug containing medium (2µg/µl) and observing for growth during incubation for 3 weeks at 37⁰C. The organism is considered resistant to TCH if growth on the drug containing medium is equal to or greater than 1% of that observed on the drug free control medium.

2.10.4.3 Pyrazinamidase test

The deamination of pyrazinamide to pyrazinoic acid and ammonia is helpful in distinguishing *M. bovis* from *M. tuberculosis*. *M. tuberculosis* is positive for pyrazinamidase activity within 4 days while *M. bovis* is pyrazinamidase negative even at 7 days of incubation. On top of the butt of two tubes of substrate medium (Dubose broth base, pyrazinamide, pyruvic acid and agar) were inoculated with a heavy loopful of growth from an actively growing culture. After 4 days of inoculation at 37⁰C, 1ml of freshly prepared 1% ferrous ammonium sulphate was added to each of the tubes. A pink band in the upper part of the agar butt is indicated in a positive test subsequent to 30 minutes of incubation at room temperature. A positive and negative control slopes inoculated with *M. tuberculosis* (ATCC 35836) and *M. bovis* (RIVM 12716) respectively were included alongside each test.

2.11 Molecular techniques

2.11.1 DNA extraction

Genomic DNA was extracted from mycobacterial isolates according to the van Soolingen protocol (van Soolingen *at al*, 1991). Initially two loopfuls of bacterial growth in 400µl of 1xTE pH 8.0 were incubated at 80⁰C for 20 minutes in a water bath. Fifty microliter of Lysozyme (Sigma, Saint Louis, USA) (10mg/ml) was added to digest bacterial cell wall. After incubation over night at 37⁰C in a water bath, 70µl of 10% SDS and 6µl of proteinase K (Sigma, Saint Louis, USA) were added to the above mixture. 5M sodium chloride and subsequently a pre-warmed cetyltrimethylammonium bromide (CTAB)/sodium chloride solution were added in a 100µl amount before incubating at 65⁰C for 10 minutes. Chloroform/isoamyl alcohol (24:1) extraction of proteins is followed by centrifugation at 12,000 *rpm* (Centrifuge 5415; H. Jurgens & Co., Berman, Germany) for 5 minute. DNA was precipitated by isopropanol after the supernatant was transferred to a new eppendorf tube. This was placed for 30 minutes at -20⁰C and spun for 15 minutes afterwards. The resultant pellet was washed with 70% ethanol and treated with 10mg/ml RNase at 37⁰C for 1 hour. Finally, the pellet was dried and suspended in 1x TE buffer. Spectrophotometer measurement of DNA concentration was performed and DNA was kept at 4⁰C for immediate use or stored at -20⁰C. Similar protocol was used for extraction of DNA from biopsy samples with some modification at the initial stages of the protocol where 1.5ml of the homogenized sample was centrifuged for 15 minutes at 12,000 *rpm*. After discarding the supernatant, 500µl of 1xTE buffer was added and boiled for 15 minutes. The rest of the protocol is followed as in the cases of genomic DNA extraction from mycobacterial culture.

2.11.2 PCR

Genus and species specific primers were used for identification of isolates both from culture and directly from samples. The following primers were used:

Genus specific

MT1 5'-TTCCTGACCAGCGAGCTGCCG-3'

MT2 5'-CCCCAGTACTCCCAGCTGTGC-3'

Amplifies a 506 sized base pair targets of Antigen 85a (Rv3804c) and Antigen 85b (Rv1886c) genes.

The cycling parameters include 35 cycles of 94°C for 1 min for denaturation, 71°C for 1.5 min for annealing, and 72°C for 2 min for extension and a final incubation at 72°C for 10 min (Kidane *et al*, 2002).

Species specific

RD10flankF 5'-CTGCAACCATCCGGTACAC-3'

RD10intR 5'-GAAGTCGTAACCTACCCGGGA-3'

RD10flankR 5'-AAGCGCTACATCGCCAAG-3'

If RD10 region is present (i.e. *M. tuberculosis*), a product with a size of 308 base pair will be amplified; if it is deleted (*M. africanum* and *M. bovis*), a PCR product with a 202 base pair will be detected.

The cycling parameters include initial denaturation at 95°C for 5 minutes and 40 cycles of denaturation at 94°C for 30 seconds, annealing at 65°C for 1 minute and extension at 72°C for 10 minutes (Parsons *et al*, 2002).

RD4intF 5'-ACACGCTGGCGAAGTATAGC-3'

RD4flankR 5'-AAGGCGAACAGATTCAGCAT-3'

RD4flankF 5'-CTCGTCGAAGGCCACTAAAG-3'

If RD4 is present (i.e. *M. tuberculosis* and *M. africanum*), a 335 base pair product will be amplified; if it is deleted (*M. bovis*), a product 446 base pair long will be amplified.

The cycling parameters include initial denaturation at 95°C for 5 minutes and 40 cycles of denaturation at 94°C for 30 seconds, annealing at 65°C for 1 minute and extension at 72°C for 10 minutes (Parsons *et al*, 2002).

The total PCR reaction mixture was 25µl with all the primers and Ready-To-Go-beads obtained from Amersham Pharmacia Biotech, Freiburg, Germany. The reaction is carried out in a thermal cycler (Gene Amplification PCR system 9700, PE Biosystem, Norwalk, CT).

2.11.3 Analysis of PCR products

A 1.5% agarose gel was used to analyze the amplification product by staining the gel with 1µl of 10mg/ml ethidium bromide (Sigma, Chem. Corp. USA) to visualize the PCR product at the expected position in comparison to the 1 Kilo base DNA ladder (Sigma, Chem. Corp. USA) as size marker and PCR products of known DNA as positive controls (DNA extracted from culture of reference strains of *M. tuberculosis* (ATCC35836) and *M. bovis* (RIVM 12716)). Negative controls were also included in each assay.

2.12 HIV screening

Patients with lymph node tuberculosis were tested for HIV antibody as per the national algorithm used to diagnose HIV positive cases. The algorithm utilizes three rapid HIV test kits: Determine™ (Abbott Laboratories, USA), Capillus™ (Trinity Biotech, USA) and Uni-Gold™ (Trinity Biotech, USA). Determine is used as a screening test whereas Capillus is required to confirm the initial diagnosis made with Determine. Uni-Gold is utilized as a tie-breaker when the screening and confirmatory test results are discordant.

2.13 Definition of outcomes

There will be five possible outcomes:

- Proportion of bacteriologically confirmed cases of *M. tuberculosis* amongst patients with a clinical diagnosis of lymph node TB.
- Proportion of bacteriologically confirmed cases of *M. bovis* amongst patients with a clinical diagnosis of lymph node TB.
- Diagnosis of TB by a positive smear with presence of acid fast bacilli demonstrated by ZN staining and /or presence of caseation, granulomas and giant cells on microscopic examination (Nambuya *et al*, 1988).
- Suggestive diagnosis of TB by presence of macroscopic caseation (= presence of cheesy, lumpy aspirate or seen on cut surface of the lymph node, creamy or grey in color).
- No TB.

2.14 Statistical analysis

Data were entered on case record forms. After compilation of the forms, data were double entered in Epi-Info version 6.04 (CDC/WHO) by two different data entry clerks; inconsistencies were checked against the raw data. Statistical analysis was done using STATA version 7 software. Sensitivities, specificities, positive and negative predictive values were calculated for the laboratory methods used in the study with culture as the gold standard. Associations between socio-demographic and clinical variables were determined with p values less than 0.05 being considered statistically significant.

2.15 Ethical considerations

Ethical clearance was obtained from AHRI/ALERT, Addis Ababa University and the National Ethical Review Committees. Letters of collaboration were obtained from the regional health offices of the respective study sites. All patients or guardians gave written informed consent prior to enrolment to the study and HIV screening. Results of laboratory investigations were reported back to the physicians for treatment initiation or decision as early as available within 10 days on average.

Part III

3. Results

3.1 Characteristics of the study population

3.1.1 Socio-demographic characteristics

The study population consisted of 150 patients from four study sites, who were clinically diagnosed as having LNTB following the 2002 national algorithm. Out of these 117 were found to be culture (FNA and /or biopsy) proven cases of TBLN of which 64 (55 %) were male and 53 (45%) female. Their age ranged from 6 to 62 years with a median of 24 years. The mean age was 25 (\pm SD 10.7) years. Most of the patients were young with 97/117 (82%) under 35 years of age, as presented in Table 1. The majority (64%) of the study participants constituted of farmers (26%), students (22%) and housewives (16%). Forty one percent of the patients were illiterate and 59% had a primary to secondary school education. Rural dwellers constituted 54 %. A total of 47 (41%) of the culture proven lymph node tuberculosis patients were single (see Table 3). The ethnic composition included 61% Amhara, 25% Oromo and the rest belonging to several ethnic groups. The main religion was Christianity (72%), whereas 28% were Muslims.

Table.1 Age distribution of the total study participants from Bahr Dar, Addis Ababa, Dire Dawa and Harar investigated for LNTB, October 2004 –August 2005.

Age group (years)	Non-LNTB (%)	LNTB (%)	Total (%)
5-14	4 (12)	13 (11)	17 (11)
15-24	8 (24)	50 (43)	58 (39)
25-34	8 (24)	34 (29)	42 (28)
35-44	6 (18)	13 (11)	19 (13)
>45	7 (21)	7 (6)	14 (9)
Total	33 (22)	117 (78)	150 (100)

3.1.2 Characteristics by study sites

The total number of patients recruited from each site includes 50 patients from Addis Ababa in the centre, 50 from Bahr Dar in the North and the remaining 50 from Harar and Dire Dawa, both in the East. Due to socio-geographic proximity of Dire Dawa and Harar, they are treated as one group. The age distribution stratified for the three study sites is presented in Table 2 showing that more than 63% of the patients were between the ages of 15 and 35 years. Male preponderance is seen in Addis Ababa (69%) and Harar / Dire Dawa (54%) whereas females constituted the majority of the patients seen in Bahr Dar (55%). In Bahr Dar, farmers (15) and housewives (10) together made up 25/45 (56%) of the total culture proven lymph node tuberculosis cases seen in this site (see Table 3). Though students (25%) and farmers (25%) represented the majority of the LNTB patients identified in Addis Ababa, students (31%) dominated in Harar / Dire Dawa though a considerable number of housewives (18%) and farmers (18%) were seen as well. The majority of tuberculous lymph node patients in Addis Ababa (56%) and Bahr Bar (40%) were single whereas this was found to be the reverse in Harar / Dire Dawa. Fifty four percent of the patients were Oromo in Harar / Dire Dawa and Amhara made up for 43% in Addis Ababa and 100% in Bahr Dar, rendering the Amhara the predominant ethnic group within the study population

Table. 2 Age and sex distribution of culture proven LNTB patients from Addis Ababa, Bahr Dar, Harar / Dire Dawa , October 2004 – August 2005.

Site	Sex	Age group distribution in years (%)					Total (%)
		5-14	15-24	25-34	35-44	>45	
Addis Ababa	F	1 (3)	5 (15)	4 (13)	-	-	10 (31)
	M	3 (9)	12 (38)	4 (13)	3 (9)	-	22 (69)
Bahr Dar	F	-	10 (22)	7 (15)	5 (11)	3 (7)	25 (55)
	M	2 (4)	9 (20)	8 (17)	1 (2)	1 (2)	21 (45)
Harar/Dire Dawa	F	2 (5)	7 (18)	5 (13)	2 (5)	2 (5)	18 (46)
	M	5 (13)	7 (18)	6 (15)	2 (5)	1 (3)	21 (54)

Table.3 Socio-demographic characteristics of culture proven LNTB patients from Addis Ababa, Bahr Dar, Harar / Dire Dawa, October 2004 – August 2005

Socio-demographic variable*		Addis Ababa (%)	Bahr Dar (%)	Harrar Dire Dawa (%)	Total (%)	p-value (X² test)
Address (n=116)	Urban	16 (50)	11 (24)	26 (67)	53 (46)	0.2
	Rural	16 (50)	34 (76)	13 (33)	63 (54)	
Occupation (n=116)	Unemployed	5 (16)	2 (4)	1 (3)	8 (7)	0.8
	Government employee	1 (3)	4 (9)	2 (5)	7 (6)	
	Student	8 (25)	6 (13)	12 (31)	26 (22)	
	Housewife	1 (3)	10 (22)	7 (18)	18 (16)	
	Farmer	8 (25)	15 (33)	7 (18)	30 (26)	
	Others	9 (28)	8 (18)	10 (26)	27 (23)	
Educational level (n=113)	Illiterate	7 (23)	26 (59)	13 (33)	46 (41)	0.1
	1⁰ school	17 (57)	11 (25)	18 (46)	46 (41)	
	2⁰ school	6 (20)	7 (16)	8 (21)	21 (18)	
Marital status (n=114)	Single	18 (56)	17 (40)	12 (31)	47 (41)	0.1
	Married	12 (38)	16 (37)	15 (39)	43 (38)	
	Others	2 (6)	10 (23)	12 (31)	24 (21)	
Ethnicity (n=114)	Oromo	8 (27)	-	21 (54)	29 (25)	0.2
	Amhara	13 (43)	45 (100)	11 (28)	69 (61)	
	Others	9 (30)	-	7 (18)	16 (14)	

*Some variables were missing because data were not reported

3.2 Clinical picture

Classical symptoms of tuberculosis reported by the culture proven LNTB patients included fever in 75 (65%), cough in 24 (21%), weight loss in 66 (58%), poor appetite in 58 (50 %) and night sweats in 64 (55%) cases. The median duration of neck swelling was 5 months (range from 2 weeks to 182 months) and mean duration of 14 ± 106 months. Thus 59 (50%) of the lymph node tuberculosis cases had the neck swelling for 5 months or less. A history of previous treatment either in the form of traditional or modern form was recorded for 78 (68%) of the patients. One or more course of broad spectrum antibiotics was taken by 74 (63 %) of the patients. A previous history of anti-tuberculosis treatment was observed in 6 (5%) patients, of whom one had discontinued treatment during the intensive phase. A history of contact with a tuberculosis patient was recalled by 29 (25%) patients. Family members constituted 23/29 (82 %) of the contact cases with a contact period of greater than 4 weeks in 25/29 (86 %) of the patients. Raw milk ingestion and direct contact with livestock were reported in 51/117 (44%) and 47/117 (40%) lymph node TB cases, respectively.

Physical examination of culture proven LNTB patients revealed that 74 (64%) had unilateral cervical lymphadenopathy with 42 (61%) having a right side involvement. The anterior cervical triangle was involved in 22 (19%), posterior triangle in 15 (13%) and supraclavicular region in 7 (6%) of the patients. Multiple nodes were affected in 51 (44%) persons whereas involvement of a single node was seen in 20 (17%) of the patients. Sixty nine patients (60%) had the largest node measuring 1 to 4cm. In 101 (88%) of the patients the nodes were mobile. They were matted in 58 (54%), fluctuant in 31 (30%) and draining with sinuses in 27 (25%) patients.

3.3 Laboratory diagnosis

One hundred and forty nine FNA smears and 146 excision biopsy smears were available for Ziehl-Neelsen staining. Cytology examination of 148 FNA slides was carried out and 146 lymph node biopsy tissue specimens were prepared for histopathological diagnosis. Similar numbers of tissue specimens from excision biopsy as well as 150 FNA specimens from lymph nodes were also processed for culture. Four lymph nodes were abscesses and no tissues could be obtained. Three FNA specimens were not adequate for the full range of tests (no ZN staining for one and no cytology smears for two specimens).

3.3.1 Ziehl-Neelsen staining

Out of the total of 149 FNA specimens processed for ZN staining, 50 were from Addis Ababa, 49 from Bahr Dar and 50 from Harar / Dire Dawa. Acid fast bacilli were detected in 5/50 (10%), 9/49 (18%) and 27/50 (54%) respectively, making the total detection of AFB positive slides 41/149 (28%). Similarly out of 146 biopsy smears for ZN examination, 37/146 (25%) were found to be positive for AFB, giving a slightly lower figure than for the FNA ZN results. Overall, FNA ZN-smears detected 38/117 (32%) of culture proven lymph node tuberculosis cases versus ZN-smears of biopsy specimens, that detected 32/117 (27%) (see Table 4). Against culture as the gold standard, FNA showed a sensitivity of 34% and specificity of 91%, whereas excision biopsy had a sensitivity of 29% and a specificity of 86% (see also Table 7). Positive and negative predictive values were 93% and 29% for FNA ZN and 86% and 27% for biopsy ZN examinations, respectively.

Table.4 Comparison of the diagnostic yield of the laboratory methods used for diagnosis of LNTB with culture proven (LNTB) and culture negative (non-LNTB) patients from four sites in Ethiopia, October 2004 – August 2005

Laboratory Methods (LNTB positive)		Culture		OR (95% CI)	p-value
		LNTB n=117 (%)	NLNT B n=33 (%)		
Ziehl-Neelsen +AFB	FNA	38 (32)	3 (9)	7.6 (1.8-34.1)	0.007
	Biopsy	32 (27)	5 (15)	2.1 (0.8-5.9)	0.160
Macroscopic Examination	FNA	78 (67)	11 (33)	3.7 (1.6-8.6)	0.002
	Biopsy	79 (68)	11 (33)	3.7 (1.6-8.5)	0.002
FNAC		88 (75)	4 (12)	23.6 (7.6-73.2)	0.000
Histopathology		102 (87)	3 (9)	83.1 (20.8-332.2)	0.000

3.3.2 Culture

3.3.2.1 FNA specimens

One hundred and fifty FNA specimens were cultured both on glycerol and pyruvate media (50 from Addis Ababa, 50 from Bahr Dar and 50 from Harar / Dire Dawa). From this total number 79 (52%) showed positive growth after 8 weeks of incubation and the rest were culture negative. High culture yield was seen with samples from Bahr Dar (68%) followed by Harar / Dire Dawa (60%) and Addis Ababa (30%). Five samples gave culture positive result with FNA specimen without showing growth from the biopsy samples. These include 2 samples from Addis Ababa, 1 from Bahr Dar and 2 from Harar. Twenty nine grew only on glycerol with 45 showing growth on both pyruvate and glycerol media. Five samples grew only on pyruvate media.

3.3.2.2 Biopsy

From 146 excision biopsy specimens (48 from Addis Ababa, 49 from Bahr Dar and 49 from Harar / Dire Dawa), 111 were found to be culture positive making the proportion of culture positive specimens 76%. Specimens from Bahr Dar, which showed a high yield with the FNA specimens, similarly demonstrated a much higher culture yield than the other sites. Biopsy specimens from the same region gave a culture positivity of 89% as compared to the two study sites, Harar / Dire Dawa (74%) and Addis Ababa (63%). From the 111 culture positive samples, 31 grew on only glycerol containing media while 79 grew on both pyruvate and glycerol media. There was one culture that grew on pyruvate media alone.

3.3.3 Biochemical testing

Biochemical tests were done with three sets of tests which included nitrate reductase, pyrazinamidase and resistance to TCH. For those which showed growth on both sets of LJ media (pyruvate and glycerol), variable results were seen for the nitrate reductase and pyrazinamidase tests especially on those isolates that grew on pyruvate containing media. Resistance to TCH showed less variability and out of 165 isolates tested 157 grew on TCH. The 8 isolates that did not grow on TCH were subsequently tested with PCR and showed signals for *M. tuberculosis*.

3.3.4 Fine needle aspiration cytology (FNAC)

Cytological examination of FNA smears was carried out on 148 specimens (50 from Addis Ababa, 48 from Bahr Dar, 50 from Harar / Dire Dawa). The cytological criteria for diagnosis of tuberculous lymphadenitis have been defined as being epitheloid cell granulomas with or without multinucleate giant cells and caseation necrosis. Cytological features consistent with tuberculosis were reported in 92/148 (62%) of the examined specimens. Addis Ababa and Bahr Dar each had 32 (32/50 (64%) and 32/48 (67%) respectively) cases of tuberculous lymphadenitis whereas in Harar / Dire Dawa the number of patients identified as LNTB was 28/50 (56%). The number of slides read as non-diagnostic was higher in Bahr Dar (11) and Harar / Dire Dawa (9) than in Addis Ababa (2). More cases of neck tumors were identified at Addis Ababa (7) than in the other two sites combined (2). These include 8 cases of lymphoma and a single case of carotid

body tumor. The total number of reactive lymphadenitis seen was 19 (13%) with Addis Ababa (6) and Bahr Dar (3) showing a lower figure than Harar / Dire Dawa (10). Of the total of 105 cases identified as TB with histology on excision biopsy specimens, FNAC identified 79/105 (75%) cases as tuberculous lymphadenitis. FNAC detected 88/117 (75%) cases of culture proven tuberculous lymphadenitis which gave this technique a sensitivity of 76% and specificity of 88% as is presented in Table 7. The positive and negative predictive value of FNAC for tuberculosis was found to be 96% and 52%, respectively.

Combination of FNAC and FNA ZN stain for AFB detected 76% (89/117) cases of culture proven LNTB, whereas combining FNAC and macroscopic examination of FNA specimens detected 93% (109/117) of the cases. Further combination of all the three methods increased the detection rate to 96% (112/117) of culture proven LNTB cases.

Table.5 Comparison of the diagnosis by histopathological and cytological investigations of specimens from suspected cases of tuberculous lymphadenitis seen at four study site in Ethiopia, October 2004 – August 2005

Diagnosis	Histopathology (%)	FNAC (%)
Tuberculosis	105 (72)	92 (62)
Reactive	10 (7)	19 (13)
Lymphoma & other tumors	14 (10)	9 (6)
Chronic inflammation	2 (1)	3 (2)
Non-diagnostic	13 (9)	22 (15)
Other diagnosis	2 (1)	3 (2)
Total	46 (100)	148 (100)

3.3.5 Histopathology

One hundred and forty six tissue biopsy specimens were available for histological examination (48 from Addis Ababa, 49 from Bahr Dar and 49 from Harar / Dire Dawa). Thirteen specimens were found to be non-diagnostic due to poor tissue preservation or processing. Histologic and cytomorphic classification for 133 specimens is presented in Table 5. One hundred and five (72%) samples were found to be consistent with tuberculosis as judged by their histopathologic picture (epitheloid cell granuloma, multinucleated giant cell and/or caseation necrosis). From these 105 histology proven specimens, 32/48 (67%) were from Addis Ababa patients, 41/49 (84%) from Bahr Dar and 32/49 (65%) from Harar / Dire Dawa. A total of 14 (9%) cases of neck tumour were identified, an additional 5 cases as compared to the FNAC results. The highest number of cases was found in Addis Ababa (8), similar to the FNAC results. All FNAC proven cases of lymphoma were confirmed in the histopathology examination. The number of reactive changes detected by histology (10) was lower than the total number reported with cytology (19). One hundred and two out of the 105 (97%) cases diagnosed by histology as lymph node tuberculosis were culture positive. Most of the AFB positive cases were seen in patients who had a cytomorphic picture consistent with granuloma plus necrosis or necrosis only as presented in Table 6. The sensitivity of histopathological examination of a lymph node biopsy was found to be 92% and the specificity 88%. The positive and negative predictive values were 97% and 71%, respectively.

Table.6 The yield of ZN staining of biopsy and FNA specimens according to cytomorphologic features of the samples from culture proven LNTB patients investigated at four sites in Ethiopia, October 2004 – August 2005

Cytomorphologic features	Histology (n=105)		FNAC (n=92)	
	AFB+ (%)	AFB- (%)	AFB+ (%)	AFB- (%)
Granuloma with necrosis	20 (19)	57 (54)	17 (19)	37 (40)
Granuloma without necrosis	2 (2)	7 (7)	1 (1)	7 (8)
Necrosis	6 (6)	13 (12)	7 (8)	23 (25)
Total	28 (27)	77 (73)	25 (27)	67 (73)

3.3.6 Macroscopic examination of FNA and biopsy samples

Examination of gross FNA specimens was carried out on 149 samples (50 from Addis Ababa, 49 for Bahr Dar and 50 from Harar /Dire Dawa). Caseation necrosis was observed in 89/149 (60%) cases. The highest proportion of lymph nodes with caseation was observed in Bahr Dar with 35/49 (71%) and the lowest in Addis Ababa with 26/50 (52%). Out of the initial 89 cases reported as consistent with LNTB by macroscopic examination, 78/89 (87%) were culture positive. Therefore, with culture as a gold standard, the sensitivity of macroscopic examination of FNA specimen is 67% and the specificity 64%. A significant association between the presence of caseous material and proven tuberculous lymphadenitis was demonstrated in FNA samples (OR 3.7, CI 1.6-8.6, p=0.002). The positive and negative predictive value was found to be 88% and 34%, respectively.

Similar examination of the cut surfaces of excision biopsy specimens was done for 146 tissue specimens (48 from Addis Ababa, 49 from Bahr Dar and 49 from Harar / Dire Dawa). The respective findings diagnostic for LNTB from Addis Ababa, Bahr Dar and Harar / Dire Dawa

were 26/48 (54%), 35/49 (71%) and 29/49 (59%). The sensitivity, specificity, PPV and NPV of cut surface examinations of biopsy specimens were found to be 68%, 63%, 88% and 34%, respectively. As in the case of FNA samples, the presence of caseous material in biopsy specimens is significantly associated with proven lymph node tuberculosis (OR 3.7, CI 1.6-8.5, p=0.002).

Combining macroscopic and Ziehl-Neelsen staining of biopsy specimens in our study revealed that a total of 78% (91/117) cases were diagnosed as lymph node tuberculosis whereas the combination of macroscopic inspection and AFB staining of FNA materials detected 79% (92/117) of culture positive cases.

Table.7 Sensitivity, specificity, positive and negative predictive values of the laboratory methods used for the diagnosis of LNTB patients from four study sites in Ethiopia, October 2004 – August 2005

Laboratory methods		Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Ziehl-Neelsen	FNA	34	91	93	29
	Biopsy	29	86	86	27
Macroscopic Examination	FNA	67	64	88	34
	Biopsy	68	63	88	34
FNAC		76	88	96	52
Histopathology		92	88	97	71
PCR		100	23	83	100

3.3.7 PCR

Species identification using RD10 and 4 sets of primers has identified all culture positive isolates from FNA and biopsy specimens as *M. tuberculosis* (see Figure 7). Direct amplification from biopsy specimens amplified 117 out of the 146 available for the procedure. Twenty four specimens which were found to be positive for PCR were culture negative. On the other hand 23 specimens were PCR negative but showed positive growth on LJ culture media. Direct amplification from biopsy specimens and from culture isolates together gave signal for 140 specimens, all of them being *M. tuberculosis*. With culture as the gold standard, the sensitivity was 100% and specificity 23%. The PPV and NPV values were found to be 83% and 100% respectively.

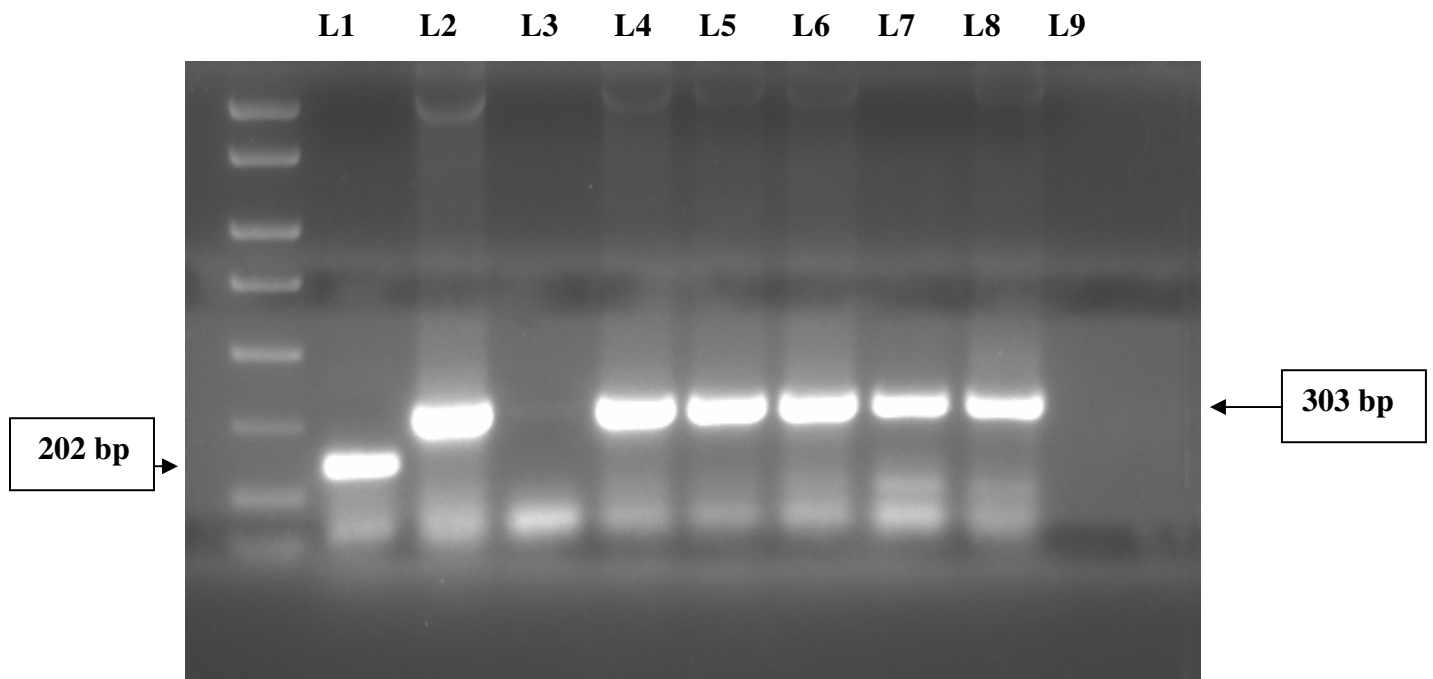


Figure.7 PCR amplification for the presence of RD10 region from culture positive samples L1 PCR marker (double stranded recombinant fragments), L2 *M. bovis* (positive control), L3 *M. tuberculosis* (positive control), L4 negative control, and L5-9 culture positive samples (all *M. tuberculosis*)

3.3.8 HIV testing

The proportion of HIV seropositive patients among the total study participants was 32/150 (21%). Similarly, the proportion of HIV seropositive patients among culture proven LNTB patients was 28/117 (24%). The socio-demographic characteristics of HIV positive and negative LNTB patients were seen in Table 8. There was an association between sex and HIV with 9 (32%) males and 19 (68%) females being HIV-positive LNTB patients ($p = 0.006$). Three (11%) were single, 12 (43%) married and 13 (46%) were in the category that included divorced, widowed and living with partner. Association between marital status and HIV was also seen with the latter groups of LNTB patients showing significant association with HIV infection ($p = 0.007$ and $p = 0.000$ respectively). Twenty (71%) of the HIV positive patients came from an urban set up where as 8 (29%) came from the rural community. Residence in an urban area was strongly associated with HIV infection ($p = 0.002$). TB and HIV co-infection peaked in the age group 25-34 with 18 (64%) of the co-infected in the age group of 15-34 years. However, no association could be confirmed between LNTB and HIV infection ($p = 0.2$). Comparable numbers of positive individuals were found in each of the educational categories. Ten (36%) had secondary school education and the number of patients who were illiterate and who had primary school education were 9 (32%) each.

From the 28 HIV positive patients 9 (32%) had AFB positive smears from excision biopsy. The number of positive AFB slides from FNA specimens was higher than those from biopsy specimens. Fifteen (54%) HIV positive individuals had AFB in their FNA samples. Macroscopic examination of FNA and biopsy specimens identified more than 50% of HIV-LNTB positive patients with 21 (75%) biopsy and 19 (68%) FNA specimens showing caseation necrosis. FNAC identified 20 (71%) HIV positive patients as having LNTB which was found to be slightly lower than the result of histology examination: 21 (75%). Macroscopic examination and AFB identification from biopsy samples together identified 23 (82%) of the HIV-LNTB positive cases. In addition, naked eye examination of FNA specimens with Ziehl-Neelsen staining of the same aspirates identified 21 (75%) of HIV positive LNTB patients. FNAC with macroscopic and AFB examination of FNA samples detected 25 (89%) while histology with macroscopic and AFB examination of biopsy specimens picked up 26 (93%) of LNTB and HIV positive patients.

Table.8 Association of socio-demographic characteristics of culture proven LNTB patients with HIV among study participants seen at four study sites in Ethiopia, October 2004 – August 2005

Socio-demographic variables*		HIV positive	HIV negative	OR (95% CI)	p- value
Residence (n=116)	Urban	20	33	4.2 (1.6-11.02)	0.002
	Rural	8	55		
Gender (n=117)	Male	9	55		
	Female	19	34	3.4 (1.3-8.7)	0.006
Marital status (n=114)	Single	3	44		
	Married	12	31	5.7 (1.4-23.3)	0.007
	Others	13	11	17.3 (3.2-94.8)	0.000
Educational level (n=113)	Illiterate	9	12		
	1⁰School	9	37	0.3 (0.1-1.1)	0.1
	2⁰School	10	36	0.4 (0.1-1.2)	0.1
Occupation (n=116)	Unemployed	3	5		
	Government employee	2	5	0.7 (0.07-6.4)	0.7
	Student	3	23	0.2 (0.03-1.58)	0.1
	Housewife	5	13	0.6 (0.11-3.91)	0.6
	Farmers	3	27	0.2 (0.03-1.35)	0.06
	Others	12	15	1.3 (0.26-6.92)	0.7
Age group (n=117)	5-14	1	12		
	15-24	6	44	1.6 (0.18-15.25)	0.7
	25-34	12	22	6.6 (0.68-63.31)	0.06
	35-44	7	6	14 (0.92-213.35)	0.01
	>45	2	5	4.8 (0.3-78.64)	0.22

* Some variables were missing because data were not reported

Part IV

4. Discussion

Extra-pulmonary tuberculosis has emerged as a prominent disease entity that is being reported from all regional health bureaus in Ethiopia. This has gone to the extent that in the last reporting year (2003/04), the number of EPTB cases notified has exceeded that of smear-positive tuberculosis for the first time. From the reported cases of EPTB, lymph node tuberculosis has taken the lion's share. Lymph node TB, especially cervical lymphadenitis, has already been seen as the dominant clinical presentation of this disease (Yassin *et al*, 2003). Recruitment of 150 consecutive cases of cervical tuberculous lymphadenitis diagnosed on the basis of the TLCP national algorithm was considered as an initial step in the evaluation of this phenomenon. Since over-diagnosis of cases is one possibility for high reporting, which could among others result from sub-optimal use of the flow chart, identification of the patients was done by strict adherence to the algorithm. Those patients who were identified as LNTB cases according to the algorithm were subsequently categorized as LNTB and non-LNTB groups in accordance to their mycobacterial culture (gold standard) results.

The age profile of those patients who were categorized as LNTB showed involvement of younger patients with the 15-35 year group being affected in more than 50% of the cases. This finding is similar to other studies that reported that the young age group, especially between 11-30 years, is the commonest age group affected by this disease (Jha *et al*, 2001). Though a considerable number of patients have been seen in the young age group, more cases of non-LNTB patients were in the greater than 35 age group (39%) than in the LNTB group (17%). This might be due to the fact that, as age increases, the chance of seeing more tumours (non-LNTB) increases as the immune system weakens with age. Nevertheless, age is not a specific parameter to distinguish between LNTB and non-LNTB.

Female gender has long been associated with tuberculous lymphadenitis (Chen *et al*, 1992). This is in contrast to our finding in that 64 (55%) of our patients were males and male preponderance was seen in two of our study sites (Addis Ababa and Harrar / Dire Dawa). Overall a male

preponderance has been attributed to the fact that females have less access to health facilities due to economic and social factors (Gupta, 2000). No significant association between sex and LNTB was observed although a young female with neck enlargement should always raise suspicion of LNTB. It is also possible that more women tended to participate in this study than men.

Lymph node tuberculosis was seen to be more common among farmers, students and housewives. As farmers constitute the majority of the population with a low nutritional and economic background, they are mainly bearing the brunt of this disease. With 63 (54%) of the LNTB patients coming from the rural part of the country, it is not surprising to find farmers to be the majority of the LNTB cases. Since prevalence of HIV in the rural community has been shown to be low (2.6%) and only increasing at a slow rate (MOH 2004, AIDS in Ethiopia-Fifth Report), HIV does not appear to be the single factor that could account for the high number of tuberculous lymphadenitis patients seen among farmers. A considerable number of LNTB cases were found to be students. This, on the other hand, may be influenced by HIV since this category of patients is in the young reproductive age group with a high prevalence of HIV infection. However, the numbers of patients are not sufficient to provide conclusive evidence of association of HIV with this group.

Overall illiterates constituted 41% of the total LNTB population which is considered to be very high, especially in Bahr Dar where more than half of the study population was illiterate. Even in the other two study sites the proportion of illiterate individuals (23% and 33% in Addis Ababa and Harar / Dire Dawa respectively) is considered substantial. The connotation of this finding is grim due to the fact that the impact of illiteracy on health in general and tuberculosis and HIV in particular is detrimental (Waalder, 2002). In addition, in those segments of the community such as farmers and housewives, where more cases of LNTB were diagnosed, illiterates constituted more than 50% with 22 (73%) farmers and 12 (75%) housewives not being able to read and write. It has to be noted in this study that there is a prominent absence of even a single case of LNTB in person with higher level of education. Singles constituting 41% of all the cases, were more common in Addis Ababa and Bahr Dar than in Harar / Dire Dawa. Compared to the

married group 43 (38%), singles if combined with another category of individuals (divorced, widowed and living with partner) form a large pool of high risk groups, vulnerable to both HIV and tuberculosis.

With regard to symptom complex of tuberculosis, cough was reported by 24 (21%) from the total of 114 LNTB cases who responded to this question. Kheiry and Ahmed reported that 35% of their Sudanese study population had cough which is found to be higher than in our finding (Kheiry and Ahmed, 1992). A number of reports have also shown that cough is a less frequent symptom, occurring in approximately 10% of patients (Dandapat *et al*, 1990; Lee *et al*, 1992). With the prominent absence of cough in the majority of the cases, the presence of abnormalities on physical examination and chest X-ray radiograph will be minimal. Indeed, in a clinical-bacteriological study of TB lymphadenitis in India, only 28% of the cases showed evidence of active pulmonary lesions or mediastinal lymphadenopathy (Aggarwal *et al*, 2001). Sputum examination which is the major laboratory method used for diagnosis of pulmonary tuberculosis could not be performed in every case of LNTB since the majority of patients had no cough or had cough that was non-productive. In Malawi, a prospective study revealed that only 1.7% (34/2026) of extra-pulmonary tuberculosis patients submitting sputum for examination were smear positive, and the proportion who were smear positive exceeded 3% only in patients with lymphadenopathy, military TB and TB meningitis (Kwanjana *et al*, 2000).

Classically, patients with *M. tuberculosis* lymphadenitis present with associated fever, weight loss, and fatigue, and somewhat less frequently with night sweat (Dandapat *et al*, 1990; Lee *et al*, 1992). From our proven LNTB patients, 75/116 (65%) and 66/114 (58%) of LNTB patients had fever and weight loss respectively. Fever and weight loss in combination was reported by 43% (50/117) of the patients. In similar studies, Patel and Mehta observed weight loss in 77% and fever in 73% of their cases (Patel and Mehta, 1987). Similarly Dandapat *et al* in India also noted weight loss in 85% and fever in 40% of their patients (Dandapat *et al*, 1990). Other authors have noted different findings, reporting that 57% of patients had no systemic symptoms (Lee *et al*, 1992) or had fewer symptoms (Jha *et al*, 2001). There was no statistically significant

association between LNTB and any individual or combination of symptoms that are part of the symptom complex of tuberculosis. The absence of such clinical markers compound the differentiation between true LNTB and non-LNTB cases and represent a challenge when attempting to design an algorithm based on signs and symptoms.

The duration of the neck swelling was 2 weeks to 182 months with mean duration of 14 months. Fifty percent had the neck swelling for 5 months or less. Weiler *et al* studied cervical lymphadenitis cases whose symptoms started between 2 weeks and 6 months before presentation with a mean duration of 6 weeks (Weiler *et al*, 2000). Longer interval prior to presentation was recorded in India by Jha *et al* whose onset of symptoms and presentation varied from 15 days to 36 months (mean 3 months) (Jha *et al*, 2001). The more delayed the presentation, the higher the progression of the disease. Progressing lesions will advance from discrete, firm and mobile nodes to matted, fluctuant nodes with frank draining sinuses. In the present study 54% (58/107) had matted nodes with 25% (27/109) showing draining sinuses. A higher percentage of matted nodes (78%) were seen by Bezabeh *et al* of whom 12% had draining sinus (Bezabeh *et al*, 2002). Abscess formation and/or draining sinus at the time of presentation was observed in 22% of cases (Cheung *et al*, 1988), a result comparable with our study.

Involvement of the posterior triangle is reported in the majority of the cases (Baskota *et al*, 2004). Twenty two (19%) of our patients had anterior cervical involvement which is higher than those who had posterior triangle affection (13%). Both triangles were affected in 27% of the cases which is also higher than involvement of any other site on the neck. Seventy four (64%) had unilateral cervical adenopathy out of which 42 (61%) had right sided involvement. A similar finding of unilateral infliction of 66% has been reported elsewhere (Ammari *et al*, 2003). Seven (6%) patients had supraclavicular tuberculous lymphadenitis. Among the group of nodes of the head and neck, supraclavicular nodes are most likely to be malignant and should always be investigated (Bazemore and Smucker, 2002).

HIV has played a key role in modifying the incidence (Mwaba *et al*, 2003), and clinical presentation of TB (Elliott *et al*, 1993). Overall TB-HIV co-infection in this study was 24%. This finding was consistent with an earlier study from Butajira where 22% of FNAC confirmed cases of LNTB were HIV positive (Yassin *et al*, 2003). This finding is in contrast to the findings in other African countries. In Malawi, out of 38 laboratory confirmed cases of LNTB, 32 (84%) were seropositive for HIV (Bekedam *et al*, 1997). Similarly in Tanzania, 75% of LNTB cases were found to be co-infected with HIV (Perenboom *et al*, 1995). A strong association between area of residence and HIV was seen in urban dwellers showing a high presence of LNTB and HIV infection. This might be due to the higher prevalence and transmission of HIV in urban areas than in rural localities. Similar findings were reported in a prospective epidemiological study of HIV-TB co-infection in the Southern region of Ethiopia (Yassin *et al*, 2004). In our study, female gender appeared to be associated with HIV in LNTB patients. This can be explained due to the fact that in Ethiopia HIV prevalence is higher in females (MOH 2004, AIDS in Ethiopia-Fifth Report). HIV-TB co-infection peaked in the age group of 25-34 years which is somewhat later than the peak for TB. This was similarly documented by Yassin *et al* who saw an additional peak in children (Yassin *et al*, 2004).

The diagnosis of tuberculous lymphadenitis has been a challenge in developing countries. Due to limited diagnostic facilities at hand, much of the diagnosis depends on clinical expertise. But there are a number of laboratory methods that could be of help as an adjunct to clinical information. AFB examination of specimens from FNA and biopsy is one of the simple and preliminary investigations that can be done in evaluation of lymph node enlargements. Several investigators have evaluated the effectiveness of these methods. Bekedam *et al* examined fine needle aspirates and biopsy smears with the Ziehl-Neelsen technique and found that this technique contributed little to the detection of tuberculosis (8% and 11% respectively) (Bekedam *et al*, 1997). Perenboom *et al* on the other hand detected AFB in biopsy and FNA smears in 53% and 35% respectively (Perenboom *et al*, 1995). Our study identified AFB in 28% (41/149) of FNA and 25% (37/146) of biopsy specimens. Though the sensitivity of this test was found to be low (33% and 29% respectively), detection of acid fast bacilli alone could justify the initiation of

anti-tuberculosis treatment. This would eliminate antibiotic trials and cumbersome follow up periods, enabling the patient to get treatment as early as possible.

Naked eye examination of FNA material and the cut half of full excision biopsy tissue could provide useful information for diagnosis of TB lymphadenitis especially in field settings where laboratory facilities are limited. Ninety patients (61%) had caseous necrosis in biopsy specimens whereas FNA aspirates contained caseation in 89 (60%) of the material from the total LNTB cases identified by the algorithm. A higher proportion was reported by Bekedam *et al*, who reported that 82% of the excised nodes showed caseation (Bekedam *et al*, 1997). A lower yield in FNA specimens as compared to our finding was seen in Zambia where 41% of the aspirates contained caseous material (Bem, 1996). Combining macroscopic and Ziehl-Neelsen staining of biopsy specimens in our study revealed that a total of 78% (91/117) cases were diagnosed as lymph node tuberculosis whereas the combination of macroscopic inspection and AFB staining of FNA materials detected 79% (92/117) of culture positive cases. The presence of caseation was significantly associated with LNTB ($p=0.002$) in both FNA and biopsy specimens, rendering these methods useful predictors of the presence of tuberculosis. It has, however, to be noted that a high predictive value from this material depends on the stage of the lymph node disease as caseation typically occurs in more advanced stages.

Culture as the gold standard is extremely useful as a diagnostic test for cervical mycobacterial lymphadenitis. Despite its long process time (2-8 weeks), LJ culture makes viable strains available for biochemical, PCR and drug susceptibility testing. In our study, a case is defined as culture positive if FNA and / or biopsy cultures are positive. One hundred seventeen (78%) cases of culture proven LNTB patients were identified from the 150 cases of LNTB clinically diagnosed according to the algorithm. Looking at the site specific context, culture positivity was found to be low in Addis Ababa for both FNA (30%) and biopsy (63%) specimens. This might be due to the fact that the types of patients seen in a central referral hospital are different from those seen at regional level. In these centres more cases of neck tumors and reactive lymph nodes are seen in addition to LNTB (Getachew *et al*, 1999). Our overall finding of 78% culture positivity was found to be higher than that reported by Bekedam *et al* (1997), but found to be

lower than the 88% reported by Perenboom *et al* (1995). Biochemical testing with nitrate reductase and pyrazinamidase tests have shown variable results which was found to be difficult to interpret. The absence of growth of non-mycobacterial strains from our specimens might indicate either low prevalence of such infections in these areas and study populations or a need to improve on our culturing system so that it could accurately identify other strains.

The impact of transmission of *M. bovis* and other mycobacterial strains to the increment of LNTB was found to be null in our study. None of the isolates were *M. bovis* on examination both by culture and with PCR. This might be due to the possibility that *M. bovis* concentrates in focal areas with characteristics that favour its transmission including the burden of bovine TB in cattle of the respective areas and dietary practices of the community. It is also possible that the conditions of culture decontamination or specimen processing did not favour *M. bovis*. These should be addressed further so that a better picture of the role of *M. bovis* in LNTB in Ethiopia is obtained. Bovine tuberculosis is an important livestock problem in Ethiopia.

Cytology examination of FNA specimens is useful for the diagnosis of lymph node tuberculosis (Patil and Bem, 1993). The diagnostic value of FNA cytology as an inexpensive and reliable tool had been studied by a number of investigators in Ethiopia (Bezabih *et al*, 2002; Getachew and Tesfahun, 1999). They have emphasized the need to include FNAC in the national algorithm as a diagnostic means instead of biopsy, which is more invasive, needs a surgical set up and is considered to be costly. In our study, FNAC identified 62% (92/149) of the total cases diagnosed as LNTB with the algorithm. Out of this, 88 cases were culture proven LNTB, giving it a sensitivity of 76% with a specificity of 88%. This is consistent with other findings that showed FNAC to have a diagnostic accuracy between 75%-85% (Ammari *et al*, 2003; Finfer *et al*, 1991).

FNAC identified 76% of culture and histology proven cases of LNTB. In addition, its sensitivity is comparable to that of culture (FNA and/or biopsy) and is considerably higher than FNA ZN stain for AFB and macroscopic examination of FNA. Combination of FNAC and FNA ZN stain

for AFB detected 76% (89/117) cases of culture proven LNTB, whereas combining FNAC and macroscopic examination of FNA specimens detected 93% (109/117) of the cases. Further combination of all the three methods increased the detection rate to 96% (112/117) of culture proven LNTB cases. FNAC in Addis Ababa alone, where the technique is performed by a pathologist has detected 28 out of the 30 culture proven cases. However, the overall low culture positivity in Addis Ababa could be explained by the fact that more cases of reactive and neck tumors were seen as patients were recruited from a central referral hospital.

The total number of slides read as non-diagnostic was high (15%) as not all the slides in the field were prepared by a pathologist due to unavailability of such a professional. Notwithstanding this shortcoming of FNAC technique, it was possible to correctly diagnose a considerable number of subsequently culture proven cases of LNTB.

Histopathology diagnosis of lymph node tuberculosis requires excision of lymph nodes, which involves trained personnel, a set up for performing minor surgery, preservation and processing of specimens and a pathologist to read the slides. It requires having an appropriate set up in place, with well equipped laboratories and trained professionals. Because of lack of such facilities, performance of excision biopsy will be cumbersome, if not prohibitively complicated in the field. However, it is undeniable that the method can provide an acceptable sensitivity and specificity for diagnosis of LNTB (92% and 88% respectively in our study). This is comparable to observations in Tanzania and Malawi where the diagnostic sensitivity and specificity was 85% and 82% respectively (Perenboom *et al*, 1995; Bekedam *et al*, 1997).

In our study, both FNAC and histology have shown that most of the AFB positive cases were seen in patients who had a cytomorphologic picture consistent with granuloma plus necrosis or necrosis only. A similar observation was made by Bezabih *et al* who examined 128 cytologically diagnosed cases of LNTB (Bezabih *et al*, 2002). This confirms the association between disease stage and presence of AFB.

4.1.1 The Algorithm of the TB and Leprosy Control Programme of Ethiopia

The national (TLCP) algorithm for the diagnosis of lymph node tuberculosis designed in 2002 aims to identify LNTB patients mainly on the basis of clinical picture and response to broad spectrum antibiotics. It involves some clinical investigations such as sputum examination and chest X-ray when signs and symptoms of tuberculosis are present. We have seen that routine examination of sputum for AFB in LNTB patients is far from being practiced, partly because most patients usually do not produce sputum or do not have a cough, as also seen in our study. The same holds true for chest X-ray, though the benefit of a chest X-ray as an adjunct to the clinical picture is undeniable. The algorithm also uses biopsy as the ultimate diagnostic means in the diagnosis of enlargement of lymph nodes. The inclusion of biopsy alone as the only confirmatory procedure without including less invasive and as effective techniques as FNAC makes the algorithm less practical and desirable to strictly adhere to, since excision biopsy can only be performed in a limited number of health facilities.

This algorithm separates patients on the basis of signs and symptoms complex into two categories: those selected for a trial with antibiotics and those routed for clinical investigations. Here the first problem emerges since only a minority of patients with LNTB has classical signs and symptoms of tuberculosis. This was confirmed in our study with only 10% (11/117) of the culture proven LNTB patients having signs and symptoms complex of tuberculosis. In fact there are no signs and symptoms that could act as a marker of LNTB. Therefore, taking the presence or absence of signs and symptoms of tuberculosis as the only criterion to put the patients on antibiotics may result in a considerable number of patients who actually do not deserve antibiotics being put on treatment. As was seen in our study, two thirds of the study participants were put on antibiotics whereas the majority were found to be culture positive for *M. tuberculosis*.

There is a need to reduce the number of patients that are put on antibiotic trials as this may result in inappropriate use of limited resources and misuse of antibiotics. Performing simple field laboratory procedures such as AFB staining of FNA materials and naked eye examination of the

specimens in addition to the clinical information gathered, will prevent some patients from being subjected to inappropriate antibiotic trials. One could argue that the sensitivity of AFB is quite low and does not detect all LNTB patients. It was seen from this study that patients outside of Addis Ababa present quite late to health facilities and that disease stage is advanced. In such cases, the likelihood of identifying AFB and caseation in aspirates is high making these procedures valuable in circumstances where delayed presentation of cases is the norm. Under such circumstances combining AFB and macroscopic examination of FNA materials could detect 79% of culture positive cases as evidenced in our study. On the other hand, in health facilities where FNAC examination is possible, it is imperative that this procedure is performed in combination with the others. Combining all the three techniques has identified 96% of culture positive LNTB patients in our study. In addition FNAC has been proven to be a simple, reliable and inexpensive tool in the diagnosis of tuberculosis. Our study showed that the sensitivity of FNAC was 76% and this can be improved to 90% with improved training in the technique of aspiration, smearing and staining of the slides (Patra *et al*, 1983).

Out of 150 patients included in the study, 117 were found to be culture positive LNTB patients. This amounts to detection rate of 78% of culture confirmed cases of LNTB. This is a considerable proportion, even though some selection bias resulting from the study physicians' practice cannot be ruled out. On the other hand, the absence of signs and symptoms complex could have shifted the patients to the route of antibiotic trial according to the algorithm and could have caused delay in initiation of treatment and allowed further progression of the disease. In addition to delay and progression, those patients that could have been identified earlier if simple aspiration procedures were performed at prior visits, were subjected to unnecessary antibiotic treatment and several visits to health facilities.

In areas where facilities for FNAC and biopsy are present, the use of the algorithm may be irrational since appropriate diagnostic tests will give a rapid clue to diagnosis and one does not need to enter into antibiotic trial. Therefore, as the algorithm is designed in such a way that its use is justifiable only in limited diagnostic settings, consistent use of it will be compromised.

Even in the field situation it has been difficult to consistently use the algorithm since it involves putting just about every patient with gland enlargement on antibiotics trial that goes against a good clinical judgment. Indeed the universal usage of the algorithm may be unrealistic unless it will be customized to different settings with due consideration of the available laboratory techniques.

Though TB-HIV co-infection in Ethiopia rates lower than in some of the other African countries, the role of HIV infection in the development of extra-pulmonary tuberculosis cannot be ignored. A major step forward in the control of the spread of HIV and tuberculosis would be to integrate these two fields in TB/HIV collaboration work. These collaborative activities can be implemented by including counselling and testing (VCT) services in the algorithm so that confirmed cases of LNTB patients as per the algorithm could have access to these services and benefit from counselling and possible Co-trimoxazole prophylaxis as well as ART.

In conclusion, it can be said that the current national algorithm has identified a substantial number of cases of LNTB patients as was proven by LJ culture (gold standard). Over diagnosis can be reduced if strict usage of the algorithm is in place even though one has to consider that this reduction is associated with the various constraints and disadvantages mentioned above. The misuse of antibiotics, delay in treatment, progression of disease, several unnecessary follow up periods and the irrationality of algorithm usage in health institutes with adequate diagnostic facilities, are some of the issues to be considered. Taking this discussion into account, a modified version of the algorithm has been proposed and is attached as an appendix II along with the previous one for the sake of comparison.

Limitations of the study

- Our study population might have been selected and may not ideally represent the general population with LNTB. This is particularly so because of the procedures involved (excision biopsy) and the need to consent for HIV testing.
- Our culturing system may have not been able to support the growth of *M. bovis*, limiting our ability to draw conclusions on the role of *M. bovis* transmission for the increase of LNTB.
- Reduction of the study site and sample size from that proposed originally might have limited our ability to analyze more samples and get more information that will further enrich our conclusions.

Conclusions

The national algorithm currently in use has, in the strict sense, identified a considerable number of subsequently culture proven cases of LNTB. We have also seen that the consistent use of the algorithm in places with good diagnostic facilities and even in health care systems with limited facilities has been difficult. The lack of a comprehensive algorithm, suitable for both settings could have contributed to a higher than expected number of cases being reported as LNTB. In addition, the use of the algorithm had a number of disadvantages which we have discussed in the previous section. The key role that laboratory investigations such as FNAC play in the diagnosis of LNTB can not be overemphasized. FNAC along with other simple lab procedures is currently not included in the algorithm. It is strongly recommended to include FNA and FNAC in the algorithm so that patients could be managed appropriately.

The role of HIV in development and progression of extra-pulmonary tuberculosis has been a prominent factor for high reporting of this form of TB in African countries. A few studies conducted in Ethiopia on HIV and tuberculosis, especially extra-pulmonary tuberculosis, showed that the percentage of co-infection is lower than in some other African countries. This relatively lower rate might not make HIV the single prime cause for increment of lymph node tuberculosis but its contribution could not be adequately determined. Its impact could, however be potentiated by socio-economic factors that work synergistically to add to the already heavy burden of tuberculosis. Poverty, which has been the mark of Ethiopian farmers, and the high illiteracy rate with alternating drought and famine, could make them more vulnerable to this type of disease. Women being the most vulnerable group of the society were found to have a high HIV-TB co-infection rate. This is compounded by the fact that females in this category have a poor educational and economic background and were from an urban setting where HIV transmission is rampant.

We could not confirm the role of *M. bovis* transmission in the epidemiology of LNTB as this micro-organism was not identified in any of the study subjects. However, we should refrain from drawing a generalized conclusion about the role of *M. bovis* transmission in LNTB in Ethiopia at

this juncture. Further studies have to be conducted in communities (including pastoralists) where bovine TB could play a more important role in LNTB. In addition, the need to improve our culture system and the reduction of the cultivation time by introduction of automated culture systems has to be dealt with to avoid a premature conclusion. *M. tuberculosis*, as the dominant and only causative agent of LNTB in this study has to be studied in terms of its similarity to *M. tuberculosis* strains involved in pulmonary tuberculosis. There is unabated transmission of *M. tuberculosis* in the community and the TB control program was unable to halt this phenomenon with detection rate of smear positive cases at 36%.

Recommendations

Based on the above discussions the following recommendations are made:

- Further expert evaluation and adoption of the proposed algorithm and rigorous adherence to its usage will improve the detection and management of LNTB in Ethiopia.
- FNAC as a simple and cost effective procedure should be adopted as primary diagnostic method in suspected LNTB cases. As part of their training, physicians should acquire the skill to perform fine needle aspiration and to have the expertise and knowledge of basic pathology that will enable them to read FNAC slides.
- Capacity building of regional laboratories with regard to their ability to carry out FNAC in terms of materials and appropriate professionals is worth encouraging.
- Setting up of tuberculosis reference laboratories at regional levels so that they are able to carry out their own diagnostic, surveillance and monitoring activities should be promoted.
- Increase case detection and decrease defaulting rates through community involvement.
- TB/HIV collaboration has to be strengthened, if control of both diseases is to be achieved.
- Strain typing of both pulmonary and lymph node isolates is necessary to understand transmission dynamics.
- Carry out further work on detection and isolation of bovine TB by expanding this work in particular to the pastoralist areas, but also to other foci with high reporting rates for TBLN.
- Introduction of new, improved and fast culture systems like the MIGT liquid culture system to appropriately equipped reference laboratories will help improve quality of research.
- Determine the drug sensitivity patterns of LNTB isolates in order to better understand transmission dynamics and treatment alternatives.

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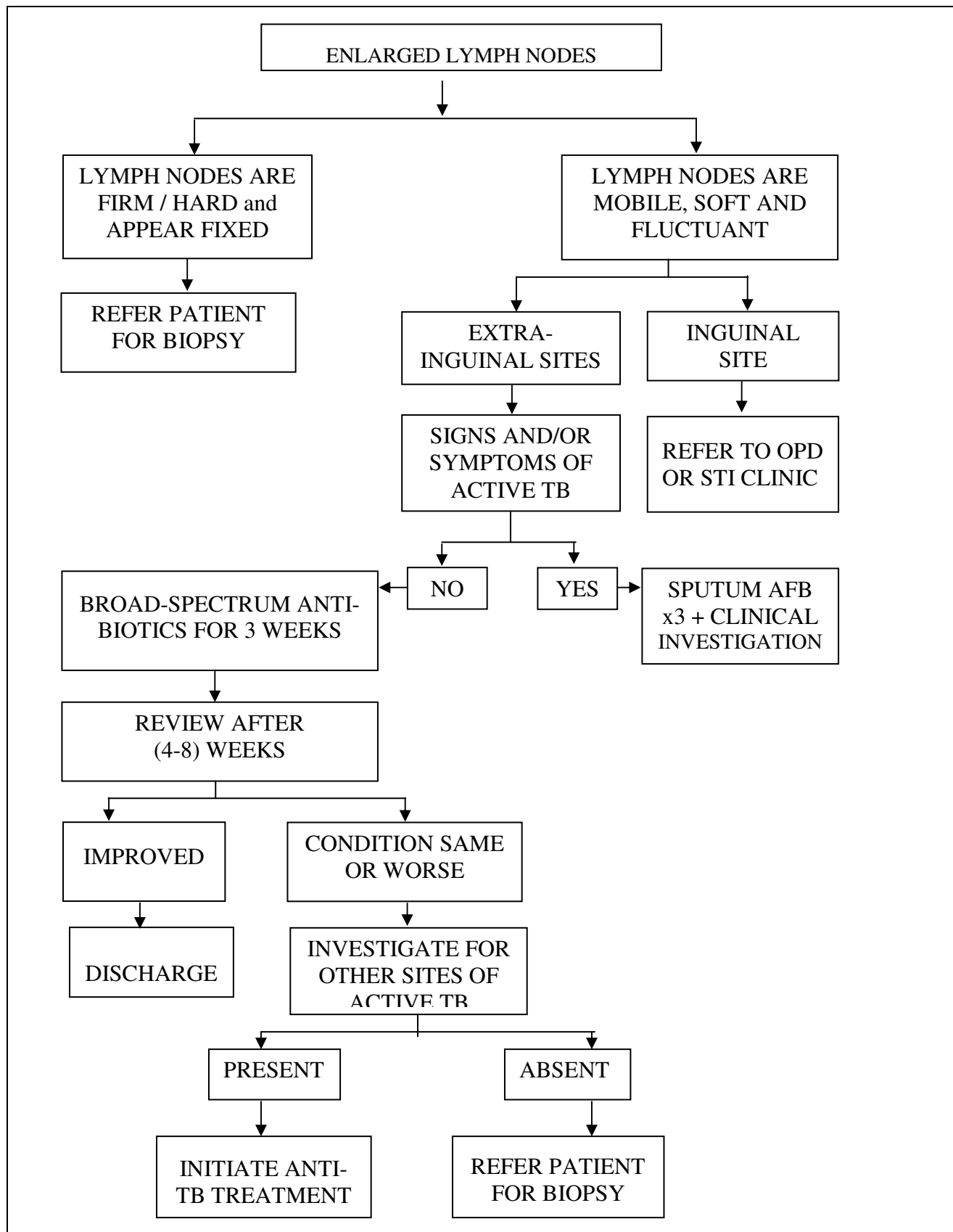
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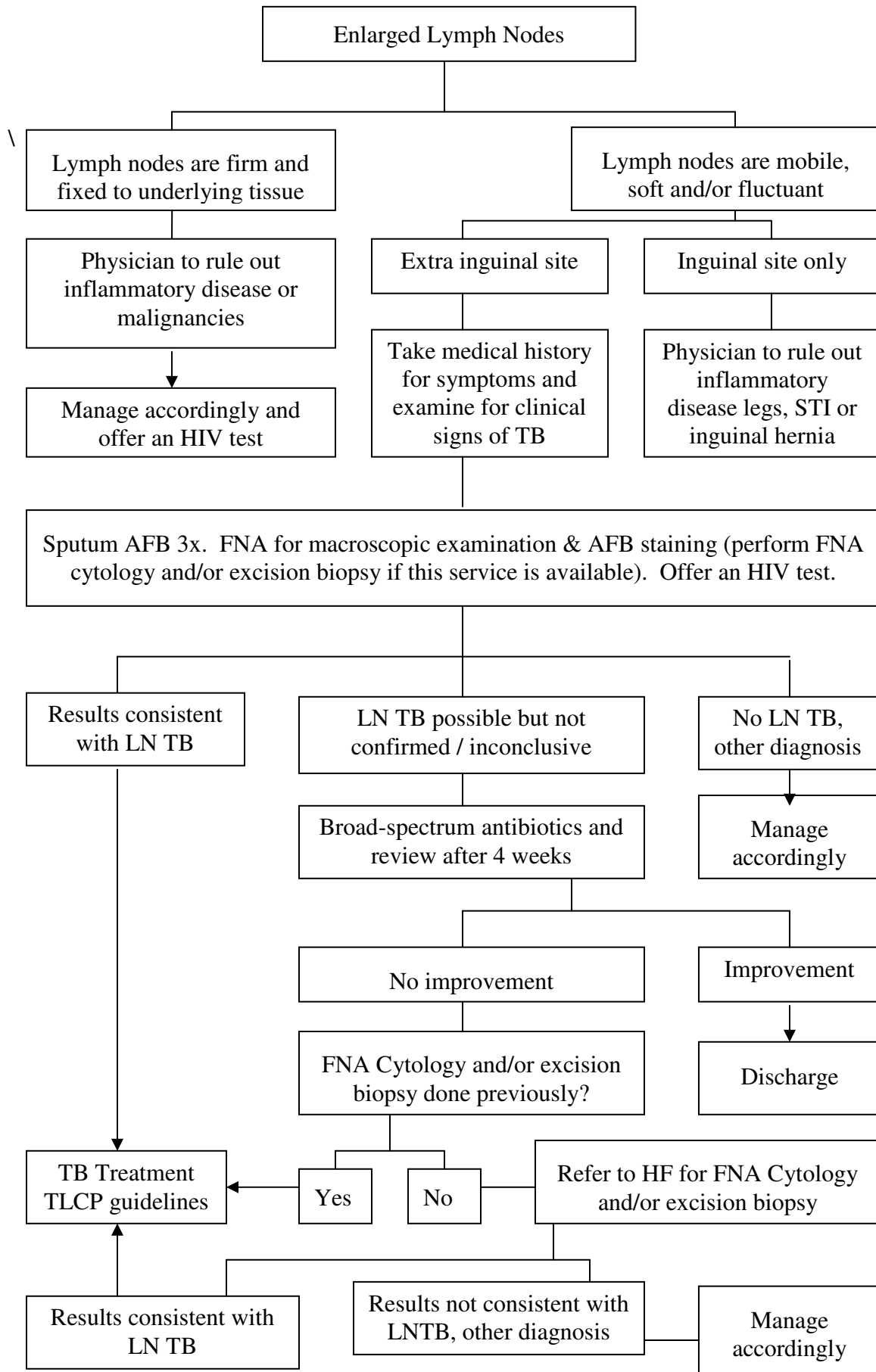
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Appendix I. The national algorithm for management of lymph node enlargement



Appendix II. Proposed algorithm for the management of lymph node enlargement



Appendix III

Case Record Form

Name of the hospital _____ Code No. _____
Card No. _____

1. Age (in years) _____

2. Sex 1. Male 2. Female

3. Address 1. Urban Kef. _____ Keb. _____
 2. Rural House No. _____

4. Occupation

1. Merchant 2. Student
 3. Housewife 4. Government employee
 5. Farmer 6. Others _____

5. Educational Level

1. Illiterate 2. Primary School
 3. Secondary School 4. Diploma
 5. Degree 6. Others _____

6. Martial status

1. Single 2. Married
 3. Divorced 4. Widowed
 5. Living with Partner

7. Ethnic group

1. Oromo 2. Amhara
 3. Tigre 4. Somali
 5. Harrari 6. Gurage

7. Others _____

8. Religion

1. Christian

1.1 Orthodox

1.2 Protestant

1.3 Catholics

1.4 Others _____

2. Muslim

Clinical data

9. Fever

1. Yes

2. No

If yes, 1. High

2. Moderate

3. Low

10. Weight loss

1. Yes

2. No

If yes, 1. Slight

2. Moderate

3. Significant

11. Night sweat

1. Yes

2. No

12. Poor appetite

1. Yes

2. No

13. Weakness

1. Yes

2. No

14. Cough

1. Yes

2. No

15. The duration of the neck swelling (in weeks) _____

16. Rate of increase of the neck swelling

1. Slow

2. Moderate

3. Fast

17. Presently felt pain at the swelling site

1. Present

2. Absent

18. Previous treatment for the swelling

1. Yes

2. No

If yes,

1. Traditional (herbal)

2. Modern

19. 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

20. History of anti-tuberculosis treatment previously

1. Yes

2. No

If yes, had they

1. finished the course

2. discontinued

21. History of contact with tuberculous patients

1. Yes

2. No

If yes, who was it?

1. Family member

2. Neighbors

3. Friends

4. Others _____

22. Contact for how long

1. Days

2. Weeks

3. Months

23. History of intake of raw milk

1. Yes

2. No

24. Living in same household with livestock (cattle, calves, sheep, goats, etc)

1. Yes

2. No

25. In regular direct contact with livestock

1. Yes

2. No

26. History of BCG vaccination (for children)

1. Yes

2. No

Physical examination

LN description (more than one variable is possible)

27. Location

1. Unilateral

1.1 Right sided

1.2 Left sided

2. Bilateral

28. Position

1. Anterior cervical

2. Posterior cervical

3. Supraclavicular

29. Tenderness

1. Tender

2. Non-tender

30. Number of nodes

1. Single node

2. Few nodes (2-4)

3. Multiple nodes (>5)

31. Size (approximation) _____ cm

32. Mobility

1. Mobile

2. Non-mobile

33. Condition of the nodes

1. Discrete 2. Soft 3. Matted
 4. Hard 5. Firm 6. Fluctuant
 7. Draining sinus 8. Other discreptions _____

34. Other pertinent findings

LABORATORY RESULTS

35. Hgb _____

36. WBC _____
& diff. _____

37. ESR _____

38. Chest X-ray

(The above results are recorded if they are available)

39. Sputum for AFB

1. Done 2. Not done

If done, 1. Positive 2. Negative

Appendix IV

Patient Consent Form

Name of the Institute _____
Card No. _____

I have been informed fully in the language I understand about a research project that aims at identifying the cause of neck swelling in patients. I have been asked to participate in the study voluntarily. If I take part in the research, fine needle aspiration will be taken from the neck swelling and the same swelling will be removed for detailed examination. It was explained to me that this procedure is the generally accepted method for accurate diagnosis of such swelling. The procedure is done free of charge and the results will be made available for my physician and I will receive treatment free if the neck swelling is due to tuberculosis. Any complication arising from the procedure will be taken care of by the projects' physician. It is also explained to me that small scar is a possible risk after excision. If I prefer not to participate in the study, I will still receive all the care required for my condition without any prejudice to me. I can also withdraw my consent at any time I want.

In addition, I have been advised to give about two table spoonfuls of blood for HIV testing, which will be made available free of charge. I will be given pre-and post-test counselling. I have the option of learning about the HIV test result if I so prefer .I have been advised this is the best thing to do. However, I have a right not to know if I do not want. The information will not be given to anyone else without my permission. If I happen to be seropositive for HIV, I will be provided with the best local standard care including treatment of opportunistic infections. I will also join the local HIV care structure for group support or any other benefit that might cross my path including the possibility of getting anti-retroviral drugs. When the system for getting anti-retroviral drug for free is established in the country, I will be linked to a governmental health institution where this provision is in place in order that I might receive this service. I am aware that this project will not provide antiretroviral medications for HIV positive individuals.

I have been asked to give my consent for the leftover materials taken from the swelling for research on TB to be stored at AHRI to answer relevant questions that might arise at a later date. The same material will be sent to the Supra-National research laboratory in the Netherlands for cross checking of results obtained at AHRI. All my clinical information will be kept in strict confidence.

I agree to participate in the study after receiving clarification for my questions and sufficient time to think about the request. By participating in this project, I have contributed to the better diagnosis and management of lymph node TB and lent a hand in the struggle that is ongoing to eradicate TB in our country.

Name of the patient

Physician signature

Signature

Witness signature

Date _____

Date _____

I do not want to know my HIV result

I wish to know my HIV result

Appendix V

**Information sheet and consent form for individuals who participate
in the research “Dilemmas in the diagnosis of lymph node
tuberculosis in Ethiopia : A study from four with
high notification of lymph node tuberculosis”**

ARMAUER HANSEN RESEARCH INSTITUTE (AHRI)

Name of the Principal Investigator	Rahel Iwnetu
Name of Organization	Armauer Research Institute
Name of sponsor	Global fund for TB, malaria and HIV AHRI Addis Ababa University

Information Sheet for the group of individuals attending the Medical out patient Department and who will participate in the research “Dilemmas in the diagnosis of enlargement of lymph node tuberculosis in Ethiopia: A study from six sites with high notification of lymph node tuberculosis”

This information sheet is prepared by the groups that are formed to investigate into the possible causes that are responsible for the rise of swelling of the neck due to tuberculosis. The group consists of Surgeons, General Practitioners, laboratory Technicians, and site coordinators from the six selected sites and the chairman of the TB & Leprosy Control and members of this team, WHO advisor for the TB & Leprosy Control team, the Head of the Medical Microbiology Department, Deputy Director of AHRI and the principal investigator is final year postgraduate student from the Department of Microbiology.

Purpose

The reason for doing this research is to find out why a high number of swelling over the neck is being reported to the Ministry of Health as tuberculosis cases. This research proposal looks into the possible causes that contribute to the above mentioned rise in our country. One major area to look into will be the flow chart that is being used to differentiate whether a person with swelling of the neck is due to tuberculosis or other diseases that presents with similar picture. If the flow chart is not able to differentiate these diseases then it will have a role in the increment of the above reports. In this case measures to improve it will be taken according to the results of the research. The project also identifies the other factors that contribute to the rise in swelling of the neck due to tuberculosis. It will use various laboratory methods to find out which will be suitable for health institution in our set up. The research also identifies the types of bacteria that are involved and to which types of drugs used in tuberculosis treatment they are affected or killed

Procedures

We cordially invite you to participate in the research project that looks into why reports of swelling of the neck due to tuberculosis are rising. The Hospitals and Health Centers of the six different regions of the country i.e. Dire Dawa, Harar, Mekelle, Gonder, Bahr Dar and Addis Ababa will be involved in the study .A total of 300 hundred people with neck swelling due to tuberculosis according to the flow chart will be asked to take part in this research project.

The study will take 18 months. If you take part in this research, the time it takes to be seen by a doctor and for laboratory evaluation is one day. Equivalent time is spent in hospital and Health Centres under ordinary circumstances. Therefore, there will not be fear of spending extra time in your part due to the project.

If you are willing to participate in the study, you will be asked to sign a consent form. You are only required to sign after you are given full explanation and received an answer to your queries. You will be asked to give consent for taking a small amount of fluid from the swelling by a needle and syringe. Under local anaesthesia the same swelling will be removed. Both the fluid and the removed swelling will be sent to Addis Ababa for further examination. We would like to explain that this

procedure is the generally accepted method that is used to identify tuberculosis of swellings of the neck.

You will also be asked to give two table spoonfuls of blood once for HIV testing. You will have counselling services before and after testing. Before that we will give you a chance to think and deliberate about it. You will be counselled by health professionals trained in this field on the pros and cons of taking the HIV test. Ultimately the decision to be tested or not is yours. If you decide to take the test, the testing and the counselling will be done free of charge .The result of the test will be kept in strict confidence. Though we encourage you to know your test result, your right to know or not to know is respected.

Consent will be asked from you for the blood and the material taken from the swelling to be stored at AHRI to answer questions that might arise at a later date and some will be sent abroad (Netherlands) for confirmation of results.

Risk and discomfort

You might feel anxious and worried during the process of taking blood and minor operation. Slight bleeding and small scar at the operation site can occur occasionally. Any other illness related to the operation will be taken care of by the doctor who is responsible for treatment and follow up free of charge.

Benefits

If you participate in this research, you will get the following benefits. There will be no payment for the laboratory expenses and for collecting the laboratory samples. If you are found to be HIV positive, you will be provided with the best local standard care including treatment for opportunistic infection. You will be encouraged and strongly advised to join the local HIV care structures for group support and any other benefit including the possibility of getting antiretroviral drugs. Although this project will not provide antiretroviral drugs for HIV positive individuals, it strongly support the initiative that is already been taken to provide the drug for free for those who cannot afford to cover the treatment expense by themselves. Your participation will improve the method used to identify swellings of the neck due to tuberculosis and to plan preventive programmes that

will help to reduce this disease to the minimum. This will lend a hand to the tuberculosis control program that is already underway in our country.

Incentive

You will not be given any incentive except for the benefits mentioned above.

Confidentiality

The information gathered about your health and the laboratory results especially that concerning HIV testing will be kept in strict confidence. Results of HIV testing will not be given to anyone else without your permission. Information about you that will be collected from the study will be stored in a file which will not have your name on it but a number assigned to it. Which number belongs to which name will only be known by the project's sponsors, principal researcher and your doctor and will be kept under lock and key.

Right to refuse or withdraw

Your participation in this research is wholly dependant on your willingness to do so. It will in no way affect your right to acquire health care and treatment if you do not wish to participate in the project. You will still have all the benefits that you would have and will not affect your treatment at this centre in any way. Your right to discontinue participating in this research and withdraw your consent will be respected. Your treatment at this centre will not be affected in any way on account of your decline from participation in this research.

Who to contact

This proposal has been reviewed and approved by the AHRI/ALERT Ethical Committee .If you want to know more about the committee, you can talk to Dr Howard Engers the Committee's chairman at telephone number 710288 Addis Ababa. It is also approved by the National Ethical Committee .The task of these committees is to make sure that research participants are protected from harm. If you wish to find out more about the National Ethical Committee, contact the chairman Dr Yemane Teklaye, Telephone 511447, Addis Ababa.

If you have any questions you may ask now or later. If you wish to ask questions later, you may contact any of the following

Dr Abraham Assefa, AHRI, Telephone 710288, Addis Ababa

Dr Birhane Kidanemariam, TB & Leprosy Control Team, Telephone 530508, Addis Ababa

Dr Omer Mohamed, TB & Leprosy Control Team, Telephone 530508, Addis Ababa