

**ADDIS ABABA UNIVERSITY, COLLEGE OF HEALTH SCIENCE,
SCHOOL OF GRADUATE STUDIES, DEPARTMENT OF
MICROBIOLOGY, IMMUNOLOGY, AND PARASITOLOGY**



**Detection and Characterization of *Mycobacterium tuberculosis* in
Stool of HIV Sero-positive Patients with Suspected Pulmonary
Tuberculosis**

By: Gizaw Eshetu

March, 2015

Addis Ababa, Ethiopia

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List of Acronyms

AAU: Addis Ababa University

AFB: Acid Fast Bacilli

AIDS: Acquired Immunodeficiency Syndrome

ALIPB: Akililu Lemma Institute of Patho-Biology

ART: Antiretroviral Therapy

ASTU: Adama Science and Technology University

BAL: Bronchoalveolar Lavage

BCG: Bacillus Calmette-Gue´rin

BMI: Body Mass Index

Bp: Base pair

CAS: Central Asian

CFP: Culture Filtrate Protein

CSF: Cerebrospinal Fluid

CT: Cycle Threshold Values

CXR: Chest x-ray

DNA: Deoxyribonucleic Acid

dNTP: deoxynucleotide Triphosphate

DOT: Directly Observed Therapy

DST: Drug Susceptibility Testing

EDTA: Ethylene Diamine Tetraacetic Acid

ELISPOT: Enzyme Linked Immunospot

ESAT: Early Secretory Antigen Target

FNAC: Fine Needle Aspiration Cytology

G-C: Guanine-Cytosine

H: Haarlem

HHI: Health and Health Related Indicators

HIV: Human Immunodeficiency Virus

HRZE: Isoniazid, Rifampicin, Pyrazinamide and Ethambutol

IFN: Interferon

INH: Isoniazid

IPT: Ionized Prevention Therapy
IQR: Inter Quartile Range
IS: Insertion Sequence
L-J: Löwenstein-Jensen media
LTBI: Latent TB Infection
MDR: Multi-drug Resistant
MGIT: Mycobacteria Growth Indicator Tube
MIRU-VNTR: Mycobacterial Interspersed Repetitive Units-Variable Number of Tandem Repeats
MTB: *Mycobacterium tuberculosis*
MTC: *Mycobacterium tuberculosis* Complex
NAA: Nucleic Acid Amplification
NGOs: Non-governmental Organization
NPV: Negative Predictive Value
PBMCs: Peripheral Blood Mono-nuclear Cells
PBS: Phosphate Buffered Saline
PCR: Polymerase Chain Reaction
PLHIV: People Living with Human Immunodeficiency Virus
PPD: Purified Protein Derivative
PPV: Positive Predictive Value
PTB: Pulmonary Tuberculosis
PZA: Pyrazinamide
QFT: Quantiferon
RD: Region of Difference
RFLP: Restriction Fragment Length Polymorphism
rRNA: ribosomal Ribonucleic Acid
RvD: H37Rv related Deletions
SD: Standard Deviation
SITs: shared International Types
SNPs: Single Nucleotide Polymorphisms
SPSS: Statistical Packaging for Social Science
TB: Tuberculosis
TCH: Thiophen-2-carboxylic acid Hydrazide
TDR: Totally Drug Resistant
TST: Tuberculin Skin Test
UV: Ultraviolet light
WHO's: World Health Organization's
XDR: Extensively Drug Resistant
ZN: Ziehl-Neelsen stain

Abstract

Background: Tuberculosis (TB) in Africa is increasing because of the human immunodeficiency virus (HIV) pandemic, and in HIV / AIDS patients, it presents atypically. Smear-negative pulmonary tuberculosis (PTB) is more common in HIV-infected patients and leads to diagnostic delay, which increases morbidity and mortality in people living with human immunodeficiency virus (PLHIV).

Objective: To detect and characterize *Mycobacterium tuberculosis* in stool of HIV sero-positive patients with suspected pulmonary tuberculosis.

Method: Institutional based cross-sectional study of PTB diagnosis among PLHIV from stool sample was carried out from January 2014 – July 2014. During the data collection period, a total of 117 PLHIV suspected to be suffering from PTB was recruited. Microscopic examination, culture, and PCR were performed to detect and characterize *M. tuberculosis*. The presence of *M. tuberculosis* was compared in sputum and stool samples simultaneously collected from patients suspected for pulmonary tuberculosis. Data was entered and cleared using EpiData version 3.1, then exported to SPSS version 20 for analysis.

Results: Of the 117 patients suspected of having PTB, 33 (28.2%) were sputum culture positive. Of these, 10 (30.3%) were sputum and stool culture positive for *M. tuberculosis*. Of the 84 sputum, culture negative cases, three (3.6%) were stool culture positive with measure of agreement 0.328. Eleven of 117(9.4%) patients were positive by sputum smear. Of 11 (9.4%) sputum, smear positive, three (27.3%) were both sputum and stool smear positive for *M. tuberculosis*. Of the 106-sputum smear negative, stool smear permitted the diagnosis of one (1%) PTB patient though the sensitivity (12.1%) was very low and kappa value of 0.18. As compared to sputum culture, the sensitivity of stool PCR was 69.7% with substantial agreement 0.67. Sputum PCR detected additional 4/81 (4.9%) from the bacteriologically negative PTB patients. We did spoligotyping and T family (43.5%), family 33 (41.3%), CAS family (4.3%), H family (4.3%), H37Rv family (4.3%) and Beijing family (2.2%) were isolated.

Conclusion: *M. tuberculosis* was detected in stool of PLHIV. Hence, examination stool sample along with sputum sample increases diagnoses of PTB in PLHIV.

Chapter One

1. Introduction

1.1. Background

Tuberculosis (TB) remains a major global health problem and human immunodeficiency virus (HIV) infection has contributed to a significant increase in the worldwide incidence of TB. It causes ill-health among millions of people each year and ranks as the second leading cause of death from an infectious disease worldwide, after the HIV (WHO, 2013). The latest report indicates that there were 8.6 million (equivalent to 122 cases per 100 000 population) new TB cases in 2012. Most of the estimated number of cases occurred in Asia (58%) and the African Region (27%); smaller proportions of cases occurred in the Eastern Mediterranean Region (8%), the European Region (4%) and the Region of the Americas (3%). The five countries with the largest number of incident cases in 2012 were India (2.0 – 2.4 million), China (0.9 –1.1 million), South Africa (0.4–0.6 million), Indonesia (0.4 –0.5 million) and Pakistan (0.3 –0.5 million). India and China alone accounted for 26% and 12% of global cases, respectively. Of the 8.6 million incident cases, an estimated 0.5 million were children and 2.9 million occurred among women (WHO, 2013).

Worldwide, 13% of TB patients have HIV co-infection, and as many as 37% have HIV coinfection in parts of African Region, which accounted for 75% of TB cases among people living with HIV worldwide. Ethiopia is among the countries most heavily affected by the HIV and TB. The World Health Organization has classified Ethiopia 11th among the 22 high burden countries with TB and HIV infection in the world (WHO, 2013).

There were an estimated 12 million prevalent cases of TB in 2012, equivalent to 169 cases per 100 000 population. By 2012, the prevalence rate had fallen by 37% globally since 1990. There were an estimated 1.3 million TB deaths (just under 1.0 million among HIV-negative people and 0.3 million HIV-associated TB deaths). Most of these TB cases and deaths occur among men, but the burden of disease among women is also high. In 2012, there were an estimated 2.9 million

cases and 410,000 TB deaths among women, as well as an estimated 530,000 cases and 74,000 deaths among children. Approximately 75% of total TB deaths occurred in the African and South-East Asia Regions in 2012 (both including and excluding TB deaths among HIV-positive people). India and South Africa accounted for about one-third of global TB deaths (WHO, 2013).

The number of TB deaths per 100,000 population averaged 13 globally in 2012 and 17.6 when TB deaths among HIV-positive people are included. There is considerable variation among countries, ranging from under 1 TB death per 100,000 population (examples include most countries in western Europe, Canada, the United States of America, Australia and New Zealand) to more than 40 deaths per 100,000 population in much of the African Region as well as three high-burden countries in Asia (Bangladesh, Cambodia and Myanmar). Mortality rates appear to be falling in most of the 22 high-burden countries, although there is considerable uncertainty about the level of and trends in mortality in some countries, notably Mozambique, Nigeria, South Africa and Zimbabwe (WHO, 2013).

Tuberculosis is the commonest opportunistic infection and the number one cause of death in HIV patients in developing countries, and accounts for about 40% of all manifestations seen in HIV patients (Pape, 2004). HIV/ AIDS fuels the TB epidemics in many ways, such as promoting progression to active tuberculosis by weakening their immune system, increasing the risk of reactivation of latent tuberculosis infection, as well as increasing chance of TB infection once exposed to tubercle bacilli. This means that any delay in the diagnosis of active TB in HIV co-infected patients is more likely to result in clinical deterioration and greater morbidity and mortality (Harries *et al.*, 2001).

Though sputum smear has traditionally been used as the method for making an early diagnosis of PTB, smear-negative PTB is more common in HIV-infected patients and leads to diagnostic delay (Saranchuk *et al.*, 2007). Sputum culture is a more sensitive method of diagnosing PTB in such cases, but can take up to 8 weeks before a result is available. The patient's condition invariably deteriorates during this interval. Other factors contributing to diagnostic delay are that patients with HIV-associated PTB present more commonly with atypical or normal chest radiographs (Hudson *et al.*, 2000). This diagnostic delay also results in increased hospitalization and increased

costs to the health system. It has also been proposed that delay in the initiation of TB treatment may accelerate HIV infection (Lawn *et al.*, 2001).

It is imperative that efforts be made to expedite the diagnosis of TB in HIV-infected people. Therefore, examination of stool might offer an alternative method for TB diagnosis when sputum is difficult to obtain from people living with HIV (PLHIV). Tuberculous bacteria are believed to be present in stool when bacteria are transported from the lungs to the Oropharynx, are swallowed and then transit through the gastrointestinal tract (Cordova *et al.*, 2010, Wolf *et al.*, 2008). The examination of stool specimens may therefore facilitate PTB diagnosis in PLHIV who are unable to produce sputum.

1.2. Literature Review

1.2.1. *Mycobacterium Tuberculosis* Complex

In 1882, in a remarkable feat of microbiology, Robert Koch isolated *M. tuberculosis* for the first time, and conclusively demonstrated in the guinea pig that this slow growing Mycobacterium was the agent of a human disease (Koch, 1882). Together with other highly related bacteria, *M. tuberculosis* forms a tightly knit complex, a single species as defined by DNA/DNA hybridization studies, which is characterized by a singular lack of diversity in the bulk of its genes (Sreevatsan *et al.*, 1997). The complex comprised of closely related organisms namely: *M. tuberculosis*, *M. africanum*, *M. bovis*, *M. bovis* BCG vaccines strains, two rarely seen members, *M. microti*, and *M. canettii* (Linda *et al.*, 2002). Despite their diversity in terms of their host tropism, metabolism, growth, environmental niche, epidemiology and pathogenicity, the group has 99.9% similarity at nucleotide level and identical 16s RNA sequence (Huard, *et al.*, 2003). The genotypic grouping of MTC, after sequencing, revealed that only two loci; KatG codon 463 CTG (Leu) and gyrA codon 95A ACC (Thr) were present at high frequency. Accordingly, based on the combination of polymorphism located at these sites; all isolates of *M. africanum*, *M. bovis* and *M. canettii* had the characteristics of group 1 (KatG463 CTG (Leu) and gyrA95 ACC (Thr). Whereas, *M. tuberculosis*, in addition to group 1, fell in to group 2 and 3; KatG 463 CGG (Arg) and gyrA95 ACC (Thr), and KatG463 CGG (Arg) and gyrA95 AGC (Ser), respectively (Sreevatsan *et al.*, 1997). The primary causative agents of TB are mainly: *M. tuberculosis*, cause vast majority of human TB cases; *M. africanum*, an agent of human TB in sub-Saharan Africa; *M. microti*, the agent of TB in voles and rarely infect humans; *M. bovis*, which infects a very wide variety of mammalian species including humans and BCG (an attenuated variant of *M. bovis*); and *M. canettii*, variant that produces smooth and glossy colonies that is very rarely encountered but causes human disease (Sreevatsan *et al.*, 1997).

There are other Mycobacterium species that do not belong to the MTC group. These groups include: *M. avium*, *M. paratuberculosis*, *M. intracellulare*, *M. scrofulaceum*, *M. kansasii*, *M. xenopi*, *M. malmoense*, *M. cheloniae* and *M. fortuitum* among others (Miller *et al.*, 2002).

1.2.2. *Mycobacterium tuberculosis* genome

Based on the relative broad host range of *M. bovis*, there was speculation that *M. tuberculosis* had evolved from *M. bovis*. However, the assumption was no more worthy after the whole genome of *M. tuberculosis* strain (H37Rv) and *M. bovis* strain (BCG) were sequenced and presence or absence of direct repeats (DRs), H37Rv related deletions (RvD) and specific deletion 1 (TbD1) in species of MTC were identified (Cole *et al.*, 1998). The genome consists of 4,411,532bp, with 3,924 open reading frames and has a mean G-C content of 65.6%. The genome contains approximately 4,000 genes distributed evenly between the two strands and accounting for > 91 percentage of the potential coding capacity (Camus *et al.*, 2002). Presence or absence of *M. tuberculosis* specific deletion (TbD1) is the base for the division of *M. tuberculosis* strains into “ancient” and “recent”. The ancient deletions occurred at different stages in the speciation process and are widespread whereas the recent deletions have a more restricted distribution. The later including representative of the major epidemics as Beijing, Haarlem and West Africa 2 *M. tuberculosis* cluster (Brosch *et al.*, 2002.). In contrast to these recent deletions, the absence of regions RD7, RD8, RD9 and RD10 from *M. microti*, *M. bovis* and BCG which are still present in all *M. tuberculosis*, seems to be a much older event in evolutionary terms (Brosch *et al.*, 2002, Sreevatsan *et al.*, 1997).

From close inspection of the DNA sequences bordering these RD regions it is apparent that deletions occurred within coding regions. Genes that are present in *M. Tuberculosis* in full length have been disrupted in BCG, *M. bovis* and *M. microti* at exactly the same location, whereas these coding sequences are still intact in *M. tuberculosis* and *M. canettii* strains. This finding rules out the possibility of the DNA in these regions having been acquired by *M. tuberculosis* but, instead, argues strongly in favor of loss of the corresponding genetic material by the other species. Based on the presence or absence of such conserved RD regions, a degree of relatedness to the last common ancestor of the *M. tuberculosis* complex was proposed that shows that the lineages of *M. tuberculosis* and *M. bovis* separated before the *M. tuberculosis* specific deletion TbD1 occurred. From this analysis, it is clear that *M. bovis* cannot have been the ancestor of *M. tuberculosis* but, rather, appears to be descended from *M. tuberculosis* or to have emerged independently (Brosch *et al.*, 2002).

Some of these regions, primarily RD9 and TbD1 but also RD1, RD2, RD4, RD7, RD8, RD10, RD12 and RD13, represent very interesting candidates for the development of powerful diagnostic tools for the rapid and unambiguous identification of members of the *M. tuberculosis* complex (Brosch *et al.*, 2002)

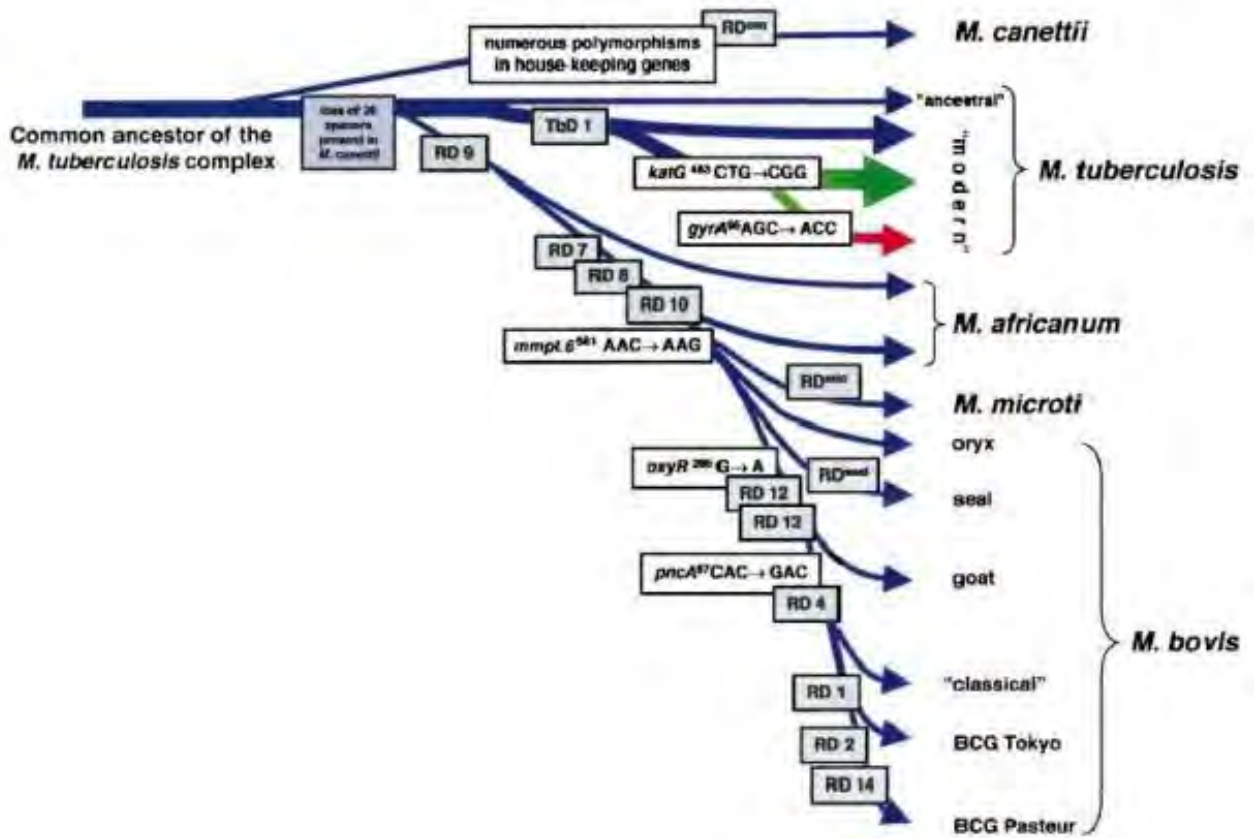


Figure1.1. Evolutionary Pathway of Tubercle Bacilli, illustrating successive loss of DNA in certain lineages (gray boxes), as proposed by Sreevatsan *et al*, 1997 and sketched by Brosch *et al*, 2002. Blue arrows indicate that strains are characterized by *katG*463.CTG (Leu), *gyrA*95ACC (Thr), typical for group 1 organisms. Green arrows indicate that strains belong to group 2 characterized by *katG*463.CGG (Arg), *gyrA*95ACC (Thr).The red arrow indicates that strains belong to group 3, characterized by *katG*463.CGG (Arg), *gyrA*95.AGC (Ser).

The genotyping of *M. tuberculosis* strains is important for TB control because it allows the detection of suspected outbreaks and the tracing of transmission chains. It is also important to monitor species diversity, as well as to identify secondary infections (Kremer *et al.* 1999, Mihret *et al.*, 2012). Insertion sequence (IS) 6110 restriction fragment length polymorphism (IS6110RFLP) is thought of as the gold standard genotyping method for *M. Tuberculosis* strain genotype identification (Kremer *et al.*, 1999). However, the method is time-consuming, labor-intensive, and costly. Furthermore, it is difficult to compare results between laboratories. Spacer oligonucleotide typing (spoligotyping), which is based on the analysis of polymorphisms of DR regions comprised of 36-bp DRs interspersed with 35 to 41bp unique spacer sequences, is a good alternative to traditional IS6110RFLP fingerprinting because of its simplicity, speed, and reliability (Kamerbeek *et al.*, 1997). Spoligotyping is useful for classifying *M. tuberculosis* strains into spoligotype families and subfamilies according to the presence or absence of spacer regions (Brosch *et al.*, 2002).

1.2.3. Natural Course of Tuberculosis

M. tuberculosis is transmitted in airborne particles called droplet nuclei that are expelled when persons with pulmonary or laryngeal TB cough, sneeze, shout, or sing. The tiny infectious particles can be carried by air currents throughout a room or building. Infection can occur when an individual exposed to an infectious case of TB inhales particles (<5 µm in size) containing the tubercle bacilli (Edwards and Kirkpatrick, 1986). If the bacilli reach the pulmonary alveoli, they may be ingested by alveolar macrophages, the first line of defense against *M. tuberculosis*. Surviving tubercle bacilli multiply within the macrophage and eventually undergo hematogenous spread to other areas of the body. In HIV infection, defective macrophages function in response to TB infection, which may in part increase susceptibility to TB disease (Patel *et al.*, 2009). Despite this, there is no conclusive evidence that HIV seropositive persons are more likely to acquire TB infection than HIV seronegative individuals, given the same degree of exposure. Once infection does occur, however, the risk of rapid progression is much greater among persons with HIV infection (Meltzer *et al.*, 1990).

1.2.4. Epidemiology

In 2012, 1.1 million (13%) of 8.6 million people who develop TB worldwide were HIV-positive. The African Region account 75% of estimated number of HIV positive incident TB cases (WHO, 2013). WHO reported 1.3 million TB-related deaths, of which 0.3 million were HIV-infected patients (WHO, 2013). Ethiopia is considered to have one of the highest coinfection prevalence rates of HIV/AIDS/TB in the world (WHO, 2013). There have been several studies of the prevalence of HIV in TB patients in different parts of Ethiopia, with prevalences of 6.6% among soldiers (Kefene *et al.*, 1990), 7.5% in North Ethiopia (Wondimeneh *et al.*, 2012), 22% in Harar (Mitike *et al.*, 1997), 44.4% in Southern Ethiopia (Gellete *et al.*, 1997), and 45.3% in Addis Ababa (Demissie *et al.*, 2000). Elsewhere in Africa, prevalence of 9%, 10.5%, 33.2%, and 43.6%, were reported in Kenya (Azevedo *et al.*, 2010), Nigeria (Iliyasu and Babashani, 2009), Chad (Tosi *et al.*, 2002), and Tanzania (Range *et al.*, 2007), respectively.

1.2.5. Impact of HIV Infection on the Pathogenesis of Tuberculosis

Immunocompetent individuals infected with *M. tuberculosis* have approximately a 10% lifetime risk of developing TB, with half of the risk occurring in the first 1-2 years after infection (Hopewell *et al.*, 2000). In contrast, HIV-infected individuals with latent TB are approximately 20-30 times more likely to develop TB disease than those who are HIV uninfected, at a rate of 8-10% per year (WHO, 2011). HIV co-infection also increases the risk of progression of recently acquired infection to active disease (Whalen *et al.*, 20011). In several outbreak settings, 35-40% of HIV-infected patients exposed to TB in health care or residential settings developed active TB disease within 60-100 days of exposure (Di Perri *et al.*, 1989, Daley *et al.*, 1992).

Infection with *M. tuberculosis* in an immuno-competent person is thought to confer significant protective immunity against exogenous reinfection (Hopewell *et al.*, 2000). However, re-infection has been reported in both HIV seronegative (Nardell *et al.*, 1986) and HIV seropositive individuals (Small *et al.*, 1993), although its incidence is not known. DNA fingerprinting on paired isolates of *M. tuberculosis* from 17 patients who repeatedly had positive cultures at a single hospital in New York City found 4 patients to have acquired a new, drug-resistant strain of

M. tuberculosis through exogenous reinfection, probably as a result of nosocomial transmission (Small *et al.*, 1993).

Tuberculosis can occur early in the course of HIV infection and throughout all stages of HIV infection. The risk of TB increases soon after infection with HIV; in a South African study of gold miners, the risk of TB doubled during the first year after HIV seroconversion (Gagneux *et al.*, 2006). Although TB can be a relatively early manifestation of HIV infection, it is important to note that the risk of developing TB, and of disseminated infection, increases as the CD4 cell count decreases. Even with effective immune reconstitution with ART, the risk of TB generally remains elevated in HIV-infected patients above the background risk of the general population, even at high CD4 cell counts (Moore *et al.*, 2007, Van Rie *et al.*, 2011).

The presentation of TB also is affected by the extent of HIV related immunosuppression. In patients with CD4 counts of >350 cells/ μ l, the clinical and radiographic presentation is similar to that of patients without HIV infection. However, as immunosuppression advances, the radiographic presentation becomes less typical, extrapulmonary, and disseminated disease become more common. In several studies of HIV-infected patients with PTB, the median CD4 count was >300 cells/ μ l. However, in patients with primarily extrapulmonary involvement or disseminated disease, the CD4 cell count may be much lower (De Cock *et al.*, 1992).

1.2.6. Diagnosis

Active pulmonary tuberculosis is difficult to diagnose, especially in children and PLHIV who have weakened immune systems. To determine if a patient has active TB disease, the following tests may be used:

1.2.6.1. Smear Microscopy

Acid-Fast Staining remains the initial step for evaluation of TB using direct microscopic examination of the acid-fast bacillus (AFB) in a smear. Because it is cheap and fairly rapid, it is the only diagnostic test for TB, particularly in developing countries (Truffot-Pernot *et al.*, 2006). Two methods are available for the direct examination: conventional staining with carbol-fuscin [Ziehl–Neelsen (ZN)] or Kinyoun stain using light microscopy and auramine-based stains

(auramine-O or auramine–rhodamine) based on fluorescent microscopy. Both methods rely on the retention of stain following the application of acid, resulting from tight binding of the stain to mycolic lipids in the cell wall of the bacillus. The bright fluorescence of stained bacteria under UV microscopy increases the sensitivity of detection at relatively low microscopic power by fluorochrome dye rhodamine (or rhodamine-auramine) staining and is useful for screening (Swaminathan *et al.*, 2010, Sharma *et al.*, 2005). Various methods of concentrating sputum based on centrifugation have been shown to increase diagnostic yield when used prior to microscopy. Whether the increase in sensitivity holds for HIV patients remains to be defined, particularly in those who are smear negative (Steingart *et al.*, 2006).

1.2.6.2. Culture

Traditional solid-phase culture techniques such as L-J culture remain the gold standard diagnostic test for TB in most resource-poor countries. Six weeks or longer on solid media and 7-21 days with liquid culture media will take the organism to grow. The development of early, manual broth-based culture systems such as BACTEC 460 (BBL; Becton Dickinson Microbiology Systems) which measured growth radiometrically have now been largely superseded by newer, fully automated non-radiometric systems. They include systems based on fluorescence such as BACTEC 9000 (Becton Dickinson) and MGIT (Mycobacterial Growth Indicator Tube Becton Dickinson), those that use a colorimetric CO₂ sensor such as MB/BacT (BioMe´rieux) and the ESPII system (TREK Diagnostic Systems, Inc) that measures the pressure changes in vial head-space. The BACTEC 9000 system has the added advantage of using a compatible medium for mycobacterial blood culture (MYCO/F lytic medium), which is of particular use in detecting dissemination of mycobacteria in blood of HIV patients, who have an increased rate of bacteraemia (Mendelson, 2007).

1.2.6.3. Biochemical test

The differentiation of MTC by Biochemical analyses includes colony morphology, niacin accumulation test, growth in the presence of thiophen-2-carboxylic acid hydrazide (TCH; 2µg/ml), nitrate reduction on modified Dubos broth, and growth characteristics on Lebek medium and on bromocresol purple medium (induction of a pH-dependent change of color from blue to

yellow). Oxygen preference in Mycobacterium isolates on Lebek (a semisolid medium) can be described as aerobic (growth on the surface) and microaerophilic (growth below the surface) (Normung, 1986). Nitrate reduction and niacin accumulation are the characteristics of *M. tuberculosis*. *M. bovis* is intrinsically resistant to pyrazinamide (PZA), major criterion for differentiation. However, susceptibility to PZA among isolates of *M. bovis* has been reported in some studies (Niemann *et al.*, 2000). Biochemical tests have now been replaced with molecular techniques for the identification and classification of MTC.

1.2.6.4. Tuberculin Skin Test (TST)

The tuberculin skin test has been in existence for over 100 years. The test works by injecting a small amount of liquid containing dead TB cells into the lower part of the arm. The injection site must then be evaluated by a trained healthcare professional 48-72 hours later. The TST measures the delayed type hypersensitivity response to a purified mix of mycobacterial antigens, purified protein derivative (PPD). Purified protein derivative comprises antigens that are found not only in *M. tuberculosis*, but also in *M. bovis*-BCG and other mycobacteria. As such, the response to TST lacks specificity in defining both latent TB infection (LTBI) and active disease (Pai *et al.*, 2004). In addition, the TST is compromised in HIV infection, where immunosuppression commonly leads to anergy, thereby reducing sensitivity. However, the TST remains a useful determinant of which patients should receive isoniazid prophylaxis to reduce progression of LTBI to active disease (60% reduction in progression to active TB in people who are TST positive) (Jasmer *et al.*, 2002).

1.2.6.5. Interferon Gamma Release Assays

Recent developments of immune based assays to detect *M. tuberculosis* infection are a significant advance (Pai *et al.*, 2004). The 6-kDa early-secreted antigenic target (ESAT-6) and culture filtrate protein 10 (CFP-10) are two proteins encoded by the RD1 genomic segment of *M. tuberculosis*, which is absent from all BCG strains and the vast majority of environmental mycobacteria. It has been shown that ESAT-6 and CFP-10 can stimulate peripheral blood mono-nuclear cells (PBMCs) from patients with tuberculosis to secrete specific interferon-gamma (IFN- γ). As a result, Quantiferon (QFT) and ELISPOT assays that detect IFN- γ release in response to these

antigens differentiate between *M. tuberculosis* infection and immune sensitization by BCG vaccination or exposure to environmental mycobacteria (Sorensen *et al.*, 1995).

1.2.6.6. Nucleic acid amplification tests

Nucleic acid amplification (NAA) is a rapidly evolving improvement in the detection targeted regions of the *M. tuberculosis* genome by amplifying specific regions of mycobacterial DNA and identification of MTB which requires strong laboratory capacity and good quality control procedures and is relatively expensive (Robin, 2007).

1.2.6.6.1 Region of Difference based analysis

Various biological and molecular mycobacterial characteristics have been utilized to identify MTC isolates but have limited applicability as MTC taxonomical tools. A series of classical tests based upon growth, phenotypic, and biochemical properties have been traditionally used to segregate members of the MTC (Niemann *et al.*, 2000). However, together these tests can be slow, cumbersome, imprecise, non - reproducible, and time- consuming, and they may not give an unambiguous result in every case and many not be performed by every laboratory. To complement the classical tests for determination of MTC species, well - defined MTC lineage and subspecies restricted single nucleotide polymorphisms (SNPs) have been used to specify certain MTC groupings through sequence analysis and/or digestion of PCR products followed by restriction fragment length polymorphism (PCR- RFLP) analysis (Niemann *et al.*, 2000). More recently, however, comparative genomics studies employing several different genetic hybridization strategies revealed regions of difference (RD) representing the loss of genetic material in *M. bovis* BCG compared to *M. tuberculosis* H37Rv (Brosch *et al.*, 2002).

1.2.6.6.2. Genus typing-Multiplex PCR

The genus typing (multiplex PCR protocol) uses six different primers. Firstly, it targets a sequence region within the 16S rRNA gene specific for the Mycobacterium genus. The two primers MYCGEN- F and MYCGEN- R are designed to amplify a specific PCR product from genomic DNA of all known mycobacteria. Secondly, the PCR mix also includes primers that are

specific for a hyper variable region of the 16S rRNA gene of *M. intracellulare* (MYCINT - F) and *M. avium* (MYCAV - R), giving one additional PCR product if the DNA template is any of these two species. Thirdly, species from the *M. tuberculosis* complex can also be identified due to the two primers (TB - F, TB- R) that target the MPB70 gene, specific for MTC (Katoch, 2004).

1.2.6.6.3. Spoligotyping

Spoligotyping is the most commonly used PCR- based technique to differentiate sub- species of *M. tuberculosis* strains (Groenen *et al.*, 1993). MTC strains contain different chromosomal region with multiple direct repeats (DRs) of 36 bp interspersed by 35 to 41 bp DNA sequences of unique spacer based on which the detection of the 43 interspersed spacer sequences (initially ascertained in laboratory strain H37Rv and *M. bovis* BCG vaccine strain P3) in the genomic DR region of MTC. This typing method relies on determination of binary result (the presence or absence) of spacers in the in vitro amplified DNA by hybridizing with labeled PCR amplified DR locus of the tested strain to multiple membranes spotted 43 synthetic spacer oligonucleotide covalently bound to a filter (Kamerbeek *et al.*, 1997). Results can be detected by chemiluminescence, and interpreted by computerized database.

1.2.6.6.4 GeneXpert

The GeneXpert® system is an advance in the field of MTC diagnosis via PCR amplification of MTC DNA. In addition to identifying MTC, Xpert MTB/RIF test GeneXpert® can also identify common rifampicin (RIF) drug-resistant alleles so that may indicate MDR-TB. By incorporating DNA extraction and amplification in a single, sealed cartridge, this system avoids many of the necessary laboratory and biosafety infrastructure and skills requirements typically required for NAAT-based diagnosis of TB. A sample is first liquefied and chemically inactivated to kill MTC cells. An aliquot of this is placed into the cartridge, which is then sealed and inserted into the machine for analysis. The GeneXpert® automatically extracts the DNA from the sample, performs nested real time PCR, analyses the data, and gives a result in under 2 hours. The GeneXpert® unit that has been tested for the Xpert MTB/RIF cartridge has four test modules that operate independently so that tests may be run individually on the same instrument (WHO, 2012).

1.2.7. Co-existence of Tuberculosis and HIV/AIDS

The interaction between the HIV epidemic and the TB epidemic is lethal where TB adds to the burden of illness of people with HIV and shortens their life expectancy, while the HIV epidemic spurs the spread of TB. Tuberculosis may be an independent matter of advanced immune suppression in HIV infected patients and it could also be a cofactor in accelerating the courses of HIV infection (Fact sheet, 2002). It was pointed out that, the containment of tubercle bacilli within a granuloma requires the generation of cytokines, including tumor necrosis factor alpha. And this tumor necrosis factor alpha may probably enhance HIV replication that in turn may increase the viral burden leading to a faster evolution to a clinical AIDS and death (Valerians, 1997). Another study pointed out that TB can accelerate the progression of HIV by accelerating the decline of CD4 T-cell counts and creating 6 - 7 folds increasing in the viral load as compared to those without TB (Jansa and Serrano, 1998). According to cohort study in Aquitaine, France, TB diseases affect survival but not occurrence of subsequent opportunistic infection or rate of CD4 lymphocyte count (Valerians, 1997). With regard to survival, HIV infected patients with TB appear to have shorter survival than HIV negative patients despite an adequate anti TB therapy (Styblo, 1991). Thus co-infected patients have the same probability of developing TB in one year as HIV – negative TB patients have in their entire lives. The HIV pandemic has substantially altered the epidemiology of TB. Various investigations have documented that persons co-infected with MTB and HIV have a 5.8% annual risk of developing active TB (Jansa and Serrano, 1998).

HIV/AIDS epidemic is reviving an old problem in developed countries and exacerbating an existing one in the developing world. Hence, HIV pandemic will worsen TB situation in developing countries in three ways over and above the existing situation. These are, reactivation of a latent TB infection among dually infected person, newly infection with tubercle bacilli and rapid progression to active diseases in HIV infected person, and by increasing the number of cases in the general population whose infection and disease is the result of transmission from HIV individual developing TB by either reactivation or recent infection. On the other hand, the impact of HIV infection on the epidemiology of TB depends on several factors (Styblo, 1991).

1.2.8. Challenges in Diagnosis of TB in HIV patients

Making a diagnosis of PTB in HIV-infected individuals can be challenging. HIV patients have higher rates of sputum smear-negative disease. Smear-negative, culture-positive TB is more common and occurs more frequently with advanced immunosuppression. Rates of AFB smear-negative disease vary widely but have been reported as high as 66%. In general, the rate of smear positivity correlates with the extent of radiographic disease. For example, patients with cavitory lesions caused by active TB will more commonly have positive smear results, whereas a negative smear result in a patient with minimal disease on chest radiograph would not be unusual, and would not rule out active TB. However, in HIV-infected patients, positive smear results may be seen with relatively little radiographic evidence. Diagnosis of TB in HIV infection also is made more difficult by the higher rates of extra pulmonary disease and the need to distinguish TB from other infectious and neoplastic complications of HIV (Hassim *et al.*, 2010).

Symptom-based screening tools are of limited utility in establishing a diagnosis of TB in HIV-infected individuals, given the many infectious complications of HIV that can cause symptoms and the diverse manifestations of TB disease in HIV-infected patients. In one study, the presence of cough of any duration, fever of any duration, or night sweats lasting 3 or more weeks in the preceding 4 weeks was 93% sensitive for TB, but only 36% specific (Cain *et al.*, 2010). However, use of a targeted symptom screen may help to exclude the diagnosis of TB in HIV-infected patients who are initiating antiretroviral therapy (ART) or isoniazid (INH) treatment for latent TB infection. One meta-analysis demonstrated that absence of fever, night sweats, weight loss, and cough of any duration had a 97.7% negative predictive value to exclude active TB infection (Getahun *et al.*, 2011).

It is recommended that at least 2 sputum samples be collected for AFB smear and culture in persons with suspected PTB. The incremental yield of third sputum for AFB smear is limited, as low as 2% in one study (Monkongdee *et al.*, 2009). Given the high rates of AFB smear-negative disease, culture can be essential to confirm the diagnosis of TB in HIV coinfection. In one series, use of 1 broth-based MGIT culture identified 71% of TB cases, and use of 3 MGIT cultures had the highest yield of strategies evaluated, identifying 98% of TB cases. In terms of incremental yield, a second MGIT culture identified 17% more TB cases, whereas the third MGIT culture had

yielded 10% more cases than the second culture (Monkongdee *et al.*, 2009). When expectorated sputum specimens are AFB smear negative, further evaluation may be indicated. Bronchoscopy with bronchoalveolar lavage and transbronchial biopsy may be useful in the evaluation of persons with abnormal chest radiograph imagery when sputum smear results are negative. In this setting, a rapid presumptive diagnosis of TB, based on histology and AFB smear of specimens obtained by bronchoscopy, can be made in 30-40% of individuals; that is similar to the yield of bronchoscopy in HIV-uninfected cases with smear-negative PTB (Kennedy *et al.*, 1992). Positive cultures for *M. tuberculosis* provide a definitive diagnosis of TB. However, approximately 15% of reported TB cases are culture negative (Mtei *et al.*, 2005).

Testing of specimens from extrapulmonary sites is required to establish a bacteriologic diagnosis of disseminated or extrapulmonary TB. HIV patients with extrapulmonary symptoms or signs of TB should have samples taken from the appropriate anatomic site(s) to increase the likelihood of TB diagnosis. Lymph node biopsy with AFB culture yielded a 42% rate of culture-confirmed TB in one series of HIV/TB-coinfected subjects (Monkongdee *et al.*, 2009). Blood culture also may be high-yield in patients with CD₄ counts of <100 cells/ μ l, with a rate of 49% blood culture positivity in one study (Jones *et al.*, 1993). Chest radiograph suggestive of TB and clinical symptoms of fever and cough were uncommon findings in HIV/TB coinfecting patients. Tuberculosis can occur at any stage of CD₄+T cells depletion (Bernard *et al.*, 2008). HIV-associated TB is more difficult to diagnose even at high CD₄ cell counts of >500 cells/ μ l, suggesting early impact after HIV seroconversion (Gupta *et al.*, 2013).

The study carried out in a tertiary care general hospital during 2010-12 in Mysore, Karnataka show that, of 416 HIV patients, 162 patients with features of pulmonary/extrapulmonary TB but smear negative were included in the study. Variety of samples were processed as per standard protocol and Mycobacteria were recovered in 76 samples (26 sputum, 12 stool, 18 blood, 7 pleural fluid, 1 CSF, 9 FNAC, 1 ascitic fluid, 1 pus, 1 ear discharge). *M. tuberculosis* (95%) was the predominant species isolated followed by *M. avium* complex (5%). Most HIV patients with TB are left undiagnosed for reasons of no advanced/high cost techniques in resource constrained settings. Thus culture could be used as a tool in diagnosing smears negative TB (Umamaheshwari and Sumana, 2013).

Tuberculin skin test can be used as an initial screening test among PLHIV to identify those at highest risk of active TB disease. Performing MTB culture for all TST-positive PLHIV is important but access to MTB culture or other sensitive tests to exclude TB disease is urgently needed to improve TB screening and prevention in resource-limited settings (Phanuphak *et al.*, 2012). Both sputum and bronchoalveolar lavage (BAL) sample evaluated in HIV-infected patients, AFB smear and serology had very low sensitivities but PCR of BAL with cycle threshold values (CT) value 32 had improved specificity to diagnose active PTB. Sputum AFB smear and the serological test had sensitivities of 66.7% and 0%, respectively. PCR with CT 40 has 96.4% sensitivity and 52.3% specificity. PCR with CT 32 had sensitivity of 85.7% and specificity of 90.9% to diagnose PTB in BAL (Kibiki *et al.*, 2007).

1.2.9. Treatment and Prevention

1.2.9.1. Treatment

Standard regimens for new TB patients presumed, or known, to have drug- susceptible TB for the Intensive phase treatment includes isoniazid, rifampicin, pyrazinamide and ethambutol (HRZE), and HR are recommended for the continuation phase for 4 months. Dosing frequency for new TB patients can be daily or three times per week with acceptable alternative provided that the patient is receiving directly observed therapy (DOT) and is not living with HIV or living in an HIV-prevalent setting (WHO, 2009). The current challenge with the treatment and prevention of the deadly disease, TB is the development of multi-drug resistant TB (MDR-TB) and extensively-drug resistant TB (XDR-TB) (WHO, 2010). An Isolate that is resistant to at least the two main first- line TB drugs RIF and isoniazid (INH) is said to be MDR-TB whereas an MDR isolate which is further resistant to fluoroquinolone and at least one of the second - line injectable agents: amikacin, kanamycin (Mukantabana) or capreomycin is XDR- TB (WHO, 2009).

The first cases of totally drug resistant TB (TDR- TB), resistant to all first line and second line anti-tuberculosis drugs, were reported from India (Udwadia *et al.*, 2012). The detection of drug resistant TB by drug susceptibility testing (DST) in liquid or on solid media is laborious and takes

several weeks to months to complete. The development of rapid molecular methods, actually endorsed by WHO, targets on specific molecular mutations associated with resistance against individual drugs and are able to perform within one or two days, important for the timely identification of drug resistance TB (WHO, 2010). Some commercially available methods like Xpert MTB/RIF and GenoType MTBDR, which are detecting based on lack of probe hybridization to wild type loci, indirectly indicating presence of mutations are currently in use (Hillemann *et al.*, 2009).

1.2.9.2. Vaccine

When the hypothesis was set out by Drs. Calmette and Gue´rin, to test that oral administration of bovine tubercle bacillus could transmit PTB (Calmette and Gue´rin, 1909), they serially sub cultured *M. bovis* in ox bile medium and isolated BCG for the first time (Calmette and Gue´rin, 1909, Liu *et al.*, 2009). However, after passage of 39 to 200 times, the strain was neither able to cause disease nor kill experimental animals, and did not revert also to virulence (Calmette and Gue´rin, 1909). The genomic analysis showed some deletions of RDs unique to the BCG strains that attributed to non- virulence of the bacterium. The three regions of difference identified when Mahairas *et al.*, using subtractive hybridization, compared the genomic sequences of *M. bovis* and BCG for the first time were RD1, RD2, and RD3 (Mahairas *et al.*, 1996). Later on, 16 other additional deletions, present in the genome of other *M. tuberculosis*, were recognized. Of these 11 were unique to *M. bovis* and 5 were only deleted from BCG (Behr *et al.*, 1999). The specific deletion to all sub- strains of BCG, the region of difference labeled RD1 (9,454 bp), is the one currently in use for vaccine of TB worldwide (Behr *et al.*, 1999). It has been concluded that the hypotheses, BCG was generated from the primary attenuating mutation of RD1, was reasonable. The low protective efficacy of the vaccine, which ranges from 0 to 80%, was thought to be due to the heterogeneity of BCG strain (Behr, 2002). Tuberculosis vaccination strategies follow two different approaches: pre- exposure vaccination in order to prevent disease in individuals that have so far not encountered *M. tuberculosis* and post- exposure vaccination that aims at inhibiting disease outbreak in individuals that are already infected (Brennan and Thole, 2012).

1.3. Statement of the Problem

Tuberculosis is the most prevalent disease in HIV positive people. The majority of the people at risk of HIV and TB are living in Sub Sahara Africa. The risk of developing active TB in HIV positive individuals is increased many fold despite antiretroviral chemotherapy (Lawn *et al.*, 2005). Tuberculosis may occur at any stage of HIV disease and is frequently the first recognized presentation of underlying HIV infection (Sonnenberg *et al.*, 2007). As compared to people without HIV, PLHIV have a 20-fold higher risk of developing TB (WHO, 2011) and the risk continues to increase as CD4 T cell counts progressively decline (Sonnenberg *et al.*, 2007).

Unlike the straightforward diagnosis and typical presentation of PTB in HIV seronegative individuals, the diagnosis of PTB in HIV / AIDS is more difficult (Batungwanayo *et al.*, 1992). This might be associated with inability or difficulty for patients to produce sputum sample due to diminished inflammatory response, a problem that is particularly common in young children and HIV-positive patients (Hartung *et al.*, 2002). Induced sputum techniques, nasopharyngeal aspirates or fiber-optic bronchoscopy, may all be used to retrieve pulmonary secretions from patients unable to provide a sputum sample but may cause logistical, cost, or biosafety challenges (Vargas *et al.*, 2005).

A study carried out in France showed that, out of 134 patients suspected to be suffering from PTB the sensitivity and specificity found to be 37.5 % and 100% respectively for the microscopic examination of stools; 54.2 % and 100% for culturing; and 100 % and 97.3% for real-time PCR (Khe'chine *et al.*, 2009). Of 228 PLHIV with culture confirmed TB from any site, 101 (44%) had a positive stool culture; of these, 91 (90%) had PTB in a study carried out in New York (Oramasionwu *et al.*, 2013). A pilot study conducted in Red Cross War Memorial Children's Hospital, Cape Town, South Africa, suggests that, stool Xpert testing from 115 children with suspected PTB detected 8/17 (47%) culture-confirmed TB cases, including 4/5 (80%) HIV-infected and 4/12 (33%) HIV uninfected children (Nicol *et al.*, 2013).

Therefore, detection of TB in stool of PLHIV may be strongly associated with PTB. So, stool may be used to diagnose PTB in PLHIV and alkali is most widely used protocols for the decontamination of stools for the recovery of mycobacteria (Oberhelman *et al.*, 2006). These study was conducted indicate if stool could be used as non-invasive and/or cheap alternative procedure in particular interest for patients who cannot expectorate in conjunction with sputum testing or as an alternative specimen upon which to base the diagnosis of PTB by AFB direct microscopy, culture or molecular techniques.

1.4. Significance of the Study

The prevalence of TB has declined by more than 37% worldwide. Regionally, prevalence rates are declining in all six WHO regions (WHO, 2013). However, there are difficulties in achieving the goal of halving TB burden by 2015 compared with a baseline of 1990 worldwide (WHO, 2013) due to a number of challenges such as unusual clinical picture, increase in smear negative acid fast bacilli, and atypical findings on chest radiography in HIV infected patients (Jones *et al.*, 1993). With advanced immunosuppression, PLHIV are more likely to have smear-negative making it increasingly difficult to diagnose PTB by sputum microscopy due to inappropriate production of sputum specimen. Stool specimen could be used as an alternative specimen due to convenience and non-invasive nature of collection in people living with HIV.

Therefore, we carried out this study in order to evaluate the utility of stool as an additional or alternative specimen for diagnosing of PTB in HIV-positive patients who do not give sputum, using smear, culture and PCR methods.

Chapter Two

Objective

2.1. General objective

To detect and characterize *Mycobacterium tuberculosis* in stool of HIV sero-positive patients with suspected pulmonary tuberculosis.

2.2. Specific objectives

1. To compare detection rate of *M. tuberculosis* in stool and sputum samples collected from HIV sero-positive patients suspected with PTB.
2. To evaluate performance of different diagnostic methods for detection of *M. tuberculosis* in stool and sputum samples collected from PLHIV.
3. To identify species of Mycobacterium isolated from stool and sputum samples from PLHIV.
4. To characterize *M. tuberculosis* isolates from stool and sputum culture

Chapter Three

Method and Materials

3.1. Study Area

The study area, Addis Ababa, administrative region, is the capital city of Ethiopia covering an area of 540 sq. km. The total population of the city is about 3.3 million with 5046 peoples per square kilometer, more of slum and overcrowded. The Administrative region has 10 sub cities and 106 woredas (districts). According to Addis Ababa health bureau report of 2010, there were 49 hospitals of which 13 were government owned, 5 NGOs and 31 are private, 36 public health centers, and 130 public health stations, 700 different levels private clinics are found in Addis Ababa city Administrative region (HHI, 2007). The study was conducted in three different health facilities (Polices Hospital, Zewuditu Hospital, and Teklehymanot Health center) conveniently selected which provide voluntary counseling and testing, antiretroviral therapy, tuberculosis diagnosis and treatment.

3.2. Study Design and Period

Institutional based cross sectional study was conducted from January 2014– July 2014 G.C.

3.3. Source and Study Populations

The source population was all PLHIV who visited the selected health facilities (Zewuditu hospital, Police hospital, and Teklehymanot health center), Addis Ababa, Ethiopia during the data collection period. The study populations were PLHIV suspected with PTB.

3.4. Sampling Techniques and Sample Size

3.4.1. Sampling Technique

We recruited all volunteer individuals consecutively until we reach our calculated sample size.

3.4.2. Sample Size

The estimated sample size was 117 PLHIV assuming 7.5% prevalence of TB among PLHIV in North Ethiopia (Wondimeneh *et al.*, 2012). Sample size derived using the following formula:

$$n = \frac{Z_{\alpha/2}^2 PQ}{d^2}$$

- Where: n = sample size; $Z_{\alpha/2}$ = standard normal distribution abscissa corresponding to 95% confidence interval (1.96); P = prevalence of TB in HIV sero-positive in study noted above (7.5%); $Q = (1-P)$; and d = desired level of precision (5%).
- **Note:-** Contingency of 10% were added on calculated sample size

3.5. Selection Criteria

Inclusion Criteria: HIV reactive with one or more typical sign and symptom (a history of prolonged fever, weight loss, cough for more than 2 weeks), or any other features suggestive of PTB and age above 18 years.

Exclusion Criteria: HIV sero-negative patient, HIV sero-positive patients with no features of PTB, HIV sero-positive patients who are on isoniazid prevention therapy, patients who are on anti-tuberculosis treatment and ART, those who were suspected of having gastrointestinal TB.

3.6. Variables

Independent Variables

Age, sex, sign and symptom, CD4 T cell count, occupation, marital status and body mass index.

Dependent Variables

Presence of MTC in sputum and stool

3.7. Data Collection Tools

Data were collected by pre-tested questionnaire and laboratory diagnosis

3.8. Data Collection Methods and Laboratory Diagnosis

3.8.1. Data Collection Methods

Structured and standardized questionnaires were used to collect information about socio-demographic and clinical feature of the study participants. One nurse and laboratory personnel were selected from each health facility and training were given on the objective, benefit of the study, individual's right, informed consent and techniques of the interview for the collection of data and specimens. The selection of participant was based on the inclusion criteria.

3.8.2. Laboratory Diagnosis

3.8.2.1. Sample Collection, Transportation, and Processing

Routine history and physical examination was performed at enrollment. All participants received baseline chest radiography and HIV testing. Each patient was instructed to provide sputum and stool at early morning for AFB smear, culture, and PCR. Morning sputum specimen and morning stool specimen were included in this study. Participants were oriented to bring 10ml of sputum and one gram of stool specimens, and then specimen was transported to Akililu Lemma Institute of Patho-Biology (ALIPB) tuberculosis laboratory within 48 hours of collection and processed for concentrated Ziehl-Neelsen (AFB), culture, and PCR.

Sputum specimens were digested and decontaminated by the NALC-NaOH method and centrifuged at 3000 rpm for 15 min. Two drops of phenol red indicator were added to the sediment after the supernatant was discarded and 2N HCl was added to neutralize the content. Neutralization was deemed to be achieved when the color of the solution was changed from purple to yellow. Then the sediment was inoculated immediately onto prepared L-J culture medium.

One gram of stool specimen was emulsified with sterile glass beads in 10 ml Tris buffer, 0.05 M, pH 7.2. The preparation was then shaken thoroughly in order to mix the sample with the buffer solution and the suspension was filtered into a 50 ml conical centrifuge tube. About 5 ml of the stool filtrate was mixed with 3 vols 1% chlorhexidine digluconate (Sigma), vortexed for 15 min at room temperature, washed in phosphate buffered saline (PBS) and centrifuged at 3000 g for 20 min at room temperature. The pellet was suspended in 1 ml PBS for analysis.

3.8.2.2. Microbiological Procedures

Smear Microscopy

After specimen processing and culture inoculation, smear was prepared, stained (Ziehl-Neelsen), and graded according to WHO recommendations. The actual number of AFB observed on smear was documented for all positive smears. Smears was then documented and reported using the following scale: negative (no AFB seen per 100 fields), + 1 (10–99 AFB per 100 fields), +2 (1–10 AFB per field in at least 50 fields), and +3 (10 AFB in at least 20 fields). Smears documented as having 1 to 3 AFB per 100 fields was recorded as negative for the purpose of analysis based on the known low correlation of 1 to 3 AFB and positive cultures. Smears with 4 to 9 AFB per 100 fields and those reported as +1, +2, or +3 was classified as positive.

Culture

Two sets of Löwenstein-Jensen slants; one supplemented with 0.4% Sodium pyruvate (L-J pyruvate) and the other with glycerol (standard L-J) were prepared. After appropriate labeling and inoculation of the culture with 0.2 - 0.4ml (2-4 drops) of the centrifuged sediment, it was incubated aerobically at 37°C for 2 month. The culture was examined daily for up to 1 week to

check for any contamination, then twice weekly for 2 months to check for any growth of colonies. Identification was based on morphology, color, rate of growth, and the acid-fastness (confirmed by ZN staining). Thereafter, isolates from the positive cultures was preserved with freezing media while at the same time heat killed in water bath at 80°C for 1 hour. The frozen and heat killed isolates were stored at -20°C for further Mycobacteriological and molecular typing analysis.

DNA Extraction

A filtered stool specimen and sputum specimen (250 µl) were separately mixed with 500 µl 1×TE buffer (Tris – EDTA), and transferred to 1.5 ml of eppindrof tube. The supernatant was discarded after centrifuging at 1200 rpm for 20 minutes. By repeating pipetting, pellets were re-suspended in 500µl of TE buffer, and 50µl of 10 mg/ml lysozyme was added, mixed well and incubated for 1h at 37°C. Seventy micro-liter sodium dedocyl sulphate (Sigma, St. Louis, Mo.) with concentration of 10g/ml and 6µl of 10 mg/ml proteinase K (VWR international Ltd., poole, BH151TD, England) were then added, mixed, and incubated for 10 min at 65°C. Afterwards, 100 µl of 5 M NaCl was added and vortexed and following the addition of 80 µL of pre-warmed Cetyl trimethyl ammonium bromide (CTAB) /NaCl (Sigma, St. Louis, Mo.) in pure water, and the mixture was incubated at 65°C for 10 min. Approximately equal volume (700 - 800 µl) of readymade phenol:chloroform:isoamyl alcohol (VWR international Ltd., poole, BH151TD, England) in proportion of 25:24:1 was added, after vortexed for at least 10 seconds and centrifuging for 10 min at 12,000 rpm. The resultant upper phase was transferred to a clean tube with 0.6 volume of isopropanol and mixed gently. The tubes were then moved slowly upside down to precipitate the nucleic acid, and would be incubated at -20⁰c overnight. Spun in a Microfuge for 15 min at 12,000 rpm, the precipitate was washed by 70% cold ethanol and the supernatant was removed. The pellet was permitted to air dry for 15 minutes and above. Finally, it was re-suspended in 1XTris-EDTA (Sigma, St. Louis, Mo.) solution (from 20µ to 50µ) based on the size of the pellet for PCR amplification. One positive control (*Mycobacterium tuberculosis* H37R) and three negative controls were used in the whole procedure.

Molecular Diagnosis

Region of Difference Based Deletion Typing

Heat killed isolates were investigated by PCR for the presence or absence of RD9 using specific primers. The PCR amplification mixture used for RD9 typing was as follows: the Hot Star Taq Master Mix (Qiagen, United Kingdom) was used for PCR, with primers described below Table 3.1. The reaction mixture contained 10 µl of Hot Star Taq Master Mix, 0.3 µl x 3 of each primer (flank R, F and Int), 2 µl DNA template and 7 µl distilled water to a final volume of 20 µl. The internal control Known *M. tuberculosis* was included in every PCR in order to check for the presence of PCR inhibitors while Qiagen water was used as negative control. The mixture were heated in Programmed Thermal Controller (Eppendorf, Hamburg, Germany) using an initial hot start of 95°C for 10 minutes followed by 35 cycles of 95°C for 1 minute; 55°C for 1 minute; and 72°C for 1 minute; a final extension step of 72°C for 10 minutes to complete the cycle. PCR products were electrophoresed in 1.5% agarose gel in 1xTAE running buffer. Ethidium bromide at ratio of 1: 10, 100bp DNA ladder and blue 6x loading dye at a ratio of 1:5 were used in electrophoresis. The gel was visualized in Multi-image UV light cabinet (Epi Chemi II Dark Room). The result was interpreted as MTB (RD9 present) when a band of 396bp was observed comparing to commercially available ladder, divided by 100bp.

Table 3.1: Oligonucleotide primers used for RD9 typing of Mycobacterium isolates (Qiagen, United Kingdom)

Locus	Primer name	Primer sequence	Present	Absent
RD9	RD9_FlankF	AACACGGTCACGTTGTCGTG	396	575
	RD9_FlankR	CAAACCAGCAGCTGTCGTTG		
	RD9_IntenalF	TTGCTTCCCCGGTTCGTCTG		

Genus Typing Multiplex PCR

Multiplex PCR was run if species was not identified by deletion typing. Five primers, with concentration of 10µM were used (Table3.2). DNA amplifications was done in thermocycler with 20 µl reaction volumes consisting: 2 µl of genomic DNA as a template, 10 µl Hotstar Taq Master Mix (MgCl₂, dNTP, Taq polymerase and PCR buffer) (Qiagen, United Kingdom) for each sample, 0.3 µl internal primer per sample, 0.3 µl forward and reverse. The reaction mixture was then heated in Programmed Thermal Controller (Eppendorf, Hamburg, Germany) cycle using the following amplification program: 95°C for 10 minutes for enzyme activation; 95°C for 1 minute for denaturation; 65°C for 0.5 minute 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 in 1.5% agarose gel in 1xTAE running buffer. Ethidium bromide at ratio of 1: 20,000, 100bp DNA ladder, and blue 6x loading dye, at a ratio of 1:5, was used in gel electrophoresis. After running electrophoresis, the gel was visualized using Multi Image Light Cabinet (Epi Chemi II Dark Room) and photograph was taken. All members of the Mycobacteria genus gave a PCR product of 1030bp with the primers MYCGEN-F/MYCGEN-R. *M. avium*, or subspecies including *M. paratuberculosis*, generated a PCR fragment of 180bp (primers MYCGEN-F/MYCAV-R) in addition to the 1030 bp genus product. Isolates from the *M. tuberculosis* complex produced a PCR fragment of 372bp (primers TB1-F/TB1-R) beside the 1030bp genus product. The quality control for this multiplex PCR was as follows: The positive controls for *M. avium*, and *M. tuberculosis* (H37Rv) always give two bands; the genus specific PCR product and the respective species-specific PCR product.

Table 3.2: Primers, and primer sequence for multiplex PCR (Qiagen, United Kingdom)

Primer Name	Primer Sequence	Present	Interpretation
MYCGEN-F	5'-AGA GTT TGA TCC TGG CTC AG-3'	1030bp	Genus Mycobacterium
MYCGEN-R	5'-TGC ACA CAG GCC ACA AGG GA-3'		
MYCAV-R	5'-ACC AGA AGA CAT GCG TCT TG-3'	180bp	<i>M. avium</i> Complex
TB1-F	5'-GAA CAA TCC GGA GTT GAC AA-3'	372bp	MTC
TB1-R	5'-AGC ACG CTG TCA ATC ATG TA-3'		

Spoligotyping

Spoligotyping was carried out using the commercially available kit from Ocimum Biosolutions, India, according to the manufacturer's instructions. Briefly, the direct-repeat (DR) region was amplified with primers DRa (biotinylated at the 5' end) and DRb, and the amplified DNA was hybridized to inter-DR spacer oligonucleotides covalently bound to a membrane. DNA from *Mycobacterium bovis* BCG and *M. tuberculosis* H37Rv were used as positive controls, whereas autoclaved ultrapure water was used as a negative control. The amplified DNA was subsequently hybridized to a set of 43 oligonucleotide probes by reverse line blotting. The presence of spacers was visualized on film as black squares after incubation with streptavidin-peroxidase and detected with the enhanced chemoluminescence system detection liquid (Amersham, Little Chalfont, United Kingdom)

Database Comparison

The spoligotyping results were prepared in octal and binary formats into Microsoft Excel spreadsheets; spoligotype patterns were designated as 43-character-long strings consisting of white squares and hyphen representing the presence or the absence of an individual spacer, respectively. The spoligo patterns which were prepared in binary and octal were entered and determined by comparing the spoligotyping results with already existing designations in the international spoligotyping database, SpolDB4.0 (Brudey *et al.*, 2006)(<http://www.pasteur-guadeloupe.fr:8081/SITVITDemo/>). In this database, two or more patient isolates sharing identical spoligotype patterns are defined as SIT (spoligotype international type) whilst single spoligo patterns are defined as "orphan" isolates. Patterns that were not found in SpolDB4.0 were assigned to families and subfamilies using the SpotClust program, which was built on the SpolDB3 database (<http://cgi2.cs.rpi.edu/bennek/SPOTCLUST.html>).

Over all Laboratory procedures

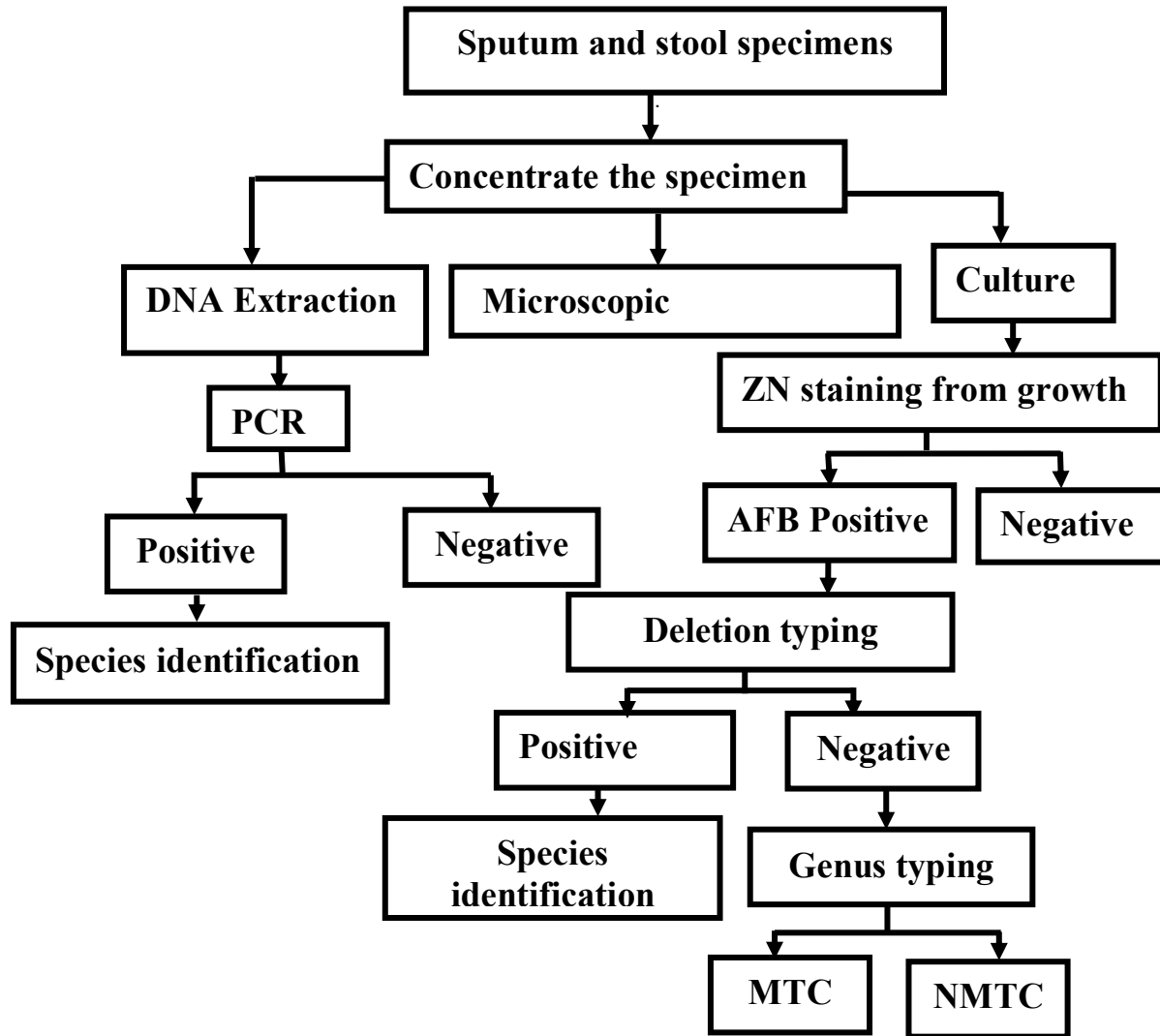


Fig.3.1 Chart showing overall procedures of the lab

3.9. Data Quality Control

The quality of data was controlled starting from the time of questionnaires preparations. The questionnaires were developed by reviewing relevant literatures on the subject to ensure reliability. Every day the questionnaires was viewed and checked for completeness and its relevance by principal investigator. The necessary feedback was given every morning before the actual procedure and analysis was started by using proper method for variables under study. An extensive quality control procedure was implemented in each collection site and laboratory to reduce the possibility of false-positive results, including from cross-contamination. Sputum and stool specimens were obtained from all enrolled, analyzed patients. Laboratories evaluated the specimen quality (e.g. physical appearance, volume) of all specimens submitted. When specimens of poor quality were submitted, laboratories requested new specimens; if patients were unable to provide specimens of higher quality, the originally submitted sputum was processed. Care was taken in preparing L.J medium, and then it was put at 37°C for 48 hours to check for contamination. A positive and negative control was run together with all steps of DNA extraction and PCR amplification procedures. The area of PCR mix was cleaned with “DNA away” before and after each mix. We used a separate mix room and amplified the mix in a different room to prevent contamination.

3.10. Statistical Analysis

After the completion of the data collection, the questionnaire was checked for its completeness, unrecorded values and unlikely responses and then was manually cleaned up on such indication. The test result was written on the laboratory data collection format sheet. Descriptive statistics was used to describe participant’s characteristics. Chi-square test and kappa value were used to compare different method used. Data was entered and cleared using Epi Data version 3.1. Then it was exported to SPSS software version 20 for analysis.

3.11. Ethical Considerations

Ethical clearance was obtained from Departmental Ethical Review and Research Committee (DERC) of department of Microbiology, Immunology and Parasitology (DMIP), College of Health Science, Addis Ababa University, ALIPB Ethical Committee, and Ethical Committee of Addis Ababa health Bureau. Formal letter was written to the selected health facilities. Before collecting sample the study participants were informed about the purpose, merit and demerit of the study in local languages, written informed consent was obtained from all participants. In addition the confidentiality was kept. Any participants who are not volunteers were not enforced to be included as study subject.

3.12. Result Communication

Positive results were reported to respective health facilities and all participants who were positive for pulmonary TB to be treated with first line anti TB drugs through their physician.

Chapter Four

Results

4.1. Socio-demographic Background of the Study Participants

A total of 117 eligible participants presenting to out-patient clinics were enrolled from January, 2014 to July, 2014. The participants' age ranges from 19-61 years with a mean of 34.5 ± 8.89 and with male to female ratio of 0.63:1. Most of the participants were in the age category of 28-37 years 66(56.4%). Majority of the patients were married 78(66.7%) and reside in Addis Ababa 95(81.2%). Concerning the level of education and occupation of the respondents, most of them were in high school 67(57.3%) and governmental employee 56(47.9%)(Table 4.1).

Table 4.1. Socio-demographic background of the study participants, January 2014 to July 2012, selected health facilities, Addis Ababa, Ethiopia

Variables	Frequency n (%)	Variables	Frequency n (%)
Sex		Location	
· Male	45(38.5)	· Urban	95(81.2)
· Female	72(61.5)	· Rural	22(18.8)
Age		Occupation	
· 18 - 27	17(14.5)	· Government employed	56(47.9)
· 28 - 37	66(56.4)	· Housewife	24(20.5)
· 38 - 47	22(18.8)	· Daily laborer	28(23.9)
· 48 - 57	9(7.7)	· Merchant	6(5.1)
· > 58	3(2.6)	· Farmer	3(2.6)
Marital status		Educational status	
· Single	22(18.8)	· Non-educated	11(9.4)
· Married	78(66.7)	· Elementary school	28(23.9)
· Divorced	6(5.1)	· High school	70(59.8)
· Widowed	11(9.4)	· Higher education	8(6.4)

4.2. Clinical Data

The clinical data of 117 suspected cases of TB were available for analysis. Of 117 cases, all had cough complaint and majority of the study participants had night sweat 111(94.9%) followed by fever 99(84.6%). All patients diagnosed as PTB complaints cough, fever, night sweat, and difficulty in breathing and none of them were positive for enlarged lymph node. Ten of 40 patients with PTB had abnormal CXR. The median body mass index was 20 (IQR=18.3-22.0). Among PTB confirmed patients most of them, 13(32.5%), were found to belong to WHO stage II (Table 4.2).

Table 4.2. Clinical variables associated with active pulmonary tuberculosis in PLHIV from January, 2014 to July, 2012, selected hospital, Addis Ababa, Ethiopia

Variables	Subjects without PTB (n=77)	Subjects with PTB (n=40)
Symptoms		
Cough	77(100)	40(100)
Fever	59(76.6)	40(100)
Diarrhea	30(39.0)	27(67.5)
Nausea or vomiting	4(5.2)	36(30.8)
Abdominal pain	22(28.6)	10(25.0)
Night sweat	71(92.2)	40(100)
Difficulty in breathing	45(58.4)	40(100)
Chest pain	54(70.1)	37(92.5)
Weight loss	44(57.1)	39(97.5)
Enlarged lymph node	6(7.8)	0
Chest x-ray (abnormal)	0	10(25.0)
WHO stage		
I	32(33.3)	7(17.5)
II	13(27.3)	13(32.5)
III	16(22.2)	10(25.0)
IV	16(17.1)	10(25.5)

4.3. Detection rate of Pulmonary Tuberculosis

A total of 117 sputum and 117 stool specimens were analyzed for the presence of *M. tuberculosis* organism in all patients. Overall 40 of the 117 (34.2%) suspected PTB patients were confirmed to be positive from sputum and 28(23.9%) from stool specimens by direct PCR examination (with mean age: 30.1 years; sex ratio male/female: 0.6:1). Pulmonary tuberculosis was excluded in 77 patients (with mean age: 34.5 years; sex ratio male/female: 0.64:1). The rates of detection *M. tuberculosis* from both sputum and stool were 12(10.2%), 36(30.8%), and 40(34.2%) by smear, culture and PCR, respectively. Out of 12(10.2%) patients positive for smear microscopy, three (25.0%) patients were positive from both specimens, 8(66.7%) from sputum and 1(8.3%) from stool specimens only. Of 36(30.8%) patients positive for culture, 23(63.9%) patients were positive by sputum culture, three (8.3%) were positive by stool culture and the rest 10(27.8%) were positive by both sputum and stool culture. Of 40(34.2%) PCR positive cases both stool and sputum PCR detected MTB in 28(70.0%) patients but 12(30.0%) patients were positive by sputum PCR only (Table.4.3).

Table 4.3. Rate of pulmonary tuberculosis by different tests carried out in study

Sample(s)	Mycobacterial detection rate from specimens		
	Microscopy positive	L-J positive	PCR positive
Sputum (n=117)	11(9.4%)	33(28.2%)	40(34.2%)
Stool (n=117)	4(3.4%)	13(11.1%)	28(23.9%)
Both sputum & stool	12(10.2%)	36(30.8%)	40(34.2%)

4.4. Laboratory Findings

a) Region of Difference Based Species Identification and Multiplex PCR Genus Typing

Both sputum and stool were processed for smear microscopy, culture and DNA extraction. PCR were conducted to differentiate the species among MTC by presence or absence of RD9 from culture isolate and extracted sediment (Fig. 4.1).

For those who could not be diagnosed and identified by deletion typing, genus typing was done with MycoF, MycoR, Intra genus F, *M. avium* R, TB1F, and TB1R primers to performing multiplex PCR and produced band at 396bp comparable with standard 100bp ladder and known positive control(Fig. 4.2).

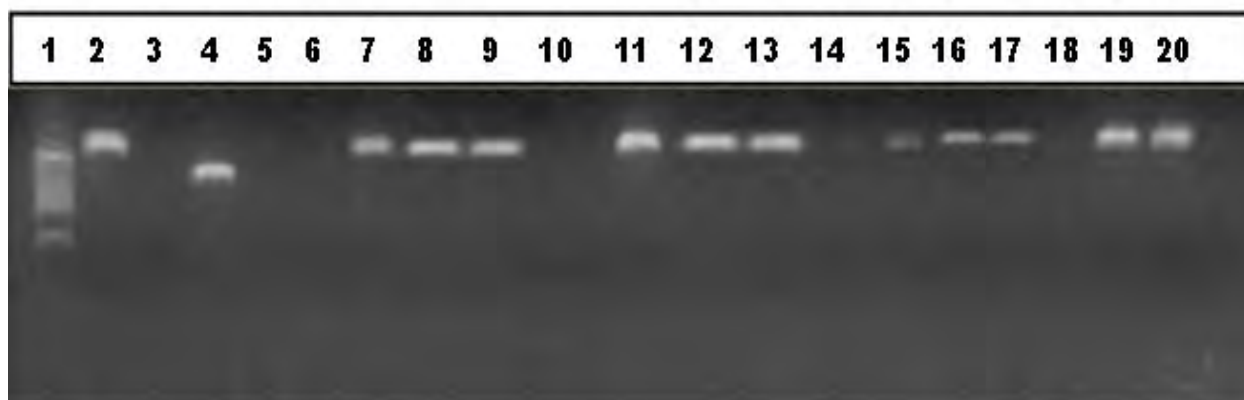


Figure 4.1. Gel picture showing isolates of Mycobacterium characterized for species identification with RD9 Primer. Description; Lane 1 = ladder (100bp), lane 2= positive control (H37Rv); lane 3=negative control; lane 4=*M. bovis*; lane 5-20 stool isolate

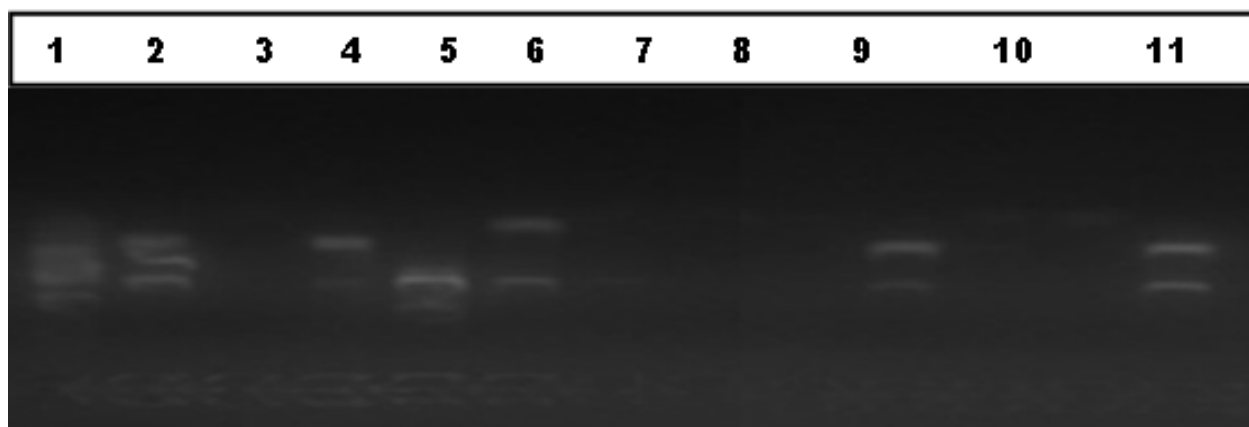


Figure 4.2. Gel picture showing Isolates of Mycobacterium characterized for genus identification with genus MycoF, MycoR, Intra genusF, *M. avium* R, TB1F, and TB1R primers. Description: Lane 1 = ladder (100bp), lane 2= H37Rv; lane 3=negative control; lane 4=*M. bovis*; lane 5=*M.intr*; lane 6=*M. avium*; lane 9 and 11*M. tuberculosis* (396bp) form stool isolate.

b) Sputum Finding

Of the 117 patients who provided sputa, PTB were confirmed bacteriologically (either by smear and/or culture) in 33 patients, while for 84 patients PTB were not confirmed by either of these methods. From the 33 bacteriologically confirmed PTB patients, we obtained 33 sputum samples, and 11 (9.4%) patients were both smear and culture positive. The remaining 22 (18.8%) sputa were smear negative but culture positive. Between smear and culture systems, the mycobacterial isolation rate of culture was statistically significantly higher than that of the smear method ($\chi^2 = 27.1$, $p < 0.001$). The PCR positivity could reach as high as 40(34.2%) in sputum specimen. The mycobacterial detection rate of the PCR systems was comparatively higher than that of the culture methods ($\chi^2 = 84.4$, $p < 0.001$) (Table 4.3). All sputum culture positive samples were positive by PCR, and 22(55.0%) samples have discordant result between sputum smear and culture and 7(6.0%) patients were positive by sputum PCR. The measures of agreement (kappa value) of sputum smear and PCR with sputum culture were 0.42 and 0.86, respectively (Table 4.4).

Table 4.4. Comparison of sputum smear and PCR with sputum culture in diagnosing pulmonary TB in PLHIV

Sputum	Culture		Sens. %	Spec. %	PPV %	NPV %	Kappa value	
	Positive	Negative						
Smear	Positive	11	0	33.3	100	100	79.2	0.42
	Negative	22	84					
PCR	Positive	33	7	100	91.7	82.5	100	0.86
	Negative	0	77					

c) Stool Finding

Stool samples from 117 suspected PTB patients were subjected to smear, culture, and PCR examinations. Of these, 13 (11.1%) were culture-positive by L-J method. However, the culture isolation rate was significant (45.5%) if the pulmonary samples from the same patient were also smear and culture positive as compared to those patients whose pulmonary samples were smear negative, culture negative, and PCR positive (42.8%). This difference was significant ($\chi^2 = 14.5$,

p<0.001). From 4 of 117 patients confirmed to be positive by stool smear, it is also important to note that approximately 4.5% of patients were found stool smear positive as compared with sputum smear negative, culture positive and PCR positive. Therefore, the cumulative total PTB confirmed cases increased from 11/117(9.4%) to 12/117 (10.2%) and 33/117(28.2%) to 36/117(30.8%) by using stool smear and culture, respectively (Table 4.5).

With the application of PCR, overall 28 of 40 (70%) sputum confirmed PTB patients from stool were found PCR positive. Stool PCR positivity could reach as high as 81.8% in sputum smear-positive cases. Even in those patients whose pulmonary samples were negative by smear and culture, the stool PCR was positive in 71.4% of patients. However, the application of PCR for stool samples was most rewarding in sputum smear and/or culture confirmed patients as compared with smear and /or culture unconfirmed patients ($\chi^2 = 41.9, p < 0.001$) (Table 4.5).

Table 4.5. Mycobacteria detection rate of various in vitro diagnostic methods applied to stool specimen from pulmonary tuberculosis patients

Suspected PTB cases(n=117) (and result of sputum sample)	Detection rate of mycobacteria in stool		
	Microscopy	L-J culture	PCR
Smear, culture and PCR positive(n=11)	3(27.3%)	5(45.5%)	9(81.8%)
Smear negative, culture and PCR positive(n=22)	1(4.5%)	5(22.7%)	14(63.6%)
Smear negative, culture negative and PCR positive(n=7)	0	3(42.8%)	5(71.4%)

Stool smear was less sensitive (12.1%) and more specific (100.0%) as compare with gold standard sputum culture. Ten of 33 (30.3%) sputum culture positive samples were positive by stool culture, and 3 of the remaining 84(3.4%) sample has concordant result between stool culture as compare with sputum culture with sensitivity (30.3%) and specificity (96.4%). Twenty three of 33 (69.7%) sputum cultures positive samples were positive by stool PCR, and 5 of 84 patients were positive by stool PCR with sensitivity (69.7%) and specificity (94.0%). The measures of agreement between stool smear, culture and PCR as compare to sputum culture were 0.18, 0.33, and 0.67, respectively (Table 4.6).

Table 4.6. Comparison of stool smear, culture and PCR with sputum culture for the diagnosing pulmonary TB in PLHIV

Stool	Sputum culture		Sens. %	Spec. %	PPV %	NPV %	Kappa Value	
	Positive	Negative						
Smear	Positive	4	0	12.1	100.0	100.0	74.3	0.18
	Negative	29	84					
Culture	Positive	10	3	30.3	96.4	76.9	77.9	0.33
	Negative	23	81					
PCR	Positive	23	5	69.7	94.0	82.1	88.7	0.67
	Negative	10	79					

d) Body Mass Index and CD4T cell

Body mass index and CD4 T cell were compared with sputum and stool results. Of 13 patients with CD4 T count <99 cell/mm³, 10(76.5%) were stool culture positive cases whereas 8(61.5%) positive for sputum culture. Of 15 PTB confirmed cases that posses BMI <18.5 , 9(60.0%) were positive by stool culture and 8(53.3%) were positive by sputum culture. The probability of stool culture positivity increases as CD4 T cell count and BMI decreases. Most patients with CD4 T <99 cell/mm³were positive for stool PCR as compared with CD4 T count >199 cell/mm³(84.6% vs 52.6%). Majority of patients with BMI <18.5 were positive for PCR as compared with patients BMI 18.5-24.99(80.0% vs 64.0%) (Table 4.7).

Table 4.7. Association of body mass index and CD4T-cell count against detection rate of mycobacteria in sputum and stool among confirmed PTB cases (n=40).

Detection rate of mycobacteria	CD4 T cell/mm ³			BMI	
	<99 (n=13)	100 - 199(n=8)	>199 (n=19)	<18.5 (n=15)	18.5 - 24.99 (n=25)
Sputum					
Smear	2(15.8)	4(50.0)	5(26.3)	3(20.0)	8(32.0)
Culture	8(61.5)	6(75.0)	19(100)	8(53.3)	25(100)
PCR	13(100)	8(100)	19(100)	15(100)	25(100)
Stool					
Smear	2(15.8)	2(25.0)	0	3(20.0)	1(4.0)
Culture	10(76.9)	3(37.5)	0	8(53.3)	5(20.8)
PCR	11(84.6)	7(87.5)	10(52.6)	12(80.0)	16(64.0)

4.5. Genetic Diversity and Family Assignment

Spoligotyping of the 46 *M. tuberculosis* isolates produced 24 different spoligotyping patterns were observed. Based on these spoligotypes, distinct families of TB were identified which includes T family, Family 33, CAS family, H37Rv family, Haarlem family and Beijing family. Among the 46 typed isolates, 33(71.7%) were classified into one of the 13 shared international types (SITs) according to SpolDB4.0. The remaining 13 isolates generated 11 different spoligotypes with 1 new shared spoligotypes that had not been previously described in the database. Among the total 24 spoligotype patterns characterized in the present study, 8 patterns (including the 1 new clustered patterns) corresponding to clusters with 2–11 isolates per cluster were identified, accounting for a very high clustering rate of 30/46(65.2%). Out of the 16 patterns that did not form clusters, 10 represented true orphan patterns that did not previously exist in SpolDB4.0 and 6 represented pseudo-orphans which were present as singles in this study but found in SpolDB4.0 (Figure 4.3).

Genotype assignment revealed that the most predominant family in our study were the T family 20(43.5%) which includes T1, T3 and T4 with distribution of 8(17.4%), 11 (23.9%) and 1(2.1%), respectively followed by family33 19(45.6%). Other families found were CAS family 2(4.3%), H37Rv family 2(4.3%), Haarlem 1 family 2(4.3%) and the Beijing family 1(2.2%).

Webdings format	Spoligotype43	SIT	Family	Prob.	No. Isolation
.....	0000000000003771	1	Beijing family	0.99	1
.....	77777774020771	47	H1 family	0.99	2
.....	77777477760771	451	H37Rv family	0.74	2
.....	77776777760731	336	T1 family	0.6	7
.....	777000377760771	149	T3-ETH	0.99	11
.....	777640007760771	249	T4 family	0.99	1
.....	577347777761771	Orphan	T1 family	0.99	1
.....	57777777763771	1096	Family33	0.99	1
.....	77777777777331	1149	Family33	1	1
.....	77777777763771	54	Family33	0.99	1
.....	77777777762771	1690	Family33	0.99	1
.....	77777777767771	2731	Family33	0.99	2
.....	47777774137771	415	Family33	1	2
.....	7777777777771	523	Family33	1	2
.....	77777777771771	Orphan	Family33	0.95	1
.....	77747777763771	Orphan	Family33	0.99	2
.....	77747777766771	Orphan	Family33	0.99	1
.....	57734777763771	Orphan	Family33	0.99	1
.....	575347477742771	Orphan	Family33	0.99	1
.....	7577777777761	Orphan	Family33	1	1
.....	77747777773771	Orphan	Family33	0.99	1
.....	7774777777771	Orphan	Family33	1	1
.....	50377740003171	Orphan	CAS family	0.99	1
.....	70377740001671	Orphan	CAS family	0.99	1

Figure 4.3. Spoligotype pattern of clustered *M. tuberculosis* strains. The hyphen and white boxes indicate the absence and presence, respectively, of the specific spacer at position 1 to 43 in the DR locus. CAS=Central Asian; H=Haarlem

Family Distribution of Stool and Sputum isolates

The highest prevalent strains isolated from sputum were T family (32.6%) followed by Family 33 (28.3%) whereas family 33 was predominant in stool isolate (13.0%). CAS family and Beijing family were not isolated from stool whereas Haarlem family only isolated from stool (Table 4.8).

Table 4.8. Distribution of all isolates (n=46) genotypes based on their spoligo pattern.

Specimen	T-family		Family-33		CAS family		Beijing		H37Rv		Haarlem	
	No.	%	No.	%	No.	%	No	%	No.	%	No.	%
Stool	5	10.9	6	13.0	0	0	0	0	1	2.2	2	4.3
Sputum	15	32.6	13	28.3	2	4.3	1	2.2	1	2.2	0	0

Chapter Five

Discussion

In this study, we had assessed utility of stool specimen as compared with sputum specimen by different diagnostic methods from volunteers living with HIV who are suspected for pulmonary tuberculosis. Of 117 patients suspected of pulmonary tuberculosis *M. tuberculosis* were diagnosed in 10.3%, 30.8%, and 34.2% using smear microscopy, conventional solid culture and PCR, respectively from both sputum and stool specimen and excluded in 65.8% patients. The performance of most easily available clinical sample, i.e., stool was evaluated against sputum specimen by different diagnostic methods. We found that *M. tuberculosis* were identified in 13(11.1%) and 4(3.4%) of 117 PLHIV with suspected PTB using culture and smear, respectively. Of the 84 sputum culture negative cases, three (3.6%) were stool culture positive which indicated that, if available, stool cultures may increase the number of persons diagnosed with TB in PLHIV. This value must be counterbalanced against the increased processing requirements and higher culture contamination rates associated with culturing stool (Oramasionwu *et al.*, 2013, Wolf *et al.*, 2008). Not only culture but also stool smear along with sputum smear methods also improved the mycobacterial detection rate. Molecular identification of AFB in stools was critical because *Mycobacterium avium* complex, *Mycobacterium florentium* and *Mycobacterium gordonae* have been previously detected in patients' stools (Khe'chine *et al.*, 2009, Colebunders *et al.*, 1990). Also, MTC organisms can be detected in the stools of patients with digestive tuberculosis (Farid *et al.*, 1999), a situation that was not addressed in this study.

The microbiological diagnosis of PTB in HIV infected patients was inefficient by conventional method and specimen. Sputum scarcity in HIV-infected individuals (especially with low CD4 T counts) hampers the diagnosis of PTB by conventional smear (Kibiki *et al.*, 2007). Thus, lack of sputum and the paucibacillary nature of TB in HIV infection (Hassim *et al.*, 2010) can result in misdiagnosis or in classifying patients as smear-negative PTB. According to our finding parallel examination of stool and sputum smear increase diagnosis of PTB in HIV patients. With respect to the diagnosis of pulmonary tuberculosis, as compare to sputum culture, microscopic analysis of sputum had a sensitivity of 33.3% which is slightly lower than the 37.5 % previously reported

(Khe'chine *et al.*, 2009). Specificity was 100%, the PPV was 100%, and the NPV was 79.2% with measure of agreement kappa 0.42. In one patient, stool smear permitted the diagnosis of PTB as compared to sputum smear though the sensitivity (12.1%) was very low. Our finding was concurrent with a study conducted in France (Khe'chine *et al.*, 2009). In a series of TB patients with HIV, stool cultures grew *M. tuberculosis* in 44% and smear microscopy was positive in 11% (Oramasionwu *et al.*, 2013), as compared to 13(11.1%) stool culture positivity and four (3.4%) stool microscopy positivity in our study, however, the sensitivity may have been higher because most patients had advanced immune suppression indicated by acquired immune deficiency syndrome or clinical stage C disease. The specificity of stool smear microscopy as compared to stool culture was 100%. Its PPV was 100% and NPV was 90% with moderate agreement kappa 0.441.

In this study, *M. tuberculosis* organisms grew in 33(28.2%) sputum and 13(11.1 %) stool specimens. As compared with sputum culture, stool culture had a sensitivity of 30.3 %, a specificity of 96.4 %, a PPV of 76.9% and an NPV of 77.9% with fair agreement (kappa) 0.328. The diagnosis of three participants (2.6%) for whom stool culture was the only culture-positive specimen for TB indicates that, if available, stool cultures may increase the number of persons living with HIV diagnosed with TB. Our finding was far up from study conducted on PTB patient in France which showed that, MTC organisms grew in 14.9% sputum and 9.7% stool specimens (Khe'chine *et al.*, 2009). Other study of pediatric patients in Peru found that stool culture detected (3/15) for the diagnosis of pulmonary tuberculosis (Oberhelman *et al.*, 2006). The authors noted that culture of a larger volume of stool and improved decontamination/ concentration techniques could improve the sensitivity of stool culture. Alkali is most widely used among protocols proposed for the decontamination of stools for the recovery of Mycobacteria (Oberhelman *et al.*, 2006). However, in this study, we used the more efficient chlorhexidine method as previous study reported in France (Khe'chine *et al.*, 2009). The sensitivity of 30.3% is too low to suggest that stool specimen should replace sputum specimens for TB diagnosis, and is lower than the sensitivity reported in other studies (Khe'chine *et al.*, 2009, Oramasionwu *et al.*, 2013).

This study evaluated PCR for direct detection of *M. tuberculosis* from sputum and stool samples. Stool PCR detected 28 (24 %) *M. tuberculosis* DNA in stool specimen and showed sensitivity of 69.7%, specificity of 94.0%, PPV of 82.1% and NPV of 88.7% as compared with sputum cultures

as the gold standard. Positive agreement between sputum culture and stool PCR was observed in this study with kappa value 0.67. The PCR results of stool was also compared with sputum and show that 70% sensitivity, 100% specificity, 100% PPV and 86.5% NPV with substantial agreement of 0.754. In a pilot accuracy study in South Africa showed that, stool Xpert testing detected 8/17 (47.1%) of children with definite tuberculosis, including 4/5 (80%) HIV–infected and 4/12 (33%) HIV-uninfected children and sputum Xpert detected 11/17 (65%) cases (Nicol *et al.*, 2013). Another study in France show that real-time PCR detected MTC DNA in 28/134 (20.9 %) sputum and 27/134 (20.2 %) stool specimens. Its sensitivity and specificity for stools were 100% and 97.3 %. The PPV was 88.9% and the NPV 100 % (Khe'chine *et al.*, 2009). Use of PCR results had better sensitivity than use of culture, which may explain why we found in consistent sensitivity to the study carried out in France (Khe'chine *et al.*, 2009). In our finding stool PCR missed 10.2% patients as compared to finding conducted in France which missed 0.7% patient. The PCR internal control indicated that 12/117 (10.2 %) stool specimens were partially inhibited, a high value when compared with a reported 2.2% rate of inhibition using stool specimens (Khe'chine *et al.*, 2009). This could be due to the DNA extraction protocol we adopted in this study; it could be inefficient in removing PCR inhibitors from stools.

In the present study, we compared the CD4+ T cell of three patients with only stool culture growing *M. tuberculosis* with 23 patients with only sputum cultures positive. People living with HIV with positive stool culture had a lower median CD4+ T cell count than those with TB by sputum culture (83.5 vs 211.1 cells/ μ l). This finding in line with study conducted in Cambodia, Thailand and VietNam that patients with stool culture positive had less CD4+ T cell count as compared to culture positive other than stool sample with mean count (75 vs 139 cells/ μ l) (Oramasionwu *et al.*, 2013). The mean BMI of patients with positive stool culture as compare with positive sputum culture was 18.2 and 20.1; respectively i.e. patients with decreased body mass index had increased probability of positive stool culture. Our finding is supported by previous study (Oramasionwu *et al.*, 2013) as the mean BMI of patients with stool culture versus culture from other site was 18 and 19, respectively.

Analysis of genetic diversity of *M. tuberculosis* strains provides insights into transmission mechanisms, emergence of drug resistance and particularly virulent strains, and information relevant to the development of new diagnostics, drugs, and vaccines. Spoligotyping was used as a

primary typing tool because of its ease of use, straightforward coding, and international database of global isolates for comparative analysis. The study conducted in France showed that distribution of the predominant clades of *M. tuberculosis* strains shows variation among different populations (Brudey *et al.*, 2006). As to our knowledge, there was no much literature on the genetic diversity of *M. tuberculosis* from stool isolate. In our study, the most frequently occurring of all tested strains was T family (43.5%) and family33 (41.3%). Although the T family is one of the most prevalent, it remains an ill-defined family of *M. tuberculosis* that is found worldwide (Brudey *et al.*, 2006). From T family, the T3ETH is the dominant sub-family (55.0%), and previous reports also showed this spoligotype present in high proportion in Ethiopia (Mihret *et al.*, 2012) and it is believed to be specific for Ethiopia and rarely reported in other countries (Mihret *et al.*, 2012, Brudey *et al.*, 2006). The family33, which is the second most frequent spoligotype in this study. The other families were CAS family, H family, H37Rv family and Beijing family comprising 2, 2, 2 and 1 isolates, respectively. All were reported from Ethiopia (Mihret *et al.*, 2012). The highest prevalent strains isolated from sputum were T family (32.6%) followed by Family 33 (28.3%) whereas family 33 was predominant in stool isolate (13.0%). CAS family and Beijing family were not isolated from stool whereas Haarlem family only isolated from stool.

Chapter Six

Conclusion and Recommendations

The finding of this study indicated that laboratory investigation of stools demonstrated potential utility for the diagnosis of TB, although they did not perform better than sputum. Sputum should remain the diagnostic specimen of choice for pulmonary TB; however, in persons who are unable to produce an adequate sputum specimen for analysis with a low CD4 count and BMI stool culture may be a useful specimen for diagnosing TB in some settings. Performing stool and sputum smear microscopy for such patient also is considered.

The PCR is a potential diagnostic tool that can be used in the diagnosis of PTB in PLHIV. However, its ultimate use in developing countries especially Ethiopia is depends on evaluation of its cost effectiveness for routine diagnosis. It is a specific technique that can be used to identify and differentiate bacteria from *M. tuberculosis* complex.

Despite the presence of predominant shared types, the diversity of the *M. tuberculosis* found in the present study is very high; 46 isolates produced 24 different spoligotypes. The families observed in this study were 8.1% of the total 62 families currently registered in the fourth international spoligotyping database, SpoIDB4.

In accordance with these conclusions, the following recommendations were forwarded:

- 1) We recommend that parallel examination of both stool and sputum culture (or smear) is good candidate assays for screening of suspected PTB in PLHIV.
- 2) There is need to carry out further studies to evaluate multiple potential specimens collected from PLHIV.
- 3) We recommend future wider studies with a better molecular method like single nucleotide polymorphisms typing and mycobacterial interspersed repetitive units (variable number of tandem repeats) to elucidate strain diversity.

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Annexes

Annex- 1: Participant Information Sheet

Date: -----

Title: “Detection and characterization *Mycobacterium tuberculosis* in stool of HIV sero-positive patients with suspected pulmonary tuberculosis.”

Background: Tuberculosis (TB) in Africa is increasing because of the HIV epidemic, and in HIV / AIDS patients it presents atypically. Smear-negative PTB is more common in HIV-infected patients and leads to diagnostic delay which increases morbidity and mortality in PLHIV.

Objective: To detect and characterize *Mycobacterium tuberculosis* in stool of HIV sero-positive patients with suspected pulmonary tuberculosis.

Organizations: The study was conducted by Addis Ababa University, school of Graduate studies. Laboratory procedure is to be carried out at ALIPB and Addis Ababa regional laboratory.

Procedure: Expert (in the area) physicians were undertaken physical diagnosis. Pulmonary tuberculosis suspected patient was sending for chest x-ray and laboratory examination. Stool and sputum specimens were collected for the microscopic examination, culture and PCR and blood sample was collected for CD4 count.

Participation: The procedure is to be carried out after getting your willingness to participate. All volunteer patients with suspected PTB, fulfilling inclusion criteria, were included.

Risks associated with sample collection: You were experience mild pain during blood specimen collection, although that is not due to participation in this study.

Benefit: You were benefited from the study; because advanced technique and multiple samples will be analyzed as compare to routine sputum smear which is part of your diagnosis.

Compensation: You were receiving your result (only positive patients) through your physician. You were get treatment for free if you become positive for PTB. Fees for transport and elapsed time were also being compensated.

Confidentiality: From medical ethics point of view and research ethics, every part of your personal information was kept confidentially. Information to be collected and variables expressing your identity was coded secretly. The only responsible people to link your variables

(important for your follow up and treatment) with the code number are your doctor and principal investigator. However, other researchers can see your clinical information, which is without your identity. Your result and information was be used only for the mentioned purpose.

Sharing the Result: Eventually, the result, devoid of your identity was reported through publication or by other means. Have no suspicion on the confidentiality of your information, even at this time. We request your permission to use the result for reporting.

Right to refuse or withdraw: Thus, it is your right to agree or to refuse to participate in the study. Withdrawal from the study is also possible, at any time. Withdraw or refusing to participate did not have any impact on your normal diagnosis or medical follow up. You can address your problem or question through one of the addresses given bellow.

Contact Addresses:

- 1) **Gizaw Eshetu:** Address: AAU, College of Health Science, department of Microbiology, Immunology and Parasitology, Mobile: +251911-8032-55, E-mail: gizaweshetu@yahoo.com
- 2) **Dr Tamirat Abebe(BSc, MSc, PhD, Assis. Prof):** Address: AAU, College of Health Science, department of Microbiology, Immunology and Parasitology, Mobile: +251911-4472-27, E-mail: tamrat.abebe@yahoo.com
- 3) **Dr Adane Mihret (DVM, MSc, PhD, Associate Prof.):** AAU, College of Health Science, department of Microbiology, Immunology and Parasitology, Mobile: +251911-4089-84, E-mail: adane_mihret@yahoo.com
- 4) **Dr. Gobena Ameni (DVM, PhD, Associate Professor):** Address: Aklilu Lemma Institute of Patho-biology (ALIPB), Mobile: +251911-4130-73, E-mail: gobenachimdi2009@yahoo.com
- 5) **Addis Ababa University (College of Health Science):** address: Office of Associate Dean, Postgraduate Programs and Research. Tel. + 251-011-551-28-765, P.O. Box 9086, Addis Ababa, Ethiopia.

Thank you for your patience and kindness!!!

Annex- 2: Informed Consent Form

Name: -----, Age -----, Sex -----

Identification No -----, lab No -----

I read and/or well informed about the nature of the study, entitled **“Detection and characterization of *M. tuberculosis* organism in stool of HIV sero-positive patients with suspected pulmonary tuberculosis”**. Though not due to my involvement in the study, I was experience mild pain during the procedure. Then, she/he asked my willingness to allow the sputum, stool and blood sample to be collected for the research purpose. Finally, she/he told me that this was certain if I agree on the following points and signed bellow.

- a) I understood the objective of the study
- b) I understood that the sample won't be used for farther study, and after completion of the whole procedure, the leftover sample will be discarded safely
- c) I am aware of any information describing my identity, collected using questionnaire and, won't be disclosed
- d) I understood report of my result won't include my name
- e) I understood that I won't get money for being part of the study except compensation for transportation and elapsed time if only the result become positive.
- f) I clearly informed as I have the right to refuse to participate and withdraw (if I change my idea) from the study at any time
- g) I understood that my refusal to take part in this study won't have impact to the normal diagnosis and to my future medical follow up.

I have had enough time to think over it freely and I understood it well. I found it would have positive impact in the investigation of my case. My agreement to this consent is without any external enforcement, and was confirmed by my signature, below.

The information sheet explained to me by: -----, Signature -----, Phone-----

Name of participant: -----, Signature -----, Phone -----

Annex -3: Questionnaire

Addis Ababa University School of Health Science Department of Microbiology,
Immunology and Parasitology

Direction: - Please make “✓” on the answer among choices or write on the space provided

Serial number: _____

Date of interview: ____ / ____ / ____

DD / MM / YYYY

To be Filled by the Interviewer		
101.	Sex	Male <input type="checkbox"/> Female <input type="checkbox"/>
102.	Age	18-29 <input type="checkbox"/> 30-39 <input type="checkbox"/> 40-49 <input type="checkbox"/> 50 and above <input type="checkbox"/>
103.	Marital status	Single <input type="checkbox"/> Married <input type="checkbox"/> Divorced <input type="checkbox"/> Widowed <input type="checkbox"/>
104.	Educational status	Illiterate <input type="checkbox"/> Elementary school <input type="checkbox"/> High school <input type="checkbox"/> Certificate and above <input type="checkbox"/>
105.	Occupation	1) Government employed <input type="checkbox"/> 2) Housewife and daily laborer <input type="checkbox"/> 3) Merchant <input type="checkbox"/> 4) Farmer <input type="checkbox"/> 5) Student <input type="checkbox"/> 6) Others -----
106.	Place of residence	1) Urban <input type="checkbox"/> 2) Rural <input type="checkbox"/>
107.	Body mass index(BMI), kg/m ²	-----
108.	Symptoms	Cough Yes <input type="checkbox"/> No <input type="checkbox"/>
		Fever Yes <input type="checkbox"/> No <input type="checkbox"/>
		Diarrhoea Yes <input type="checkbox"/> No <input type="checkbox"/>

		Nausea or vomiting	Yes <input type="checkbox"/>	No <input type="checkbox"/>
		Abdominal pain	Yes <input type="checkbox"/>	No <input type="checkbox"/>
		Night sweats	Yes <input type="checkbox"/>	No <input type="checkbox"/>
		Difficulty breathing	Yes <input type="checkbox"/>	No <input type="checkbox"/>
		Chest pain	Yes <input type="checkbox"/>	No <input type="checkbox"/>
		Weight loss	Yes <input type="checkbox"/>	No <input type="checkbox"/>
109.	Chest radiograph with any abnormality		Positive <input type="checkbox"/>	Negative <input type="checkbox"/>
110.	Enlarged lymph nodes		Positive <input type="checkbox"/>	Negative <input type="checkbox"/>
111.	WHO stage of HIV		1) I <input type="checkbox"/>	3) III <input type="checkbox"/>
			2) II <input type="checkbox"/>	4) IV <input type="checkbox"/>
112.	CD4+ Lymphocyte count		<200Cells/mm ³ <input type="checkbox"/> 200–399 Cells/mm ³ <input type="checkbox"/> 400–599 Cells/mm ³ <input type="checkbox"/> ≥600 Cells/mm ³ <input type="checkbox"/>	
118.	Sputum bacillary burden		Minimal (smear-negative) <input type="checkbox"/> Moderate (1+) <input type="checkbox"/> Most (2–3+) <input type="checkbox"/>	

የተሳታፊዎች የጥናቱ መረጃ ቅፅ

ቀን -----

ርዕስ: “**ቲቢን በHIV ህመምተኛ አይነምድር ዉስጥ መመርመር።**”

መግቢያ: ቲቢ፣ ማይኮባክተሪዮም በምባል ተዋህስያን የምመጣ ስሆን፤ ብዙ ህመምና ሞትን እያደረሰ ያለ በሽታ ነው።

የጥናቱ ዓላማ: “**ቲቢን በHIV ህመምተኛ አይነምድር ዉስጥ መመርመር።**”

ጥናቱን የምያካሄድ ድርጅቶች: ኣዲስ አበባ ዩንቨርሲቲ፣ የድህረ ምረቃ ትምህርት ቤት ነው። የላቦራቶሪው ስራ የምካሄድበት ቦታ፣ አክሊሉ ለማ መካከ-ጥናት የምርምር ተቋም ነው።

የናሙና አወሳሰድህደት: በሙያዉ የተካኑ ላቦራቶር ባለሙያዎች የደም ናሙና ለCD4 ይወስዳሉ። የአክታና የሰገራ ናሙና በላቦራቶር ባለሙያ እንደታዘዙት ይሰበስባሉ። ማንኛውም የታዘዘሎ ምርመራ ከተሰራ በኋላ የቀረዉ ናሙና ለጥናቱ አላማ ይወሰዳል። ነገርግን ለጥናቱ አላማ ተብሎ ናሙና እንደገና አይወሰድም።

ተሳትፎ: በጥናቱ ዉስጥ ያለመሳተፍ መብትዎ የተጠበቀ ነው። ናሙናዉን የምንወስደዉ የእርስዎን ሙሉ ፍቃድ ካገኘን ብቻ ነው። መስፈርቱን የምያሟሉና ፍቃደኛ የሆኑ ሰዎች ሁሉ በጥናቱ ዉስጥ ይገባሉ።

በናሙና አወሳሰድ ጊዜ ልያጋጥሞት የምችል ችግር: በጣም አነስተኛ ህመም የደም ናሙና ስበስብ ልሰማዎ ይችላል ነገር ግን በተቻለ መጠን ጥንቃቄ ይደረጋል።

ከጥናቱ ሊያገኙ የምችሉ ጥቅም: ጥናቶች እንደምያሳዩት የቲቢ በሽታ የሚያመጣዉ ተዋስ ሰገራ ዉስጥ ይገኛል። በተጨማር ጥናቱ የምርመራዎ አንድ አካል ስለሆነ፤ የቲቢ ባክቴሪያ ከተገኘቦት፣ አሁን ላሉቦት ህመም መፍትሄ ሊሆንዎ ይችላል።

ማካካሻ: የቲቢ ባክተሪያ ከተገኘቦት ብቻ ወደ ህክምዎ ቀርበዉ መድሃኒት በነጻ ያገኛሉ። እንድሁም ለትራንስፖርት ላወጡት ገንዘብና ላባክት ጊዜ ተመጣጣኝ ገንዘብ ይሰጦታል።

ምስጥር ስለመጠበቅ: በህክምና ሙያም ሆነ በምርምር ስራት መሰረት፣ ማንኛውም ዓይነት የግል መረጃዎ በምስጥር ይጠቃል። በመጠይቅ የተሰበሰበዉ መረጃዎ ማንነትዎን በማይገልጽ

በምስጢር ኮድ ወደ ኮምፕዩተር እንድንገባ ይደረጋል። የላቦራቶሪ ወ.ጤትም ምስጢሩ የተጠበቀ ከመሆኑም በላይ የእርስዎን ስም አይዘገም። ይሁን እንጂ፣ ሌሎች ተመራማሪዎች የርስዎ ማንነት የለሰበትን ወ.ጤትም ሊያዩ ይችላሉ። መረጃዎና ወ.ጤትዎ ለተጠቀሰው አላማ ብቻ ይወላል።

የወ.ጤት አገላለፅ ህደትና መንገድ: ጥናቱ ከተጠናቀቀ በኋላ፣ ወ.ጤቱ በተለያዩ መንገድ ይገለጻል። የእርስዎን ማንነት እንደመይዘና ምስጢርዎ እንደተጠበቀ ይሆናል። ለዝህም የእርስዎን በጎ ፍቃድ እንጠይቃለን።

በጥናቱ ያለመሳተፍ መብት: በጥናቱ ውስጥ የመሳተፍም ሆኑ ያለመሳተፍ መብት መብት አለዎት። ናሙናውን ከፈቀዱ በኋላም፣ በማንኛውም ጊዜ፣ ከጥናቱ ህደት ውስጥ አቋርጠው መውጣት ይችላሉ። በጥናቱ ውስጥ አለመሳተፍም ሆነ ማቋረጥ፣ በምስጢርዎ ህክምና ላይ ምንም አይነት ችግር አያመጣም።

ተመራማሪዎችን ሊያገኙ በትየምችሉባቸው አድራሻዎች፡ ስለጥናቱ ጥያቄ ካሎትም ሆነ በምፈልጉበት ጊዜ ጥናቱን ማቋረጥ ከፈለጉ፣ ከምክተሉት ውስጥ የአንዱን አድራሻ ይጠቀሙ።

1. ግዛው እሸቱ፡ አድራሻ፡ አዲስ አበባ ዩኒቨርሲቲ፣ ሞባይል +251911803255 ፣ኢ-መይል፡— gizaweshetu@yahoo.com
2. ዶ/ር ታምራት አበበ (ረዳት ፕሮፌሰር) ፡ አድራሻ፡ አዲስ አበባ ዩኒቨርሲቲ ሞባይል +251911447227' ኢ-መይል፡- tamrat.abebe@yahoo.com
3. ዶ/ር አዳነ ምህረት(ተባባሪ ፕሮፌሰር)፡ አድራሻ፡ አዲስ አበባ ዩኒቨርሲቲ ሞባይል +251911447227 ኢ-መይል፡- adane_mihret@yahoo.com
4. ዶ/ር ጎበና አሜኒ (ተባባሪ ፕሮፌሰር)፡ አድራሻ፡ አዲስ አበባ ዩኒቨርሲቲ፣ አክሊሉ ለማመካከ ጥናት የምርምር ተቋም፣ ሞባይል +251-911413073 ኢ-ሜይል፡- gobenachimdi2009@yahoo.co.uk
5. አዲስ አበባ ዩኒቨርሲቲ፣ የህክምና ፋኩሊቲ፣ የድህረ ምረቃና ምርምር ቢሮ፣ ፖ.ሳ.ቁ. 9086. አድስ አበባ፣ ኢትዮጵያ፣ ስልክ 251-011-551-28-765

አመሰግናለሁ !!!

የስምምነት መጠየቅ ቅፅ

ስም-----፣ ዕድሜ -----፣ ፆታ -----
የሆስፒታሉ ቁጥር -----፣ የላቦራቶር ቁጥር -----

ካነበብኩት ወይም ከሰማሁት “**ቲቢን በHIV ህመምተኛ አይነምድር ዉስጥ መመርመር።**” ስለተባለው ጥናት በቂ ግንዛቤ አግኝቻለሁ። ናሙናዉ ስወስድልኝም ምንም ህመም እንደማይኖረዉ ተረድቻለሁ። የምወሰደዉ ናሙናም ከታዘዘልኝ ምርመራ የተረፈ ነዉ። በመጨራሻም ናሙናዉን መፍቀዴ የማረጋገጠዉ፤ በምክተሉት ነጥቦች ከተሰማማሁ ብቻ መሆኑ ተነግሮኛል።

ሀ. የጥናቱ ዓላማ ገብቶኛል

- ለ. ናሙናዬም ሆነ መረጃዬ ለተገለጸዉ ዓላማ ወጪ እንደማይወልድና የተረፈ ናሙና ካለም በጥንቃቄ እንደምወገድ ተረድቻለሁ።
- ሐ. በመጠየቅ የሰጠሁት መረጃዬም ሆነ የናሙናዉ ወጪት ማንነቴን እንደማይገልፅ ተረድቻለሁ።
- መ. በጥናቱ ተሳታፊ ስለሆንኩ ሳይሆን፤ በባክትሪያዉ ተጠቂ ከሆንኩ ብቻ የትራንስፖርትና ላባክንኩት ጊዜ የምሆን ተመጣጣኝ ገንዘብ እንደምሰጠኝ አወቀለሁ።
- ሠ. በጥናቱ ዉስጥ ያለመሳተፍም ሆነ ከገባሁ በኋላ የማቋረጥ መብት እንዳለኝ ተረድቻለሁ።
- ረ. በጥናቱ ዉስጥ ያለመሳተፍም ሆነ ማቋረጥ በምሰጠኝ ህክምና ላይ ምንም ችግር እንደማያመጣ አወቀለሁ።

ስለጥናቱ ካነበብኩት ወይም ከሰማሁት፣ በቂ ጊዜ ወስጄ ካሰብኩ በኋላ፣ በቂ ግንዛቤ አግኝቻለሁ። ስለሆነም፣ በጥናቱ ዉስጥ መሳተፊ ጠቃሚ ሆኖ ስላገኘሁት፣ በራሴ ወስኜ ናሙናዉን እንድጠቀሙ መፍቀዴን በፋርማዬ አረጋግጣለሁ።

የተሳ ታፋ ስም ----- ፊርማ ----- ስልክ -----
የመርጃ ተቀባይ ስም ----- ፊርማ ----- ስልክ -----

Annex -4: Declaration Sheet

Title of the study: “Detection and characterization of *Mycobacterium tuberculosis* organism in stool of HIV sero-positive patients with suspected pulmonary tuberculosis”

I, the undersigned, declare that this MSc research is my original work. It has not been for a degree in any other university. False statements cause the invalidation of this research thesis and may lead to other administrative or legal actions.

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APPROVED BY THE BOARD OF EXAMINERS

This thesis by Gizaw Eshetu is accepted in its present form by the board of examiners as satisfying thesis requirement for the degree of masters in Medical Microbiology.

Examiner:

Full name

Rank

Signature

Date

1) _____

2) _____