



Addis Ababa University

School of Graduate Studies

**MOLECULAR TYPING OF MYCOBACTERIA ISOLATED
FROM TUBERCULOSIS PATIENTS AT DEBRE BIRHAN
REFERRAL HOSPITAL, NORTH SHOA.**

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**A Thesis Submitted to the School of Graduate Studies of Addis Ababa
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MSc Thesis

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Declaration

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

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List of abbreviations

AFB	Acid Fast Bacilli
AIDS	Acquired Immunodeficiency Syndrome
ALIBP	Aklilu Lemma Institute of Pathobiology
BCG	Bacille Calmette Guerin
BTB	Bovine Tuberculosis
CD	Cluster of Differentiation
CSF	Cerebro-Spinal Fluid
DNA	Deoxyribonucleic Acid
DR	Direct Repeat
EA	Euro-America
EAI	East-Africa India
ELISPOT	Enzyme Linked Immunospot
EPTB	Extrapulmonary tuberculosis
FMoH	Federal Ministry of Health
FNA	Fine Needle Aspirate
HIV	Human Immunodeficiency Virus
IGRA	Interferon Gamma Release Assay
LJ	Löwenstein-Jensen
MA	<i>Mycobacterium africanum</i>
MTC	<i>Mycobacterium Tuberculosis</i> complex
PCR	Polymerase Chain Reaction
PTB	Pulmonary Tuberculosis
RD	Region of Difference
rRNA	ribosomal Ribonucleic Acid
ST	Spoligotype
TB	Tuberculosis
TBLN	Tuberculosis Lymphadenitis
UK	United Kingdom
USA	United States of America
WHO	World Health Organization

Abstract

Introduction: Tuberculosis (TB) is one of the major killers among infectious diseases in the world. Each year an estimated 8.9 to 9.9 million incident cases and approximately 1.55 to 2.32 million deaths due to TB occur worldwide. Ethiopia ranks 7th in the list of world's 22 high burden countries for TB with incidence estimated at 379 persons per 100,000 persons for all forms of TB. Based on preliminary assessment about the disease and the research gap in the study setting, a cross-sectional study was carried out from November 2010 to June 2011 on 99 smear positive pulmonary and 98 smear negative extrapulmonary TB patients at Debre Birhan Referral Hospital.

Objective: Molecular characterization of *Mycobacterium* strains implicated in tuberculosis.

Materials and methods: Structured questionnaire, acid fast bacilli smear staining, culture, deletion typing and spoligotyping were used in the study.

Results: The proportional distribution of TB disease and isolates were not varied substantially ($p>0.05$) either with age, sex, residence area, occupation, previous contact with TB patients, previous treatment with antibiotics or antimycobacterial drugs, habit of raw animal product consumption or with close contact with livestock for both pulmonary and extrapulmonary TB combined. Out of 99 sputum and 98 fine needle aspirate samples, 80.80% (80/99) and 36.73% (36/98) of them were culture positive respectively. Speciation of isolates using region of difference-9 (RD9) deletion typing showed that 90.00% (72/80) of the isolates from sputum and 88.89% (32/36) of the isolates from fine needle aspirate were *M. tuberculosis*, while only 1.25% (1/80) and 2.78% (1/36) were *M.bovis* respectively. Further characterization of 97 *M. tuberculosis* isolates to the strain level using spoligotyping resulted in the identification of 25 clusters that constituted 80.41% (78/97) of the isolates tested out of which 17 clusters were new to Ethiopia. The most dominant spoligotypes were spoligo international typing number (ST) 149, 53 and 37 that accounted 32.99% (32/97) of the total spoligotypes identified. Comparison of our spoligotypes with international spoligotype database, SpolDB4, showed 19 new spoligotypes which were clustered into nine clusters and have never been reported from elsewhere in the world. Analysis of non-clustered (new) spoligotypes and their source indicated that 35.71% (10/28) of the isolates from extrapulmonary sources were unique, compared with 13.04% (9/69) of the pulmonary isolates. Classifying strains on the bases of phylogeny of *M. tuberculosis* using SPOTCLUST software revealed that they belonged to Euro-American, East-African Indian and *Mycobacterium africanum* lineages. The most

prevalent lineage in the current investigation was Euro-American constituting 69.07% (67/97) of the strains analyzed.

Conclusion: This research has shown the presence of several clusters and new strains of *M. tuberculosis* circulating both in pulmonary and extrapulmonary TB patients in Ethiopia. As mapping the population structure of *M. tuberculosis* is vital to understand the transmission and disease dynamics TB and set appropriate control measure, further similar studies are recommended.

Key words:

RD9 Typing, Spoligotyping, *Mycobacterium* family, *Mycobacterium* lineage, Tuberculosis, Debre Birhan Referral Hospital.

1. Introduction

Tuberculosis (TB) is one of the major killers among infectious diseases, causing more than 8.9 to 9.9 million new cases and 1.55 to 2.32 million deaths each year [WHO, 2009]. The incidence of tuberculosis has been increasing dramatically throughout the world in the last decade. World Health Organization (WHO) estimates that one-third of the global community is infected with *M. tuberculosis* complex and also estimated 0.7 million cases and 0.2 million deaths in HIV positive people [WHO, 2009]. After HIV/AIDS, TB is the second most common cause of death due to an infectious disease and current trends suggest that it will still be among the 10 leading causes of global disease burden in the year 2020 [Corbett *et al.*, 2003].

1.1 Etiology

TB is an infectious disease caused by *M. tuberculosis* complex (MTC) bacteria which has an endemic character and worldwide distribution. The MTC comprises closely related species responsible for strictly human and zoonotic tuberculosis. The complex consists of seven species and subspecies including *M. tuberculosis*, *M. canetti*, *M. africanum*, *M. pinnipedii*, *M. microti*, *M. caprae* and *M. bovis*. Despite the different species tropisms, the MTC is characterized by 99.9% or greater similarity at the nucleotide level and possess identical 16S rRNA sequence [Dye *et al.*, 2005]. There is little or no exchange of chromosomal DNA between cells from MTC, making this group of bacteria highly clonal. In a strictly clonal population, any mutation present in an ancestral strain will be present in all descendents and can be used to identify clonal complexes [Smith *et al.*, 2006].

The genus *Mycobacterium* is non-motile, non-capsular and non-spore-forming. It is obligate aerobic thin rod usually straight or slightly curved having 1-10 μ m length and 0.2-0.6 μ m width and is facultative intracellular parasite mostly of macrophages and has a slow generation time about 15-20 hours. Its cell wall is rich in lipids that provide it the thick waxy coat which is responsible for acid fastness and hydrophobicity. This waxy coat is also greatly contributing for the bacterium resistance to many disinfectants, common laboratory stains, antibiotics and physical injuries. It probably also contribute to the slow growth rate of some species by restricting the uptake of nutrients [Palomino *et al.*, 2007].

The *Mycobacterium* envelope is composed of typical peptidoglycan layer with free lipids. It also contains free fatty acids, such as mycolic acid that causes the waxy appearance and impermeability of the envelope. Approximately 250 genes within the *Mycobacterium* genome are involved in the fatty acid metabolism. These fatty acid-carbohydrate complexes inhibit phagolysosome fusion in the host and are often considered to be indicators of the virulent strains. The bacterium also has high (61-71%) percentage of guanine plus cytosine (G+C) in the genomic DNA [Brosch *et al.*, 2002].

Once stained, the rods cannot be decolorized with acidic solutions; hence the name acid-fast bacteria. Because the mycobacterial cell wall is complex and this group of organisms is fastidious, most *Mycobacteria* grow slowly, dividing every 12 to 24 hours. Isolation of *M. tuberculosis* complex may require 3 to 8 weeks of incubation and we have to wait at least 6 weeks to be culture negative. This slow growth forms the basis for the chronic nature of tuberculosis infection and disease. Slow bacterial growth further complicates mycobacterial diagnosis and makes long-term drug treatment necessary [Cole *et al.*, 1998].

M. tuberculosis strains can be classified into a number of major clades according to defined evolutionary markers. It is hypothesised that strains comprising these clades have evolved different properties which may influence a local strain population structure. This evolved different properties growth was exclusively attributable to drug-susceptible strains. Recent evidence suggests that these differences likely reflect enhanced pathogenicity rather than transmissibility. The rapid emergence of different strains demonstrates adaptation to conditions within the study community and poses a grave challenge to future TB control [Spuy *et al.*, 2009].

Bovine tuberculosis (BTB) is caused by *M. bovis*, a *Mycobacterium* highly similar to *M. tuberculosis*. The main host of *M. bovis* is cattle but it also affects many other mammals including man. In human, it is the most frequent cause of zoonotic TB which is clinically indistinguishable from TB caused by *M. tuberculosis*. Before milk pasteurization, *M. bovis* was an important cause of human TB especially intestinal TB in children. The development of the polymerase chain reaction (PCR) and other molecular tools to identify *M. bovis* and differentiate it from other members of the *M. tuberculosis* complex have allowed the discovery of more cases in retrospective studies and have suggested new forms of transmission [Ojo and Sheehan, 2008].

Although tuberculosis may manifest itself at any tissue, the lung represents the main port of entry and is an important site for disease manifestation. Extrapulmonary tuberculosis (EPTB) develops in about 10% of all cases. Extrapulmonary sites include the pleura, central nervous system (Meningitis), lymphatic system (Scrofula of neck), genitourinary system, abdominal cavity organs, bones and joints (Pott's disease of the spine) [Murray *et al.*, 2005].

1.2 Pathogenesis

M. tuberculosis complex usually enters the alveolar passages of exposed humans and animals in an aerosol droplet where its first contact is thought to be with resident macrophages. But it is also possible that bacteria can be initially ingested by alveolar epithelial type II pneumocytes. This cell type is found in greater numbers than macrophages in alveoli, and *Mycobacterium* can infect and grow in these pneumocytes *ex vivo*. In addition dendritic cells play a very important role in the early stages of infection since they are much better antigen presenters than are macrophages and presumably play a key role in activating T cells with specific *M. tuberculosis* antigens. Since dendritic cells are migratory, unlike differentiated macrophages, they also may play an important role in dissemination of *M. tuberculosis* [Issar, 2003].

M. tuberculosis is an intracellular pathogen that able to establish lifelong infection. At the time of exposure, *M. tuberculosis* enters the respiratory airways and minute infectious particles penetrate to the alveoli where they are phagocytised by alveolar macrophages. In contrast with most phagocytised bacteria, *M. tuberculosis* prevents fusion of the phagosomes with lysosomes by blocking the specific bridging molecule called Early Endosomal Autoantigen 1 (EEA1). At the same time the phagosome is able to fuse with other intracellular vesicles permitting access to nutrients and facilitating intravacuole replication. Phagocytised bacteria are also able to evade macrophage killing mediated by reactive nitrogen intermediates formed between nitric oxide and superoxide anions through catalytically catabolising the oxidants that are formed [Murray *et al.*, 2005].

In the majority cases of primary tuberculosis, within 10–14 days a reactive inflammatory focus develops the so-called primary focus from which the TB bacteria move into the regional hilar lymph nodes where they reproduce and stimulate a cellular immune response. This in turn results in clonal expansion of specific T-lymphocytes and attendant lymph node swelling. The Ghon's complex develops between 6 and 14 weeks after infection. At the same time

granulomas form at the primary infection site and in the affected lymph nodes and macrophages are activated by the cytokine macrophage activating factor. A tuberculin allergy also develops in the host. The further course of the disease depends on the outcome of the battle between the *Mycobacterium* and the specific cellular immune defences. Post-primary dissemination foci are sometimes observed as well that is development of local tissue defect foci at other localizations typically the apices of the lungs. Liquefied caseous foci provide excellent conditions for extracellular growth of *M. tuberculosis*. Cavity formation may lead to rupture of nearby bronchi which may allow the bacilli to spread through the airways to other parts of the lung and the outside environment [Crevel *et al.*, 2002].

Mycobacteria may also be transported to other organs via the lymph vessels or bloodstream and produce dissemination foci there. The host eventually develops granulomas and foci fibrose, scar, and calcify but the infection remains clinically silent. In about 10% of infected persons the primary tuberculosis reactivates to become organ tuberculosis either within months or after a number of years. Reactivation begins with a caseation necrosis in the centre of the granulomas (also called tubercles) that may progress to cavitations and frequently stems from old foci in the lung apices. Tissue destruction is caused by cytokines among which tumour necrosis factor alpha appears to play an important role. These cytokines are also responsible for the cachexia associated with tuberculosis. The body's immune defences have a hard time in containing necrotic tissue lesions in which large numbers of *Mycobacterium* cells occur [Kayser, 2005].

1.3 Immunity

The immune response against *Mycobacterium* plays a fundamental role in the outcome of TB infection. The immune system reacts efficiently in the vast majority of infections and that is why around 90% people infected by the tubercle bacillus do not develop the disease throughout their lifetimes. Nevertheless, the risk of developing the disease increases considerably when TB infection co-exists with an alteration in the immune system such as infection with HIV [Palomino *et al.*, 2007].

Alveolar resident macrophages are the primary cell type involved in the initial uptake of *Mycobacterium* species. After this first encounter, dendritic cells and monocyte derived macrophages also will take part in the phagocytic process. Endocytosis of the bacteria involves different receptors on the phagocytic cell. During the first infection with tubercle

bacilli, certain resistance is acquired and there is an increased capacity to localize tubercle bacilli, retard their multiplication, limit their spread, and reduce lymphatic dissemination. This can be attributed to the development of cellular immunity with evident ability of mononuclear phagocytes to limit multiplication of ingested organisms and even to destroy them. With the development of specific immunity and the accumulation of large numbers of activated macrophages at the site of the primary lesion, granulomatous lesions (tubercles) are formed. These lesions consist of lymphocytes and activated macrophages such as epithelioid cells and giant cells. Initially the newly developed tissue damaging response is the only event capable of limiting mycobacterial growth within macrophages. Cell-mediated immunity is critical at this early stage. In the majority of infected individuals local macrophages are activated when bacillary antigens processed by macrophages stimulate T-lymphocytes to release a variety of lymphokines. These activated cells will aggregate around the lesion's centre and can effectively neutralize tubercle bacilli without causing further tissue destruction. T-cell immunity will develop two to three weeks after infection. Subsequent to this phase, the early logarithmic bacillary growth stops. As a result infection may become stationary or dormant. Disease may progress and haematogenous dissemination may take place after primary infection as well as months or years afterwards under conditions of failing immune surveillance [Crevel *et al.*, 2002].

During the initial steps of infection antibodies alone or in conjunction with the proper cytokines may provide important protective functions such as prevention of entry of bacteria at mucosal surfaces. Antibodies could enhance immunity through many mechanisms including neutralization of toxins, opsonisation, complement activation, promotion of cytokine release, antibody dependent cytotoxicity, and enhanced antigen presentation [De Valliere *et al.*, 2005].

Elimination of *Mycobacterium* infection mainly depends on the success of the interaction between infected macrophages and T-lymphocytes. CD4 T-cells exert their protective effect by the production of cytokines primarily interferon gamma (IFN- γ) after stimulation by mycobacterial antigens. Other T-cell subsets like CD8 T-cells are likely to contribute as well by secreting cytokines and lysing infected cells. Phagocytic cells play key role in the initiation and direction of adaptive T-cell immunity by presentation of mycobacterial antigens and expression of co-stimulatory signals and cytokines [Crevel *et al.*, 2002].

1.4 Epidemiology

TB accounts for 2.5% of the global disease burden and ranks seventh amongst causes of death worldwide. In general the global trend in TB incident rate is increasing at 1.5% per year [WHO, 2008]. The global distribution of TB cases is skewed heavily toward low-income and emerging economies. Ninety-five percent of all cases and 99 % of deaths due to TB occur in developing countries with the greatest burden in Sub-Saharan Africa and South East Asia. Africa, more specifically sub-Saharan Africa, has the highest incidence rate of TB with approximately 83 and 290 persons per 100,000 persons respectively [Soolingen, 2001]. In population-based studies from sub-Saharan Africa, where the rate of *M. tuberculosis* transmission is very high, the proportion of clustered *M. tuberculosis* isolates from patients with smear-positive TB has varied from 38 to 47%. The proportion of isolates appearing in clusters was even higher (67%) for isolates from male patients in a gold-mining community in South Africa with a particularly high incidence of TB [Wilkinson *et al*, 1997].

TB cases occur predominantly in the economically most productive 15 to 49 years old age group. Our understanding of TB epidemiology and the efficacy of control activities have been complicated by the emergence of drug resistant bacilli and by the synergism of TB with HIV co-infection which fuels the epidemic of TB on a large scale [Dye, 2005]. This risk was previously attributed mainly to an increased risk of reactivation of a latent infection. Studies using DNA fingerprinting of insertion element IS6110 of the *M. tuberculosis* genome has been showed that nearly two-thirds of *M. tuberculosis* isolates from HIV-infected patients appear in clusters suggesting recent infection. Further support for this suggestion is provided by DNA fingerprinting of nosocomial TB outbreaks including transmission of multidrug resistant strains [Soolingen, 2001].

At least one-third of HIV-infected persons worldwide are infected with *M. tuberculosis*, and 8% to 10% of them develop clinical disease every year. The African region accounted for most HIV-positive TB cases (79%), followed by the South East Asia region which had 11% of total cases. The prevalence of HIV infection among patients with TB ranges from 50% to 80% in many settings in Sub- Saharan Africa but in other parts of the world it varies from 2% to 15% [Swaminathan *et al.*, 2010].

Genotyping analysis demonstrates different clonal populations depending on the geographical region under study. Due to the consumption of raw milk in regions where AIDS is highly

prevalent, many studies concentrated on patients having lymphadenitis. Importantly, there are no reports of human infection by *M. bovis* coming from a direct environmental source. There are several reports about the incidence of zoonotic TB in Africa [Biet *et al.*, 2005].

Molecular epidemiology is a powerful approach for monitoring infectious diseases. It is particularly important in the study of chronic diseases such as TB. Moreover; it can give a unique insights into the international dissemination dynamics of TB by the comparison of isolates from widespread geographic areas and allows one to analyze evolutionary changes of pathogen populations [Supply *et al.*, 2001].

Molecular epidemiologic studies of TB have focused largely on utilizing molecular techniques to address short and long term epidemiologic questions such as in outbreak investigations and in assessing the global dissemination of strains respectively. This is done primarily by examining the extent of genetic diversity of clinical strains of *Mycobacterium* species. When molecular methods are used in conjunction with classical epidemiology, their utility for TB control has been realized. Molecular epidemiologic studies have added much needed accuracy and precision in describing transmission dynamics. They have also facilitated investigation of previously unresolved issues such as estimates of recent versus reactive disease and the extent of exogenous re-infection. More recent issues include the impact of HIV co-infection on TB transmission. In addition, there is a mounting evidence to suggest that specific strains of *Mycobacterium* species belonging to discrete phylogenetic clusters (lineages) may differ in virulence, pathogenesis, and epidemiologic characteristics [Mathema *et al.*, 2006].

M. tuberculosis complex genome is highly conserved in relation to other bacterial pathogens and this monomorphic species does have polymorphic genomic regions. They are characteristically punctuated by monomeric sequences repeated periodically (repeated units). There are two types of repetitive units, interspersed repeats (IR) which are direct repeats and insertion sequence-like repeats and tandem repeats (TR) which are head-to-tail direct uninterrupted repeats [Ferdinand, 2004].

Molecular techniques have made possible to identify risk factors for clustering and by extension for the recent transmission and rapid progression of clinical TB. Nowadays, there exist a number of different working tools which are used routinely or for special occasions like Genomic Deletion Analysis, Spoligotyping, Variable Number of Tandem Repeats Typing

(VNTR), IS6110 Restriction Fragment Length Polymorphism (RFLP) and Long Sequence Polymorphism [Supply *et al.*, 2001].

1.5 Transmission

In about 95 % of cases, TB is an airborne disease, transmitted by particles or droplet nuclei that are expelled when persons who have pulmonary TB (PTB) or laryngeal TB sneeze, cough, speak or sing [Feja, 2005]. Droplet nuclei containing between one to ten bacilli and a diameter close to 10µm are expelled with the cough, suspended in the air and transported by air currents. Normal air currents can keep them airborne for prolonged periods of time and spread them throughout rooms or buildings. Some of these droplet nuclei, usually larger than 10µm are inhaled and anchored in the upper respiratory tract [Wells, 1995]. The effective infective droplet nucleus is very small; measuring 5µm or less, it is able to avoid the mucus and ciliary system action and produce the anchorage in bronchioles and respiratory alveoli. The small size of the droplets allows them to remain suspended in the air for prolonged periods of time. Although theoretically a single organism may cause disease, it is generally accepted that about 5 to 200 inhaled bacilli are necessary for a successful infection. As in adults, childhood TB is mostly due to *M. tuberculosis* [Palomino *et al.*, 2007].

The presence of extensive pulmonary lesions such as cavities is the most important individual human factor in determining the infectious power. Since extensive pulmonary lesions are associated not only with an important concentration of oxygen that allows active bacillary multiplication but also with a rapid pathway to the external environment. The amount of bacilli released into the atmosphere under these conditions is enough to produce the transmission from person to person [Correa, 1997]. The main reservoir of *M. tuberculosis* is the patient with pulmonary tuberculosis. Such patients may have pulmonary cavities that are rich in bacilli. Patients with cavitary PTB are almost always smear positives and are the main source of infection in the transmission of tuberculosis. The number of infectious droplets projected into the atmosphere by a patient is very high when coughing or sneezing. When they come into contact with the air these droplets rapidly dry and become very light particles but still containing live bacilli that remain suspended in the air. In an enclosed space, the droplets can remain suspended for a long time and the bacilli remain alive for several hours in the dark. When a person inhales these infectious particles, the large particles are deposited on the mucous of the nasopharynx or the tracheo-bronchial tree and are expelled by mucociliary

clearance. But the smallest particles having less than a few microns in diameter can penetrate to the alveoli. The closer and the more prolonged the contact with an infectious patient is the greater the risk of infection as this is linked to the density of the bacilli in the air the individual breathes and the amount of the air inhaled [WHO, 2003].

Acquisition of *M. bovis* by humans was mainly related to ingestion of uncooked meat and unpasteurized milk from infected cows. Respiratory transmission is possible mainly in people regularly handling carcasses or offals contaminated with *M. bovis* such as slaughterhouse workers. The proportion of both paediatric and adults TB cases caused by *M. bovis* is very low. It is generally associated with close contact with cattle, and is variable from one country to another and even from region to region inside the same country. Most paediatric TB cases can be traced to a household relative contact. On the other hand, older children may become infected from an external source such as schoolmates, team leaders or young adults outside their home [De la Rúa-Domenech, 2006].

Even though effective drugs to treat the disease have been available for more than 50 years, yet every 15 seconds someone in the world dies from TB. Even more alarming: a person is newly infected with *M. tuberculosis* every second of every day. Left untreated, a person with active TB will infect an average of 10 to 15 people every year [Dye *et al.*, 2005].

1.6 Clinical manifestations

The most common clinical manifestation of TB is pulmonary distress which is insidious at onset. Patients typically have nonspecific complaints of malaise, fever, weight loss, cough, shortness of breath, chest pain and night sweats. Sputum may be bloody if cavitory and purulent [Fauci *et al.*, 2008].

Extrapulmonary TB accounts for about 10% of the disease in HIV seronegative people but is more common in HIV seropositive individuals. Cervical lymphadenitis is common in women and young children. The most serious clinical manifestation of EPTB is involvement of the central nervous system in the form of chronic meningitis as well as tuberculomas. Tuberculosis can affect any bone or joint but the spine is the most common bony structure involved marked by spinal deformity. Any portion of the gastrointestinal tract may also be affected by tuberculosis even if terminal ileum and the cecum are the sites most commonly involved. Abdominal pain, diarrhoea, obstruction, hematochezia, and a palpable mass in the

abdomen are common findings at presentation during gastrointestinal tuberculosis. Tuberculous peritonitis follows either the direct spread of tubercle bacilli from ruptured lymph nodes and intra abdominal organs or haematogenous seeding. Nonspecific abdominal pain, fever, and ascites should raise the suspicion of tuberculous peritonitis. In tuberculous peritonitis, paracentesis reveals an exudative fluid with a high protein content and leukocytosis that is usually lymphocytic. The yield of direct smear and culture is relatively low hence peritoneal biopsy is often needed to establish the diagnosis [Fauci *et al.*, 2008].

Genitourinary tuberculosis is uncommon and is difficult to distinguish from other infections of the genitourinary tract. In men, manifestations include those of prostatitis, epididymitis, and orchitis but the disease can also be presented as a painless scrotal mass. Urine analysis may show red or white blood cell or both with a negative urine culture. In women genitourinary tuberculosis is an important cause of infertility in areas with high tuberculosis incidence [Murray *et al.*, 2005].

Disseminated tuberculosis is defined as involvement of many organs simultaneously and can occur as a result of primary progressive disease or reactivation of latent infection. The clinical manifestation of pulmonary involvement is a miliary pattern rather than an infiltrate in most cases. Not all patients with disseminated disease have pulmonary involvement. Miliary TB accounts for about 1–2% of all cases of TB and about 8% of all forms of EPTB in immunocompetent individuals. The disease is more frequently encountered in immunosuppressed individuals. Mortality is high despite chemotherapy and may be related to delays in diagnosis and other commonly present underlying medical conditions [Sharma *et al.*, 2005].

1.7 Diagnosis

Often, the diagnosis is first entertained when the chest radiograph of a patient being evaluated for respiratory symptoms is abnormal. Radiographic findings suggesting TB include upper lobe infiltrates, cavitory infiltrates, and hilar or paratracheal adenopathy. In many patients with primary progressive disease and those with HIV infection, radiographic findings are more subtle and can include lower lobe infiltrates or a miliary pattern. Tests for the diagnosis of tuberculosis vary in sensitivity, specificity, speed, and cost. Patients with persistent cough lasting longer than two weeks in addition to the other signs should be assessed for TB.

Among children, important diagnostic clues are a history of previous exposure to an individual with TB or evidence of TB infection [Thomas *et al.*, 2003].

The intradermal administration of tuberculin has been used since the early 1900s to assess latent *M. tuberculosis* infection. Although the tuberculin skin test is the best available way to diagnose latent *M. tuberculosis* infection, it has limitations including low sensitivity in immunocompromised patients, cross-reactivity with Bacilli Calmette-Guerin (BCG) vaccine and environmental *Mycobacteria*, and a requirement that patients must return 48–72 hours after the test is done to have the result read [Lee *et al.*, 2002; Thomas *et al.*, 2003].

A wide variety of serological tests for the detection of antibodies in individuals suspected to have TB have also been used to detect active disease. Serology has additional advantages in situations when: the patient is unable to produce adequate sputum, sputum smear results are negative, and if EPTB. A whole blood interferon gamma release assay (IGRA) like the tuberculin skin test assesses cell mediated immunity to tuberculin. IGRA responses are diminished in HIV-infected individuals resulting in low sensitivity in this important population but they may aid in detecting latent infection among certain populations who are at increased risk. Although the IGRA is less sensitive and specific than the tuberculin skin test, responses are less affected by previous BCG vaccination. An enzyme-linked immunospot (ELISPOT) assay has recently been developed which is relatively sensitive and specific in detecting latent *M. tuberculosis* infection [Muzarek *et al.*, 2001].

Active TB is diagnosed by detecting *M. tuberculosis* complex bacilli in specimens. Although many new molecular diagnostic methods have been developed, acid fast bacilli (AFB) smear microscopy and culture on Löwenstein-Jensen (LJ) medium are still the gold standards for the diagnosis of active TB. Especially in low-resource countries, acid fast staining and culture are the only methods available for confirming TB in patients with a clinical presumption of active disease. AFB smear microscopy is rapid and inexpensive and thus is a very useful method to identify highly contagious patients. But AFB microscopy has relatively low sensitivity (40–60%) in confirmed cases of PTB particularly in children and in people living with HIV/AIDS. In AFB microscopy; fluorochrome, Ziehl-Neelsen, and Kinyoun staining methods can be used [Sharma *et al.*, 2005; Swaminathan *et al.*, 2010]. The International Union Against Tuberculosis and Lung Disease (IUATLD) and WHO recommend the Ziehl-Neelsen method under most circumstances [Rieder *et al.*, 2007; WHO, 2009]. Culture is required for definite

diagnosis and is essential for drug susceptibility testing. The organism can take 6 weeks or longer to grow on solid culture media and growth generally occurs within 7–21 days with liquid culture media [Fauci *et al.*, 2008].

Nucleic-acid amplification assays can also be used directly on clinical specimens but they are most reliable in smear positive respiratory samples from patients with previously untreated TB. In such samples, the sensitivity and specificity can be as high as 95% and 98% respectively. DNA fingerprinting can also be useful to identify laboratory cross-contamination and elucidate the epidemiology of TB [Fauci *et al.*, 2008]. One of the recent advances in molecular epidemiology is spacer-oligonucleotide typing (spoligotyping) and has become the most widely used molecular tool for the study of *M. tuberculosis* complex. It has been successfully used for classifying strains with low copies number of IS6110 element. Spoligotyping analysis is based on DNA polymorphism present at one particular chromosomal locus, the “Direct Repeat” (DR) region which is uniquely present in *M. tuberculosis* complex. This locus contains well conserved 36 base pairs (bp) DRs interspersed with non-repetitive 34-41bp DNA spacer sequence. The presence of polymorphisms related to mobility of repetitive elements along with single nucleotide polymorphism indicates that transposition and homologous recombination are the major events contributing the diversity of *M. tuberculosis* complex strains. The deletion pattern suggests that there is extensive genomic variability among different *M. tuberculosis* complex genotypes in the world. Based on the presence or absence of *M. tuberculosis* complex specific deletion (TbD1), a new evolutionary scenario for the evolution of *M. tuberculosis* complex and the origin of human TB has been suggested [Kamerbeek *et al.*, 1997; Brosch *et al.*, 2002].

1.8 Prevention and control

The best measure for primary prevention of TB is the treatment of infectious cases. Primary prevention can also be promoted through good public health practice to reduce the transmission of infection in institutions by adequate ventilation and isolation of infectious patients. As direct sunlight rapidly destroys the bacilli, letting air and sunshine into rooms where TB patients live can reduce the risk of infection for those living in contact with them. Prevention of TB also includes BCG vaccination and prescription of Isoniazid chemoprophylaxis for groups at risk [FMoH, 2008; WHO, 2009].

TB is usually treated with four different antimicrobial agents. The course of drug therapy usually lasts 6-9 months. The most commonly used drugs are rifampicin (RIF), isoniazid (INH), pyrazinamide (PZA) and ethambutol (EMB) for 2 to 3 months followed by 4 to 6 months of INH and RIF or alternative combination drugs treatment. When adherence with the regimen is assured, this four-drug regimen is highly effective. The goals of treatment are to ensure cure without relapse, to prevent death, to stop transmission, and to prevent the emergence of drug resistance. Since administration of a single drug often leads to the development of a bacterial population resistant to that drug, effective regimens for the treatment of TB must contain multiple drugs to which the organisms are susceptible. In general, regimens used for PTB are effective in the treatment of EPTB [Rieder, 2002].

TB treatment is hampered by the emergence of multidrug-resistant (MDR) strains of TB that require long-term treatment with a variety of second-line TB drugs. In some countries MDR-TB constitutes up to 10% of the TB prevalence among new cases. By contrast, resistance levels are low in sub-Saharan Africa, where routine reports indicate that MDR-TB prevalence in new TB cases is from 0.8% to 2.6% [Jones *et al.*, 2008]. However; among previously treated cases in high HIV-burden African countries, MDR prevalence is estimated to be 6.3%. Resistance to any single TB drug is close to 10% in all African countries surveyed. As drug-resistant TB has become more prevalent across the world, so too have strains that are resistant to one or several second-line drugs, the concept of extensively drug-resistant (XDR) TB has emerged to define those strains that are resistant to almost all TB drugs. The phenotypic description of XDR-TB specifies resistance to rifampicin and isoniazid among the first-line drugs (MDR-TB) as well as to a fluoroquinolone and to one or more of amikacin, capreomycin or kanamycin [WHO, 2006b].

XDR-TB patients die because there are insufficient drugs active enough to support their recovery. New anti-TB agents are urgently needed, and a new programme of TB therapeutics research must be invigorated, as in the near future it is certain that the emergence of even more drug resistant strains will heighten the need for new classes of antibiotics. Trials of new drug development include a moxifloxacin-containing treatment arm that reduces treatment length to four months, and success would represent an important development towards increasing adherence to standard treatment. However, fluoroquinolones are definitively inactive against XDR-TB strains, and there are some concerns that inappropriate sub-therapeutic doses may actually increase the propensity to develop resistance to other drug

classes. If fluoroquinolones are to become a standard part of the first-line TB therapy arsenal, this must be accompanied by a robust effort to reduce their inappropriate use for other conditions, and to strengthen the commitment to monitoring treatment completion inherent in direct observatory treatment strategy [Gillespie *et al.*, 2005].

Prophylaxis for exposure to TB can include isoniazid for 9 months, rifampicin for 4 months, or rifampicin and pyrazinamide for 2 months. Pyrazinamide and ethambutol or levofloxacin are used for 6 to 12 months after exposure to drug resistant *M. tuberculosis* complex. Randomised and case-control trials have shown consistently high protective efficacy (above 70%) of BCG vaccine against serious forms of disease in children but variable efficacy against PTB in adults. Thus, vaccination is recommended for children in high prevalence areas at birth or at first contact with health services except for children with symptomatic HIV infection [WHO, 2002].

The eradication of TB from livestock is expensive since it requires intensive surveillance of the livestock, the slaughter of the infected animals, and compensation of those who owned the infected slaughtered animals. The general lack of public resources in developing countries seriously hampers such control strategies leading to uncoordinated efforts and eventually to failure. This and the fact that rural communities have limited access to healthcare are contributing greatly to local authorities general neglect of endemic TB derived from infections in livestock and to the farmer's resistance to appropriate interventions [Marcotty *et al.*, 2009].

To control TB, IUATLD and WHO recommend the DOT strategy which has five elements: political commitment, diagnosis primarily by smear microscopy, short course treatment with effective case management by direct observation, regular drug supply, and systematic monitoring to assess outcomes of every patient started the treatment [Rieder *et al.*, 2007; WHO, 2009].

1.9 Current situation in Ethiopia

According to the 2008 WHO TB report, Ethiopia ranks 7th in the list of world's 22 high burden countries for TB with incidence estimated at 379 persons per 100,000 persons for all forms of TB and 168 persons per 100,000 persons for smear positive TB. The annual risk of TB infection is also estimated at 2.2% [WHO, 2008]. In 1997, about 30% of all new TB cases

were believed to occur in HIV positive individuals [Mitike *et al.*, 1997]. In this country where HIV infection is highly endemic, the relative importance of reactivation and recent infection is not known. But information in this field may have paramount implications for TB control policies [Bruchfeld *et al.*, 2002].

As to the Federal Ministry of Health hospital statistics data, TB is one of the leading causes of morbidity. According to the data, TB is the fourth causes of hospital admission and the second causes of hospital death in Ethiopia. TB mortality rate is estimated at 84 persons per 100,000 populations per year. The high rate of chronic malnutrition, widespread poverty, overcrowding, and high sero-prevalence of HIV infection has created an environment which made TB a formidable threat in Ethiopia. Of the total 141, 589 cases notified in 2007/2008, 138,650 (98%) were new cases. Out of the 138,650 new cases, 40,744 (29%) were pulmonary smear positive cases. The case detection rate of new pulmonary positive cases was 34% for the same year [FMoH, 2008].

Ethiopia has one of the highest incidence rates of human EPTB in the world, a clinical presentation that is often associated with transmission of *M. bovis* from cattle to humans. It is also noteworthy to mention that 36% of incident of TB cases in Ethiopia are extrapulmonary [WHO, 2008]. Although the widespread and endemic nature of BTB has been known since 1967 in this country, little information about its genotypic characteristics, epidemiology and its public health significance is available. In addition to its use in designing a more targeted control measure, the availability of such information would help study phylogenetic characteristics of the organism that in turn provide new insight into the natural history of BTB. It is estimated that 82% of the milk is supplied unpasteurized by intra and peri-urban producers to consumers while only 18% is supplied by dairy enterprises in pasteurized form. Some research conducted on *M. bovis* from cattle indicated that Ethiopian isolates are relatively more heterogeneous compared to isolates from other countries. Multiple spoligotype infection was also recorded and this may indicate the prevailing high degree of super infection [Ameni *et al.*, 2003; Ayele *et al.*, 2004; Ameni *et al.*, 2007; Berg *et al.*, 2009; Biffa *et al.*, 2010].

Infection with *M. bovis* can be transmitted from cattle to human mainly through the consumption of contaminated milk and meat products. Because of the route of infection, disease often manifests itself as EPTB. Kidane and others indicated that among 35 PCR

positive cases of human TB lymphadenitis from Southern Ethiopia, 29(82.9%) were caused by *M. tuberculosis* and 6(17.1%) were caused by *M. bovis* [Kidane *et al.*, 2002]. A recent study conducted on cattle by Berg and his associates showed that 58(48.74%) and 8(6.72%) out of 119 isolates were *M. bovis* and *M. tuberculosis* respectively [Berg *et al.*, 2009].

Another recent research carried out on cattle by Ameni and others in North Eastern Ethiopia revealed that 25 to be *M. bovis* out of 27 isolates and the remaining 2 were *M. tuberculosis* [Ameni *et al.*, 2010]. There is also a clear indication that the incidence and prevalence of BTB in Ethiopia is on the rise which found to be up to 39.6% prevalence because of the expansion of dairy farming sector. According to a research conducted by Tsegaye and his colleagues, from 11 isolates 8 were *M. bovis* and the remaining 3 were *M. tuberculosis* [Tsegaye *et al.*, 2010].

2. Statement of the problem

Even though Ethiopia is one of the countries with high prevalence of TB, information about genotypic characteristics of *M. tuberculosis* complex species and strains found in the country is limited. But this information is very vital in order to study phylogenetic characteristics of the organism which in turn will provide a new insight into the natural history of the disease in addition to its use in designing more effective control measures [Eyob *et al.*, 2002]. Therefore; intensive and wide area coverage research is needed in order to fill this information gap and get the whole picture of circulating strains in order to realize the mapping of strains throughout the country. Some recent ongoing researches on molecular epidemiology of *Mycobacteria* especially in few rural parts of Ethiopia by ALIPB and Armauer Hansen Research Institute (AHRI) are showing geographic localization of strains. However; there is no study conducted in North Shoa Zone of Amhara Regional State on tuberculosis in this regard. Accordingly, this study was carried out to molecularly characterize species and strains of *Mycobacterium* among smear positive PTB patients and clinically diagnosed EPTB patients from Debre Birhan Referral Hospital.

3. Significance of the study

Based on the above facts, it is possible to hypothesise that the genetic diversity of the *Mycobacterium* strains circulating within an endemic and epidemic area may give rise to an array of pathogenic characteristics. Such a bacterial population would be subjected to selective pressures which may give rise to phenotypic variations affecting host–pathogen interactions and, consequently, influencing the structure of the bacterial strain population within a particular host population. There is limited information about genotypic characteristics of *Mycobacteria* species and strains circulating in Ethiopia in general and in the current study area in particular as to the best of our knowledge. But there is an increasing demand of knowledge on the phylogenetic characteristics of this microorganism to have a good understanding towards the dynamics of *Mycobacteria* species and strains circulation in the population. And hence; there is a need to undertake molecular typing study throughout the country for the implementation of mapping the strains circulating in the country. In line with this, the findings of our study can play paramount role in identifying and characterizing the *Mycobacteria* species and strains responsible for the cause of different forms of TB in the study area. On top of this, it will also help in identifying the dominant *Mycobacteria* strains circulating in the zone.

4. Objectives of the study

4.1 General objective

To characterize the species and strains of *Mycobacteria* causing TB in and around Debre Birhan using molecular tools.

4.2 Specific objectives

- To identify and type the species of *Mycobacteria* causing different forms of tuberculosis at the study setting.
- To identify the dominant strains of *M. tuberculosis* circulating in the study area.
- To evaluate the contribution of *M. bovis* for the development of human TB
- To assess the risk factors of *Mycobacteria* infection in human.

5. Materials and methods

5.1 Study area and period

The study was conducted at Debre Birhan Referral Hospital, North Shoa Zone of Amhara National Regional State from November 2010 to June 2011. The study site is one of the coldest central highlands of Ethiopia and is located at 9°36 N, 39°38E, 130km northeast of Addis Ababa at an altitude of 2780 meter above sea level. The climate has a bimodal rainfall pattern consisting of a long rainy season (June - September), short rainy season (March - May) and dry season (October - January); average annual rainfall of 954 mm, and a mean minimum and maximum temperatures 1.3 and 16.3°C, respectively. The life of most of the residents of the zone depends on crop-livestock mixed farming system and has a very close contact with their livestock. The catchment population of the hospital is estimated to be 2,119,100 and it is giving service for more than 49,849 and 3,226 person as outpatient and inpatient health care annually, respectively [Esubalew, 2007].

5.2 Study design

A cross-sectional study was carried-out to characterize *Mycobacteria* isolates circulating in the Zone among TB patients. The study site was selected purposefully based on the basis of earlier observations of the disease in the Zone and the lack of documented information on the species and strains of *Mycobacterium* circulating in the area.

5.3 Source and study population

Our target population was TB patients living in the North Shoa Zone of Amhara National Regional State. All patients suspected of TB visiting Debre Birhan Referral Hospital during the study period were the study population. AFB smear positive PTB and clinically diagnosed EPTB patients were our study subjects. AFB smear positive sputum samples and all fine needle aspirate (FNA) samples recommended for AFB smear staining by physicians were collected from patients.

5.4 Inclusion and exclusion criteria

Smear positive PTB and tentatively clinically diagnosed EPTB patients who were volunteer to participate in the study and gave their consent or assent, and who were greater than or equal to 5 years old were included in the study. Those TB patients who were below 5 years of age,

who were not willing to participate in the study and could not give their consent or assent were excluded from the study. Patients clinically suspected of disseminated TB, with clinical impression of pyogenic lymphadenitis and peritonitis and who already have started anti-TB treatment were not also included.

5.5 Sample size determination

All TB patients fulfilling the selection criterion and visited Debre Birhan Referral Hospital during the study period were sampled. Sputum and body fluid FNA samples were collected from 197 informed and consented or assented patients with AFB smear positive PTB or clinically diagnosed EPTB.

5.6 Questionnaire survey

The results obtained by physical and clinical examinations and laboratory investigations were complemented by questionnaire survey. Patients were interviewed using a pre-structured questionnaire in one-visit interview during their sample submission. The focus of the issues in the questionnaire was to identify possible risk factors of acquiring TB such as the patients' previous contact with TB patients, habits of raw dairy product consumption, contact with livestock and previous treatment history for similar illnesses.

5.7 Specimen collection and processing

Sputum and FNA samples were taken by trained laboratory personnel or physician at the hospital with the maximum care and safety and questionnaires were completed at the same time for each patient. All the three spot-morning-spot sputum sample of each patient were pooled together in a universal tube and kept in phosphate buffer saline of 7.2 pH at +4°C , according to WHO, 2002 recommendations, at the hospital until transported to ALIPB laboratory for further laboratory tests. The epidemiological and clinical data were also documented at the same time for each patient. FNA samples were also collected using sterile and tightly closed test tubes and kept using phosphate buffer saline at pH 7.2 and +4°C in a similar manner to sputum samples. Finally both the sputum and FNA samples were transported to the ALIPB laboratory using ice pack within a week or two for further analysis.

5.7.1 *Mycobacteria* culture

All samples were processed and cultured as soon as arrived at the Institute according to the standard methods described by WHO, 2002. Briefly; the specimen were decontaminated by an equal volume of 4% NaOH, centrifuged at 3000rpm for 15 minutes. Then the supernatant was discarded and the sediment was neutralized with 2N HCl. The neutralization was said to be achieved when the colour of phenol red indicator was changed from red to deep yellow. And the sediment were inoculated into the conventional LJ egg slant medium containing 0.6% sodium pyruvate and glycerol and incubated for at least 6 weeks with weekly observation for the presence of mycobacterial colonies. Microscopic examinations of the colonies were performed by using Ziehl-Neelsen staining method so as to select AFB positive isolates. Heat killed cells were prepared from AFB positive isolates by mixing two loopful of colonies in 200µl distilled water and heating at 80°C for one hour. The same amounts of colonies were kept in glycerol stock at -20°C as backups for future possible related experiments. Finally the heat killed cells were used for molecular characterization.

5.7.2 Molecular typing

5.7.2.1 Region of difference based deletion typing

Heat killed cells of AFB positive samples from 189 isolates were investigated by PCR based deletion typing for the presence or absence of region of difference-9 (RD9) so as to identify *M. tuberculosis* from other species of *Mycobacteria*. The sequences of the primers used for RD9 deletion typing were as depicted in Table 1. PCR amplification of mixtures used for RD9 typing was performed using Thermal Cycler PCR machine (VWR International, UK) according to the standard procedure described by Brosch *et al.*, 2002. In short: reaction mixtures were made in a total volume of 20 µl consisting of 10 µl HotStarTaq Master Mix (Qiagen, UK), 7.1 µl distilled water, 0.3 µl of each of the three oligonucleotid primer (100 µM), and 2 µl DNA template samples or controls (heat killed cells). *M. tuberculosis* H37Rv and *M. bovis* 2122/97, and water were used as positive and negative controls, respectively. The reaction mixture was then heated using Thermal Cycler PCR machine (VWR International, UK) using the following amplification program: 95°C for 10 minutes for enzyme activation; 95°C for 1 minute for denaturation; 61°C for 0.5 minutes for annealing; 72°C for 2 minutes for extension, involving 35 cycles all in all; and final extension at 72°C for 10 minutes. The product was electrophoresed by Agarose Gel Electrophoresis System (BIO

RAD, UK) in 1.5% agarose gel in 1X TAE running buffer. Ethidium Bromide at a ratio of 1:1000, 100 bp DNA reference ladder and orange 6x loading dye were used in agarose gel electrophoresis. The gel was visualised using MultiImage Light Cabinet (Alpha Innotech Corporation, UK) and photograph was taken. The results were interpreted as *M. tuberculosis* (RD9 present) when a band size of 396 bp was observed and as either *M. bovis* or *M. africanum* (absence of RD9) when the band size of 575 bp was observed. Further strain characterization was done by spoligotyping method for randomly selected RD9 confirmed isolates.

Table 1. Oligoneucleotide primers used for deletion typing of *Mycobacterium* isolates

Locus	Primer name	Primer sequence	Present	Deleted
RD9	RD9_FlankF	5'-AACACGGTCACGTTGTCGTG-3'	396	575
	RD9_flankR	5'-CAAACCAGCAGCTGTCGTTG-3'		
	RD9_InternalF	5'-TTGCTTCCCCGGTTCGTCTG-3'		

5.7.2.2 Spoligotyping

97 randomly selected isolates, out of 182, confirmed to be *M. tuberculosis* by deletion typing were further characterized by spoligotyping techniques as originally described by Kamerbeek *et al.*, 1997 and Goyal *et al.*, 1997. In short; the DR region was amplified by Thermal Cycler PCR machine (VWR International, UK) using oligonucleotide primers derived from the DR region. A total volume of 25 µl reaction mixtures was used for PCR: 12.5 µl of HotStarTaq Master Mix ((Qiagen, UK): this solution provided a final concentration of 1.5 mM MgCl₂ and 200 µM of each deoxynucleotide triphosphates), 2 µl of each primer (2 pico mol each), 5 µl suspension of heat killed cells (approximately 10 to 50 nano gram), and 3.5 µl distilled water. Initial heating of the mixture was done at 96°C for 15 minutes and then subjected for amplification by holding 1 minute at 96°C, one minute at 55 °C, and 30 seconds at 72°C for 30 cycles. Final elongation was performed by holding at 72°C for 10 minutes. The amplified PCR product was denatured at 96°C for 10 minutes and then cold shocked on ice to get a single stranded DNA of PCR product. Then this single stranded PCR product was discharged

into cellulose membrane using Mini Blotter 1 (Ocimum Biosolutions, UK). Finally, the single stranded PCR products were hybridized using HB-100 Hybridization Oven (VWR International, UK) to a set of 43 immobilized oligonucleotides on the cellulose membrane. After hybridization, the membrane was washed twice for 10 minutes in 2X SSPE (1X SSPE is 0.81 M NaCl, 10 mM NaH₂PO₄, and 1 mM EDTA (pH 7.7), and 0.5% sodium dodecyl sulphate at 60°C and then incubated in 1: 4000 diluted streptavidin-peroxidase (HotStar, UK) for 45 to 60 minutes at 42°C. The membrane was washed twice for 10 minute in 2X SSPE and 0.5% sodium dodecyl sulphate at 42°C and rinsed with 2X SSPE for 5 minute at room temperature. Hybridizing DNA was detected by the enhanced chemiluminescence method using Hypercassette (Amersham Biosciences, UK) and by exposed to x-ray film (Hyperfilm ECL) as specified by the manufacturer.

5.8 Quality control

The qualities of field and laboratory works were assured by using well known instruments, reagents and chemicals from recognized companies on top of following recent and standard procedures. Appropriate data collection procedures and analysis software's were implemented to make sure that the data is well organized.

5.9 Data management and analysis

All data generated during physical, clinical and laboratory investigations were double entered into Microsoft Excel Database. Data verification, clearing and analysis was made using STATA 8.

5.10 Ethical considerations

Ethical clearance for current study was obtained from Addis Ababa University, College of Health Sciences, Department of Microbiology, Immunology and Parasitology Ethics and Research Committee. The purpose of the study was explained to the patients who were involved in the study using an approved study protocol. Informed written consent or assent was obtained from all study participants. Confidentiality was assured through the use of codes in records.

6. Results

6.1 Socio-demographic characteristics of the study population

A total of 197 patients were considered for current investigation out of whom 99 were reported as AFB smear positive PTB patients and 98 were tentatively diagnosed as EPTB patients and 52.28% (N=103) of them were females while 47.72% (N=94) were males. From this table one can observe that 72.60 % (N=143) of TB patients in the study area belonged to the productive age groups (18-47 years of age) and farmers constituted for 58.37% (N=115) of TB patients. History of contact with previous TB patients was recorded in 28.43% (N=56) of the study population. Out of the 197 study participants, 58.88% (N=116) of them had a history of treatment with at least one type of antibiotic for the same symptoms and 13.71% (N=27) of TB patients were retreatment cases. The socio-demographic data of the study subjects are presented in Table 2.

Table 2. Distribution of the study population by socio-demographic characteristics

Socio-demographic factor		Frequency	Percentage
Sex	Male	94	47.72
	Female	103	52.28
Age category in years	8-17	21	10.66
	18-27	60	30.46
	28-37	56	28.43
	38-47	27	13.71
	48-57	20	10.15
	58 and above	13	6.60
Occupation	Merchant	19	9.64
	Student	21	10.65
	Housewife	6	3.04
	Gov. Employee	11	5.58
	Farmer	115	58.37
	Others	21	10.65
Residence area	Rural	150	76.14
	Urban	47	23.86
Previous contact with TB patients	Yes	56	28.43
	No	141	71.57
Close contact with livestock	Yes	94	47.72
	No	103	52.28
Raw meat and/or unpasteurized milk consumption habit	Yes	101	51.27
	No	96	48.73
Previous antibiotics treatment for similar symptoms	Yes	116	58.88
	No	81	41.12
Previous treatment for tuberculosis	Yes	27	13.71
	No	170	86.29

6.2 Culture

A total of 197 samples (99 sputum and 98 FNA samples) were collected from the study participants. All sputum but only one FNA samples were reported AFB smear positives by Debre Birhan Referral Hospital. Culture positivity was confirmed in 47.41% (55/197) and 52.58% (61/197) samples within 4-6 and 7-16 weeks of incubation respectively. Finally 189 colonies were cultivated from all cultured samples. Figure 1 shows different colonies of *M. tuberculosis* that vary in colour because of difference in age at culture, from right to left from young to oldest colonies respectively. Growth of *Mycobacteria* was observed in 116 samples giving a recovery rate of 58.88%. Culture positivity rate of sputum was 80.81% (80/99) while FNA was 37.11% (36/98). Contamination was observed in one sample indicating only 0.51% contamination rate. No growth was observed in the remaining 80 samples after at least 8 weeks of incubation. A significant number of the isolates (64) were cultivated from both pyruvate and glycerol containing LJ medium while 29 and 23 isolates were grown on glycerol and pyruvate containing LJ slant media, respectively.

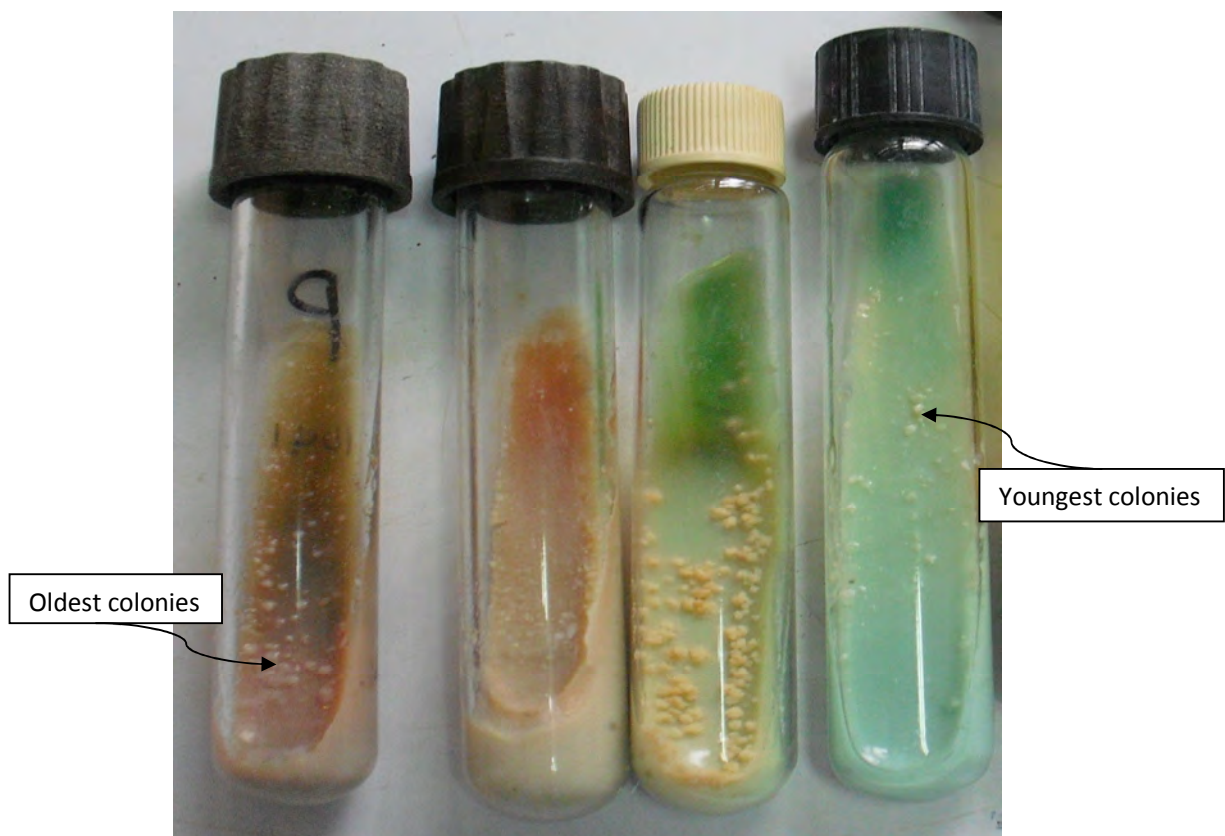


Figure 1. Characteristics of *Mycobacterium* species colonies on LJ media.

As listed in Table 3, the peak cultivation of *Mycobacterium* from FNA culture was from ascites and pleural fluid samples which constituted 44.44% (16/36) each out of the total post culture AFB positive FNA samples. The remaining 5.56% (2/36) and 2.78% (1/36) of the isolates were from cerebro-spinal and synovial fluids, respectively.

Table 3. Proportion of isolates in relation to total samples cultured in each category.

Sample	Culture positive		Culture negative		Contaminated	
	Frequency	Percent	Frequency	Percent	Frequency	Percent
Sputum (N=99)	80	80.81	18	18.18	1	1.01
Pleural fluid (N=47)	16	34.04	31	65.96	—	—
Ascites (N=41)	16	39.02	25	60.98	—	—
CSF (N=7)	3	42.86	4	47.14	—	—
Synovial fluid (N=2)	1	50.00	1	50.00	—	—
Wound swab (N=1)	0	—	1	100	—	—
Total	116	58.88	80	40.61	1	0.51

Generally, there was no statistically significant difference for being culture positive among socio-demographic parameters used in this research ($p > 0.05$). But interestingly, many more males were positive for EPTB which is 63.89% versus 36.11% females. On contrary, 55.00% (44/80) of culture positives were females in case of sputum samples. About 57.50% (46/80) of patients having habit of eating raw meat and/or drinking unpasteurized milk developed PTB while 47.22% (N=17) of patients having the aforementioned behaviour contracted EPTB (Table 4).

Table 4. Socio-demographic parameter and their respective culture result

		Culture result		X^2	p value
		Positive	Negative		
Sex	Male	59	35	1.1195	0.29
	Female	57	46		
Age category in years	8-17	13	7	4.6393	0.461
	18-27	34	27		
	28-37	32	23		
	38-47	20	8		
	48-57	12	8		
	≥ 58	5	8		
	Residence area	Urban	27		
	Rural	89	61		
Occupation	Merchant	10	9	1.3060	0.971
	Student	13	8		
	Housewife	4	2		
	Gov. employee	6	5		
	Farmer	70	45		
	Others	13	12		
Previous contact with TB patient	Yes	31	25	0.9181	0.632
	No	85	56		
Previous antibiotic treatment	Yes	63	53	2.4368	0.119
	No	53	28		
Previous Anti- TB treatment	Yes	20	7	2.9822	0.084
	No	96	74		
Raw Livestock product consumption	Yes	63	38	1.0445	0.307
	No	53	43		
Close contact with livestock	Yes	49	45	3.3890	0.066
	No	67	36		

6.3 Region of difference based deletion typing

RD9 deletion typing was carried-out on 182 AFB positive colonies from both sputum and FNA samples and RD9 was present in 169 of them. Presence of RD9 was confirmed in 72 of the 80 sputum and 32 of the 36 FNA samples which were culture and AFB positive and thus confirmed to be *M. tuberculosis* isolates (Figure 2 and 3). But RD9 deletion was observed in two samples, each from sputum and FNA samples, thus suggesting these isolates to be either *M. bovis* or *M. africanum* that should be differentiated by RD4 deletion typing. The remaining seven sputum and three FNA samples did not show any band at all and they might be non-tuberculosis *Mycobacterium* species or other acid fast bacteria. But this was not verified since we did not perform genus typing using multiplex PCR based deletion typing.

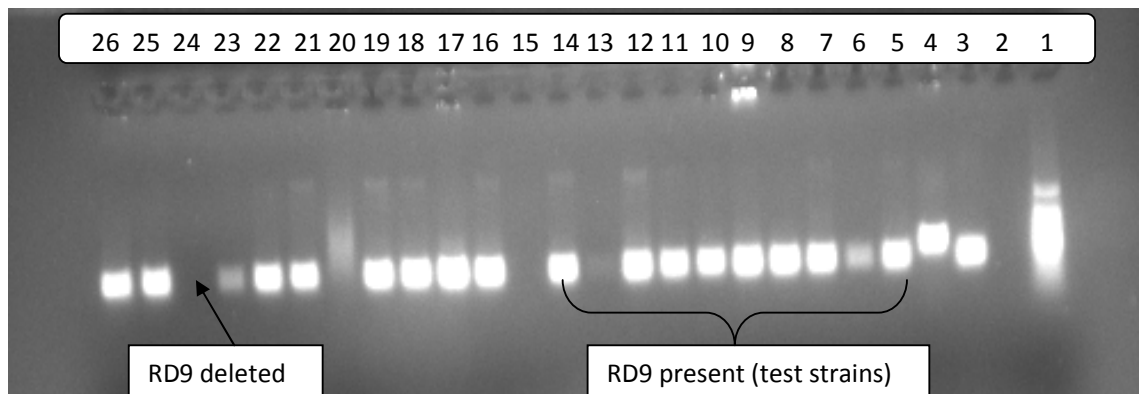


Figure 2. Selected gel showing the results of RD9 deletion typing

Gel A: Lane1= 1kb ladder, Lane2= Qiagen H₂O, Lane3=H37Rv, Lane4= *M. bovis*, Lane5=4024S, Lane6=1079, Lane7=4024G, Lane8=1093, Lane9=3006, Lane10=4032, Lane11=2049, Lane12=4036, Lane13=2096, Lane14=3098, Lane15=2020, Lane16=1079, Lane17=1062, Lane18=3093, Lane19=2096, Lane20=0616, Lane21=3020, Lane22=0767P, Lane23=0750, Lane24=1001, Lane25=3050, Lane26=4064.

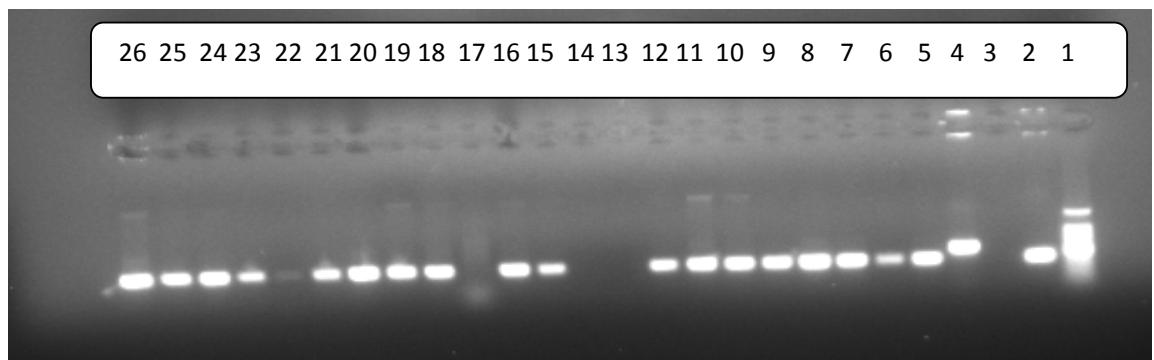


Figure 3. Selected gel showing the results of RD9 deletion typing

Gel B: Lane1= 1kb ladder, Lane2= H37Rv, Lane3= Qiagen H₂O, Lane4= *M. bovis*, Lane5=3023, Lane6=3006R, Lane7=0767, Lane8=2096R, Lane9=2095, Lane10=1093D, Lane11=0767G, Lane12=2049B, Lane13=04083, Lane14=2094Y, Lane15=8G, Lane16=3006R, Lane17=0586G, Lane18=0813, Lane19=0750G, Lane20=2057, Lane21=2093, Lane22=2094F, Lane23=1001G, Lane24=2057, Lane25=3093P, Lane26=4022G.

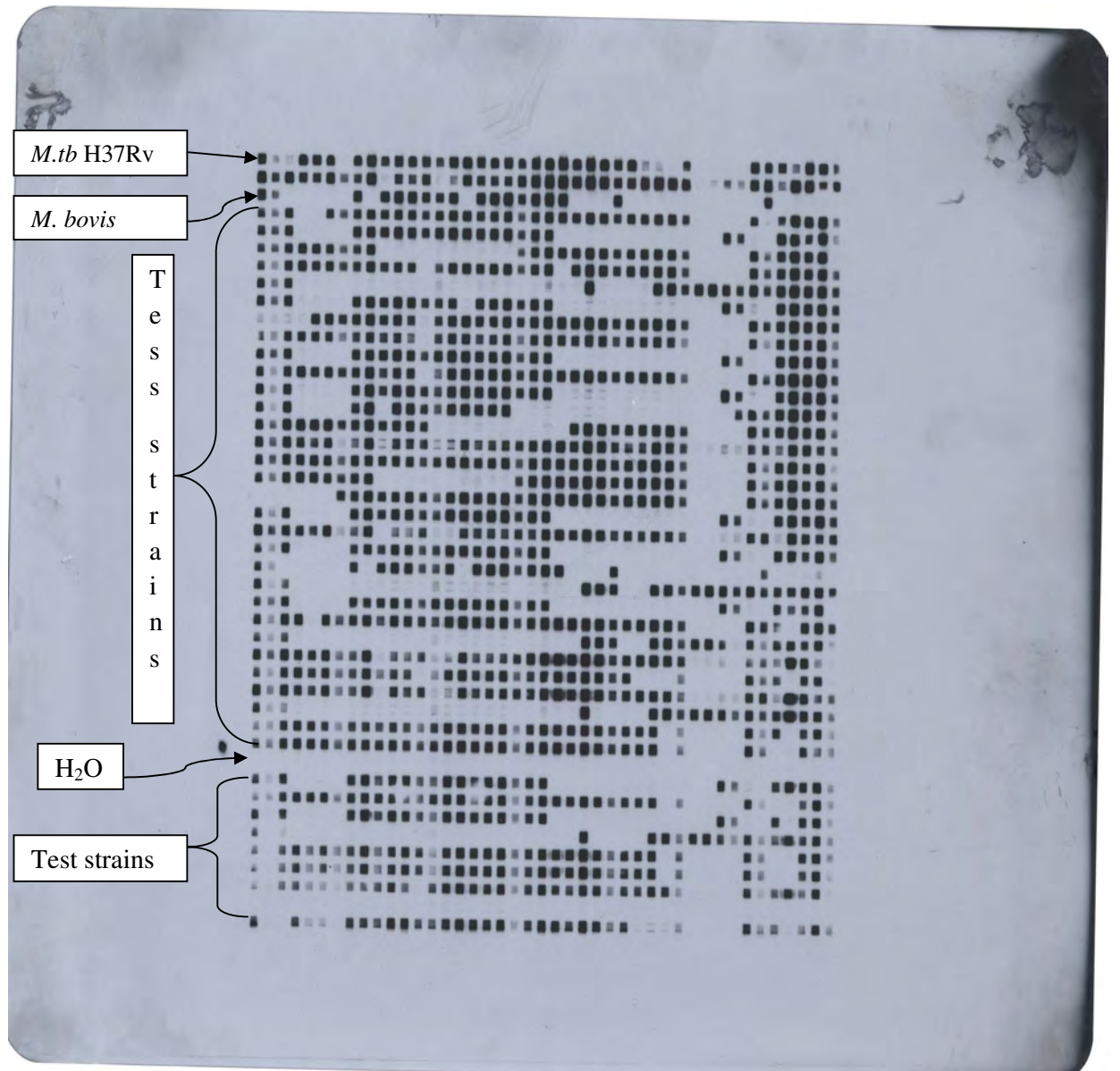


Figure 5. Radiograph of some of the spoligotypes

Table 5. Distribution of spoligotypes and their report status

ST No	PTB	EPTB	Total	Percentage	Report from Ethiopia
25	4	0	4	4.13	Yes
26	1	1	2	2.06	No
37	5	2	7	7.22	Yes
41	3	0	3	3.09	Yes
47	1	0	1	1.03	No
52	4	0	4	4.13	Yes
53	7	2	9	9.29	Yes
54	3	3	6	6.19	No
73	1	0	1	1.03	No
119	3	0	3	3.09	No
134	0	1	1	1.03	No
149	12	4	16	16.49	Yes
289	1	0	1	1.03	No
336	2	2	2	2.06	No
345	1	1	2	2.06	No
357	0	1	1	1.03	No
442	3	0	3	3.09	No
522	1	0	1	1.03	No
868	2	0	2	2.06	No
910	3	0	3	3.09	Yes
913	1	0	1	1.03	No
926	0	2	2	2.06	No
1166	1	0	1	1.03	No
1532	1	0	1	1.03	No
1580	0	1	1	1.03	No
New	9	10	19	19.59	No report from any country
Total	69	28	97	100	

Three lineages and seven families of *M. tuberculosis* were identified, using SPOTCLUST, in and around the study area. Sixty seven and 8 out of the 97 spoligotypes were from modern tuberculosis lineages called Euro-American and East-Africa Indian, respectively while only 3 of them were from the ancestral lineage known as *Mycobacterium africanum* as listed in Table 6. All of the spoligotypes that came from ancestral lineage were sourced from PTB patients. The highest family recovered in this study was *M. tuberculosis* family T1 accounting 34.02% of the total spoligotypes followed by *M. tuberculosis* family T3 that constituted 18.56%. We temporarily assigned all the new isolates together since their spoligotype pattern is unique to the international spoligotype database and it was impossible to get *M. tuberculosis* family that match with them. The dominant family from both PTB and EPTB patients was *M. tuberculosis* family T1 but the least were Haarlem1 and Haarlem3.

Table 6. *M. tuberculosis* spoligotypes distribution

Lineage	Family	EPTB(N)	EPTB(N)	Total N (%)
Euro-American	<i>M. tb</i> family 33	3	3	6 (6.19)
<i>Mycobacterium africanum</i>	<i>M.tb</i> family 36	3	0	3(3.09)
East-African Indian	<i>M.tb</i> family CAS	6	2	8(8.25)
Euro-American	<i>M.tb</i> family Haarlem1	1	0	1(1.03)
Euro-American	<i>M.tb</i> family Haarlem3	0	1	1(1.03)
Euro-American	<i>M.tb</i> family LAM9	3	0	3(3.09)
Euro-American	<i>M.tb</i> family T1	23	7	30(30.92)
Euro-American	<i>M.tb</i> family T3	13	5	18(18.56)
Euro-American	<i>M.tb</i> family X1	8	0	8(8.25)
Euro-American	New	9	10	19(19.59)
Total		69	28	97(100)

7. Discussion

The present study has identified and characterized *Mycobacteria* from TB patients attending Debre Birhan Referral Hospital. As TB is endemic to Ethiopia, it is of paramount importance to identify predominant species and strain types in different regions of the country in order to study transmission patterns within the country and to understand the epidemiology of the disease.

Based on the characterization of the species and strains of *Mycobacteria*, different risk factors were evaluated as to their association with the occurrence of TB disease. The results of such evaluations showed that the occurrence of TB disease and the frequency of the isolates of *Mycobacterium* isolated from TB cases were not affected either by age, sex, residence area, occupation, previous contact with tuberculosis patients, previous treatment with antibiotics or antimycobacterial drugs, habit of raw meat and/or unpasteurized milk consumption or by close contact with livestock in both PTB and EPTB combined ($p>0.05$). Similar observation was made by Iwnetu *et al.*, 2009. The lower case notification rates in females might be due to under-diagnosis or underreporting of TB in females as a result of various social and/or cultural factors, including the stigmatization of females with TB and their consequent impaired access to health care. It might also be due to real differences in rates of infection with *M. tuberculosis* complex reflecting social, cultural, and biological factors that influence opportunities for exposure [Dye, 2005].

The findings of the current study demonstrated that the majority of sputum samples (80.80%) have yielded *Mycobacterium* in culture. Out of 73 isolates, 72 (98.63%) were *M. tuberculosis* and the remaining was *M. bovis* or *M. africanum*. This supports the notion that states TB is caused mainly by the members of the *M. tuberculosis*. Thus *M. tuberculosis* is the most prevalent and *M. bovis* is the least prevalent causative agents of TB in individuals attending Debre Birhan Referral Hospital which is consistent with the results of previous studies in other regions of Ethiopia [Bruchfeld *et al.*, 2002; Kidane *et al.*, 2002; FMOH, 2008; Beyene *et al.*, 2009].

Most epidemiological studies on *Mycobacteria* have been focused on pulmonary infections, while extrapulmonary infections have been poorly explored. The recent global resurgence of *M. tuberculosis* infection has been matched by a rapid increase in EPTB accounting for 20 to

50% of all cases of TB in recent studies [Noussair *et al.*, 2009]. In line with this, Yassin *et al.*, 2003 indicated that TB lymphadenitis (TBLN) constituted 40% of the total TB diagnosis in Butajira, rural Ethiopia. According to the National TB and Leprosy Control Program of Ethiopia, EPTB accounts for 36.6% of TB cases from which 80% is TBLN [FMoH, 2006]. On contrary to this, the current study indicated no TBLN out of 97 tentatively diagnosed EPTB patients.

EPTB remains a challenging diagnosis for both clinicians and microbiologists since signs and symptoms are most often nonspecific, sampling requires invasive procedures and irregular distribution of bacilli that tend to clump together in the sample. EPTB samples such as pleural exudates and CSF are known to contain only few *Mycobacteria* resulting in a low sensitivity of acid-fast staining techniques. For instance pleural exudates sensitivity may be as low as 20%. Most of the time, sample sizes have been too small to draw definitive conclusions. Due to this, cases of EPTB are more often smear microscopy and culture negative than cases of PTB [Noussair *et al.*, 2009]. Our study is also not far from this fact since all except one of the extrapulmonary samples were pre-culture smear microscopy negatives and only 36 out of 97 samples yielded *Mycobacterium* in culture.

To the best of our knowledge, this is the first molecular characterization study in Ethiopia from extrapulmonary sites of ascites, pleural, cerebro-spinal and synovial fluid. Due to this, we faced difficulties to compare our results in the country. This calls for typing of isolates as in addition to epidemiological importance, it may be useful for individual patient treatment as some of the strains such as *M. bovis* are naturally resistant to pyrazinamide, one of the first-line anti-TB drugs used in the country.

Cases caused by *M. bovis* have been associated with EPTB. Human-to-human transmission of *M. bovis* is limited and anecdotal as it is believed that pulmonary patients infected by *M. bovis* are less infectious as they eliminate fewer bacilli in their sputum than those infected by *M. tuberculosis* [Mathema *et al.*, 2006]. In Ethiopia, little has been done to identify how *M. bovis* causes human TB despite the population's routine consumption of unpasteurized milk and raw meat. A skin testing study from central Ethiopia reported 13.50% as the average prevalence of bovine TB in cattle [Ameni *et al.*, 2007b]. A study carried out by Kidane and his associates in 2002 using DNA from FNA of TBLN patients from Butajira, Southern Ethiopia, indicated that 17.11% of cases were caused by *M. bovis*. In another similar research

conducted in Tanzania, *M. bovis* was confirmed in 10.77% (7 out of 65) of human cervical lymphadenitis cases [Cleaveland *et al.*, 2007]. These results, compared to our finding that pointed out only 1.72% of TB may be caused by *M. bovis* is distinctly high. But according to Beyene and his colleagues' research in 2009, no *M. bovis* was isolated from 156 TBLN cases.

Tuberculous peritonitis is a form of abdominal TB that predominantly involves the omentum, intestinal tract, liver, spleen or female genital tract in addition to parietal and visceral peritoneum. Peritoneal TB is nowadays defined to account for approximately 1.00–2.00% of all TB cases and sometimes it is seen in association with pulmonary or disseminated form of the disease [Koc *et al.*, 2006]. The present study revealed the isolation of a significant number of isolates from ascetic and pleural fluid, which is higher than the result recorded by Golden *et al.*, 2005 in USA but lower than from Razanamparany and others in 2002. However our result is similar with the result reported by Nicol *et al.*, 2005 from South Africa.

A study in USA by the year 2004 found that bones and/or joints to be the most common site of EPTB [Yang *et al.*, 2004]. By the next year a report from the same country revealed as bone and joint TB may account for up to 35.0% of cases of EPTB [Golden *et al.*, 2005]. However, we found bones and/or joints to be the least common sites since among 33 isolates only 3.03% were from synovial fluid. Our finding was comparable with 2.56% report from Madagascar [Razanamparany *et al.*, 2002].

Central nervous system TB accounts for approximately 1.00% of all cases of TB worldwide carries a high mortality and a distressing level of neurological morbidity [Rock *et al.*, 2008]. About 6.06% isolates of *Mycobacteria* were obtained from CSF in this research and it is in consistent with 5.9% report from USA [Yang *et al.*, 2004]. The proportion of patients with meningeal TB reported to be 17.2% in the Hong Kong study by Noertjojo *et al.*, 2002 is much higher than reported in the current study.

This study describes spoligotyping of 97 isolates of *M. tuberculosis* from Debre Birhan Referral Hospital, Ethiopia. A total of 78 spoligotypes were grouped into 25 different clusters. The remaining 19 isolates had unique spoligotype patterns which later categorized into 9 different clusters. The cluster formation indicates exogenous infection resulting from recent transmission and clonal expansion (Sebastien *et al.*, 2006). The three major clusters were ST37, ST53 and ST149, which constituted 32.99% of the total clustered strains. Out of the clusters, ST25, ST37, ST41, ST52, ST39, ST149, and ST910 were reported by previous

researchers from Ethiopia. These previous reports indicated that ST149 is, characterized by absence of spacers 10–19 and 33–36, the most common spoligotype identified and this substantiate the result of the present study. Large number of ST11 (East-African Indian lineage) have been reported from India, Bangladesh, Sri-Lanka, Malaysia, Indonesia, UK, Denmark, Netherlands, France, New Zealand and USA [Filliol *et al.*, 2001; Driscoll *et al.*, 2006; Ehtesham *et al.*, 2009] but none of our isolates showed the pattern of this spoligotype. Such findings could suggest the geographic structuring of the clonal population resulting in genetically and phenotypically distinct *M. tuberculosis* population within different parts of subcontinent. Such difference may also explain the geographically variable response to vaccination with *M. bovis* BCG vaccine [Baker *et al.*, 2004], or predominance of EPTB in Ethiopia since the variability is higher in EPTB than PTB patients. Nineteen out of 97 spoligotypes possesses unique spoligotype patterns that could not match with international spoligotype database.

Of the 9 *M. tuberculosis* families identified in the current work, T1 took the leading position recording 34.02% of the total spoligotypes indentified. The other most prevalent spoligotype in the present study was T3 family observed in 18 strains. This is similar with other reports elsewhere in the world [Baker *et al.*, 2004; Kulkarni *et al.*, 2005; Hasan *et al.*, 2006]. The result of this research also revealed that organisms presumably of European descents such as Haarlem1 and Haarlem3 families that rarely found were circulating in TB patients in the study area. This finding is evidenced by similar research in India [Kulkarni *et al.*, 2005].

At the phylogeny level, research results showed that although ‘modern’ strains of *M. tuberculosis* are more prevalent worldwide, the ‘ancient’ strains of *M. tuberculosis* such as East African Indian (EAI) strains are also responsible for the spread of tuberculosis [Baker *et al.*, 2004]. The most prevalent lineage in the current assessment was Euro-American lineage in which 69.07% of the strains analyzed belonged. Our data further confirm that the old ancestral lineages such as East-African Indian and *Mycobacterium africanum* are circulating in the study population even if in relatively low rate.

Researchers suggested that diverse strain types identified among *M. tuberculosis* have been related to selective advantages such as advanced mechanisms to evade *M. bovis* BCG-induced host defence mechanism [Mollenkopf *et al.*, 2004]. In support of this hypothesis, recent studies have shown that BCG protects mice less against infection with Beijing strain than

infection with the laboratory strain H37Rv. The different cytokine responses by CAS1 and Beijing strain provide information that protection against *M. tuberculosis* infection and disease caused by BCG vaccination may be influenced by the nature of infecting strains [Grode *et al.*, 2005]. Our results may have important implications for the evaluation of new candidate vaccines for the use in the control of tuberculosis.

It has been reported that mostly resistance conferring mutations are present in all lineages, which may occur independently on multiple occasions; however phenotypically antimicrobial drug resistance is significantly associated with lineage and nucleotide substitution arising under neutral, positive and negative pressure all inherited by clonal descendants (Baker *et al.*, 2004). In a country like Ethiopia where tuberculosis is endemic, the presence of many genotypes is contrary to the hypothesis that an endemic area may have few circulating strain types. Clustered isolates indicate the presence of active TB transmission and presence of isolates which aren't clustered might indicate reactivation.

8. Conclusion and recommendations

In Ethiopia, few studies were done mainly focusing on pulmonary TB and also limited researches on TB lymphadenitis but other types of EPTB did not get any attention so far. EPTB obviously poses an important hurdle for the control of TB in the country. The burden of EPTB infection indicated in this study appears to be high enough to warrant special attention since more than one third of AFB smear negative samples yielded *Mycobacterium* in culture and showed unique spoligotype patterns. None of the socio-demographic parameters used in the current study showed significant association with TB disease development. The present study indicated that *M. tuberculosis* is the most prevalent and *M. bovis* is the least prevalent causative agents of TB in individuals living in and around Debre Birhan town. *M. tuberculosis* family T1 and *M. tuberculosis* family T3 were the first and second most prevalent families observed in this study, respectively. The different spoligotypes of *M. tuberculosis* isolates that have been identified will have variability in virulence, transmissibility and response to treatment. Such variations could affect future diagnostics, drugs and vaccines development. A large proportion (80.41%) of isolates of *M. tuberculosis* identified in this study belonged to clusters, suggesting the existence of recent transmission. Spoligotyping of *M. tuberculosis* isolates from infected individuals will play significant role in tracking the source of infection and disclosing the epidemiology of TB. Thus, it is believed that the result of the present study could serve as important baseline information on the genetic diversity of *M. tuberculosis* and the current trend of TB transmission in Ethiopia.

Based on the above conclusion, the following recommendations are forwarded.

1. Nationwide studies should be carried out so as to establish the whole spectrum of strains most implicated in the disease to map the population of *M. tuberculosis* complex in the country.
2. The relationship between strain differences and their antimycobacterial drugs susceptibility should be studied.
3. Further studies on the newly identified strains of *M. tuberculosis* using more discriminatory tools are encouraged.

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10. Annexes

10.1 Annex 1- Patient information

Name of the researcher

Dr Legesse Garedeu

Department of Microbiology, Immunology and Parasitology

School of Health Sciences, College of Medicine

Addis Ababa University

Topic of the research

Molecular Characterization of Mycobacterium Species from Tuberculosis Patients at Debre Berhan Hospital, North Shewa Zone, Ethiopia.

Research objective

To Identify and characterize the species of *mycobacteria* affecting smear positive tuberculosis patients from Debre Berhan Hospital, North Shewa Zone, Amhara Regional state, Ethiopia.

Name of sponsor:

School of Post Graduate Studies, Addis Ababa University.

Study design

A cross-sectional study will be conducted at Debre Berhan Hospital, North Shewa Zone of Amhara National Regional State. The study is going to include those with AFB smear positive patient who are willing to take part in the study and can give their consent with eighteen and above years of age.

To achieve the planned objective physicians or trained laboratory technologists will take small fluid from swallow cervical lymph node or peritoneal fluid using fine needle (FNA) from extra pulmonary tuberculosis patients. From pulmonary tuberculosis patients small amount of morning sputum will be collected. For further molecular investigation only AFB smear positive samples will be taken. If recommended by physician and surgeon, biopsy may be taken from swollen lymph nodes. Both culture and molecular tests will be carried out at Akilu Lemma Institute of Pathobiology laboratory by an experienced laboratory technician and or researcher together with the initiator of this study.

Patients will be told that they will benefit from diagnosis of their condition since this is the confirmatory test and referral for free diagnosis and treatment at their nearby public health

facility. Their laboratory results will be reported to their physician or health centre. To assess potential risk for transmission, patients will be asked about their family living environment such as contact with livestock, raw milk and meat consumption and contact with known tuberculosis patients and their answers will be registered in a questionnaire format. In addition, patients will be told that their name will not be mentioned by any means in any report. All their answers will be kept confidential and will not be given to other person or institution for any purpose.

Possibility accident and pain

Sometimes little bleeding and pain may occur at the site of puncture during aspiration. Therefore; health professionals will provide them the necessary health care free of charge immediately. In the case of lymph node biopsy appropriate post operative care will be given. But as to sputum sample there will be no accident and pain associated.

Patients will be informed that if they agree to give sample, samples will be taken only once using fine needles for aspirate samples. For pulmonary tuberculosis, they are expected to give one morning sputum sample. On voluntary bases, they will be asked about their exposure to potential risk factors associated with bovine tuberculosis as well as general health history and previous contact with tuberculosis patients. This is because this information from the questionnaire is helpful for the prevention and control of tuberculosis.

Participant's right

Since participation is volunteer based, participants have the right to decide to participate in the study and can withdraw from participation under any condition and at any time of the study period. They have full right to refuse to answer questionnaire and to give specimens. Nothing will happen to them because of their refusal to participate. They will get their usual health care service at the health centre. However; if they are willing to participate, they will not be charged for laboratory tests which are important for the treatment of the disease. In addition they will be told that their participation will contribute to the effort of tuberculosis prevention and control program.

Participants' benefits

Clear explanation will be given as they will not be charged for their laboratory test results. They will know whether they get infected with bovine or human tuberculosis and enable to prevent disease from other family members. Because of their participation the status of the disease condition in the area will be known and communities in the area will be benefited.

Participants' incentives

There are no incentives because of participation. Participation is their free choice.

Agreement

Patients will be asked for signature of agreement. This is to make sure that agreement to participate in the aforementioned study. Otherwise there is no other reason for signing. The study is approved by Addis Ababa University College of Medicine Review Board. Getting signatures of agreement from participants is one of the criteria of the committee for the indication of no one can participate in the study without participant consent and agreement. Participants will make agreement on their volunteer bases. They will have full right to get full information about study procedures and other related issues with language of your choice. Their signature approves that they get satisfactory answers for their questions to the researcher.

Communication

If they need further information they can contact:

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Addis Ababa University, College of Health Sciences, School of Medicine

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Addis Ababa University, Aklilu Lemma Institute of Pathobiology.

10.2 Annex-2 - Informed consent

Research title: Molecular Characterization of Mycobacterial Species from Tuberculosis

Patients at Debre Berhan Hospital, North Shewa.

Research Initiator: Dr Legesse Garedew, Addis Ababa University, Addis Ababa, Ethiopia

Name of the patient: -----**Health centre:** -----

Card number: ----- **Patient code no. :** ----- **Address:** -----

Specimen: 1. Fine needle aspirate 2. Sputum

I have been told by researcher that my presumptive TB might be due to human and/or bovine tuberculosis and it is possible to test for TB. AFB tests can be performed at the Hospital and in addition it is possible to confirm whether it is human or bovine TB. Test for TB needs to culture specimen at laboratory for confirmation. I have been informed by researcher that I can get health services without any further laboratory tests. Thus; if I am volunteer to give specimen, it is possible to know whether I had infected with *Mycobacterial species* or not. Test will be free of charge. I may have a little pain and bleeding at the site of puncture. Since such tests are routine procedure in the Hospital, there will no major problem. The specimen will be taken only once. There is no need to come back again to the hospital to give second specimen. If test result confirms that I am TB positive, I will get free treatment services for TB whether I will participate in study or not. However if I give specimen I will know about a disease. I will not be paid because of my participation. My participation is based on volunteer base.

In addition I have been told that I will be asked about potential risk factors such as my living environment and contact with tuberculosis patients and livestock. The specimen will be tested to identify causative agent. I have been told that specimen will be taken by fine needle or sputum. In addition I have been told that sampling follow routine procedure and have no major effect except little bleeding and pain, and I will get health care from physician. More over I have been asked to give permission to make test and culture specimen at laboratory for strain molecular characterization. I have been informed that all information will be registered on case book and kept confidential. Researcher has given me brief explanation that my participation is on volunteer base and my refusal to participate has no any consequence of

punishment on me and I can withdraw from participation without any prerequisite and at any time. I can ask questions and will get brief explanation in the language of my preference.

I agree to participate and give specimen for lab test here in the Hospital and/or at Addis Ababa University, Aklilu Lemma Institute of Pathobiology for further strain characterization after culturing in laboratory and identification of TB agent. I agree to be interviewed about assessment of risk factor associated with disease transmission. In connection with this, I would like to confirm my agreement by signing.

Participant signature: ----- date -----

Witness signature: ----- date-----

Researcher signature: ----- date-----

10.3 Annex 3-Assurance of investigators

The undersigned agrees to accept responsibility for the scientific, ethical and technical conduct of the research project and for provision of required progress reports as per terms and conditions of the university's research principles in effort at the time of grant if grant is awarded as a result of this application.

Name of student	Signature	Date
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Name of advisors	Signature	Date
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10.5 Annexe 5- Physical examination

LN description (more than one variable is possible)

- 1. Location: 1. Unilateral (Right sided, Left sided) 2. Bilateral
- 2. Position: 1. Anterior cervical 2. Posterior cervical
- 3. Tenderness: 1. Tender 2. Non-tender
- 4. Number of nodes:1. Single node 2. Few nodes (2-4) 3. Multiple (>5)
- 5. Size (approximation) |__|__| cm
- 6. Mobility: 1. Mobile 2. Non-mobile
- 7. Condition of the nodes: 1. Discrete 2. Soft 3. Matted 4. Hard
5. Firm 6. Fluctuant 7. Draining 8. Other descriptions.....

Abdominal pain or discomfort condition

- 1. Ccolic 2. Abdominal discomfort 3. Sharp pain 4. Feeling of swelling 5. Felling having excess fluid 6. Feeling as having hard object in the abdomen 7. Others specify.....
.....

10.6 Annex 6-Clinical data

- 1. Coughing: 1. Yes 2. No, if yes what was duration of cough (weeks)
- 2. Blood in the sputum: 1. Yes 2. No
- 3. Fever: 1. Yes 2. No If yes, 1.High 2. Moderate 3.Low
- 4. Weight loss: 1. Yes 2. No If yes, 1. Slight 2. Moderate 3.Significant
- 5. Night sweat: 1.Yes 2. No

6. Poor appetite: 1. Yes 2. No

7. Weakness: 1. Yes 2. No

8. Duration of the neck swelling in (months):

9. Rate of increase of the neck swelling: 1. Slow 2. Moderate 3. Fast

10. Presently felt pain at the swelling site: 1. Present 2. Absent

11. Duration of abdominal pain and discomfort (in months):

12. Presently pain feeling around the abdomen 1. Yes 2. No

13. Previous treatment for the swelling/pain : 1. Yes 2. No

If yes, 1. Traditional (herbal) 2. Modern

14. Intake of antibiotics for the swelling/pain : 1. Yes 2. No

If yes, what kind (if they know the name or describe the colour, size, shape) -----

15. History of contact with tuberculosis patients: 1. Yes 2. No If yes, who was it?

1. Family member 2. Neighbour's 3. Friends 4. Others(specify).....

16. Duration of illness in (months):

17. History of anti-tuberculosis treatment previously: 1. Yes 2. No

If yes, had they: 1. Finished the course 2. Discontinued:

18. Intake of raw milk: 1. Yes 2. No; If yes: 1. Frequently 2. Rarely

19. Intake of raw meat: 1. Yes 2. No; If yes: 1. frequently 2. rarely

20. Living in same household with livestock (cattle, calves, sheep, goats, camel):

1. Yes 2. No

21. Irregular direct contact with livestock: 1. Yes 2. No

22. History of BCG vaccination (for children < 10 years): 1. Yes 2. No

23. Do you have livestock with coughing symptoms: 1. Yes 2. No

10.7 Annex 7- Case record form - AFN and sputum samples

Date:...../...../.....(G.C)

Name of the Hospital:-----

Code No..... BTBHS No..... Center Card No.....

1. Age (year):

2. Sex: 1. Male 2. Female

3. Address: 1. Urban Kef..... Keb..... H. No.....
 2. Rural PA.....

4. Occupation: 1. Merchant 2. Student 3. Housewife

4. Government employee 5. Pastoralist / Agro-pastoralist

6. Abattoir worker 7. Veterinarian 8. others (specify).....

5. Educational Level: 1. Illiterate 2. Primary School 3. Secondary

School 4. Diploma 5. Degree 6. Others (specify).....

6. Marital status: 1. Single 2. Married 3. Divorced

4. Widowed 5. Living with Partner 6. Separated

7. Religion: 1. Christian (Orthodox, protestant, catholic) 2. Muslim

3. Others; specify

Laboratory samples

1. Sputum for AFB: 1. Early morning sputum 2. Day time sputum

No. of samples 1. one sample 2. two samples 3. Three samples

2. FNA samples: 1. Lymphadenitis aspirates 2. Peritonitis aspirates

