

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



**USE OF URINE AS AN ADJUNCT SPECIMEN FOR THE**  
**DIAGNOSIS OF PULMONARY TUBERCULOSIS IN**  
**PEOPLE LIVING WITH HIV IN ADDIS ABABA**

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Addis Ababa, Ethiopia

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living with HIV in Addis Ababa**

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**A Thesis Submitted to the School of Graduate Studies of Addis Ababa University, College of Health Sciences, Department of Microbiology, Immunology and Parasitology in the Partial Fulfillment of the Requirements for the Degree of Master of Science in Medical Microbiology**

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## ABBREVIATIONS

AFB-	Acid Fast Bacilli
AIDS-	Acquired Immunodeficiency Syndrome
ALIPB-	Akililu Lema Institution Pathology
ART-	Antiretroviral Therapy
ATCC-	American Type Culture Collection
BCG-	Bacillus Calmette-Guerin
CTAB-	Cetyltrimethyl Ammonium Bromide
DNA-	Deoxyribonucleic Acid
dNTP-	Deoxynucleotide Triphosphate
DOTS-	Direct Observed Therapy Strategy
EDTA-	Ethylene Diamine Tetraacetic Acid
EMU-	Early Morning Urine
EPTB-	Extrapulmonary TB
HIV-	Human Immunodeficiency Virus
IGRA-	Interferon- $\gamma$ release assay
IRIS-	Immune Reconstitution Inflammatory Syndrome
LJ-	Löwenstein-Jensen
MDR -	Multidrug-Resistant
MGIT-	Mycobacterium Growth Indicator Tube
MTC-	Mycobacteria Tuberculosis Complex
PBS-	Phosphate Buffered Saline
PCR-	Polymerase Chain Reaction
PPD-	Purified Protein Derivative
PRH	Polis Referral Hospital
PTB-	Pulmonary Tuberculosis
PZA-	Pyrazinamide
QFT-G-IT-	QuantiFERON TB-GOLD In-Tube
SDS-	Sodium Dodecyl Sulphate
TAE-	Tris-Acetic acid EDTA
TB-	Tuberculosis

TCH-	Thiophen-2-carboxylic acid hydrazide
THC	Teklehaymanot Health Center
TST-	Tuberculin Skin Test
USCDC -	United States Centers for Disease Control and Prevention
WHO-	World Health Organization
XDR-TB-	Extensively-drug resistant TB
ZMH	Zewuditu Memorial Hospital
ZN-	Ziehl-Neelsen



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## ABSTRACT

**Background:** Tuberculosis is an opportunistic infection that increases the mortality and morbidity in human immunodeficiency virus (HIV) infected individual. The diagnosis of pulmonary tuberculosis (PTB) is usually established by examination of three Zeihl-Neelsen (ZN) stained smears but in HIV infected persons, ZN staining mostly shows negative smear, which do not preclude Pulmonary TB. Since tubercle bacilli or their nucleic acids are expected to be excreted through the kidneys, we were interested to assess spot urine as a supplementary specimen for diagnosing of pulmonary TB.

**Objectives:** The aim of this study was to evaluate urine as an adjunct specimen for the diagnosis of pulmonary tuberculosis in people living with HIV by using different diagnostic methods.

**Method:** A cross-sectional study was conducted on PTB suspected patients infected with HIV from November 2013 to January 2015. A total 234 specimens (117sputum & 117urine) were collected from 117 PTB suspected cases. The collected samples were processed for culture using Lowenstein-Jensen medium and the left were subjected to PCR using RD9 primers. The culture isolates were further analyzed using deletion typing for species identification and multiplex PCR for genus typing and the isolates were characterized using Spoligotyping.

**Result:** Out of 117 PTB suspected HIV infected individuals, sputum culture alone detected more mycobacterial isolates 33 (28.2%) than the urine specimen alone 17 (14.5%). Of the 84 sputum culture-negative cases, four (4.8%) were urine culture-positive. Among patients whose pulmonary samples were negative by all bacteriological methods, the urine PCR was positive in 5.2% of the patients. The combination of urine culture and PCR result was comparable with the result of sputum culture with the sensitivity, specificity, PPV and NPV of 87.9%, 100%, 100%, and 79.2% respectively. In our study, the majority of the isolates (86.8%) belonged to two major families: Family33(43.4%) and T family(43.4%).

**Conclusion:** PCR and culture examination of urine specimen from PTB suspected patients significantly improved the detection rate of *M.tuberculosis*. The distribution of *M.tuberculosis* isolated from both urine and sputum specimens of the same patients showed difference with 53.8% in their family.

**Key words:** Tuberculosis; Pulmonary tuberculosis; TB HIV infected, Diagnosis; Urine; PCR.

## CHAPTER ONE

### INTRODUCTION

In 2013, an estimated 1.1 million (13%) of the 9.0 million people who developed TB worldwide were HIV-positive. The African Region accounted for 78% of the estimated number of HIV-positive incident TB cases. Persons co-infected with TB and HIV is 29 times more likely to develop active TB disease than persons without HIV. Beginning in the 1980s, the HIV epidemic led to a major upsurge in TB cases and TB mortality in many countries, especially in southern and eastern Africa. The number of people dying from HIV-associated TB has been falling since 2004. However, globally there were still 360,000 deaths from HIV-associated TB in 2013, equivalent to 25% of all TB deaths (among HIV-negative and HIV-positive people) in 2013 and around 25% of the estimated 1.5 million deaths from HIV/AIDS. (WHO, 2014). In Ethiopia, the prevalence of HIV co-infection among TB patients revealed varying rates of HIV seropositivity in active TB patients ranging from 6.6% to 52.1% (Kefene H et al., 1990, Demisse M et al., 2000, Gellete A et al., 1997, Kassu A et al., 2007a, Mitike G et al., 1997, Yassin MA et al., 2004) TB disease may occur at any stage of HIV disease and is frequently the first recognized presentation of underlying HIV infection(Sonnenberg P et al., 2005). Tuberculosis and HIVco-infections are associated with special diagnostic and therapeutic challenges and constitute an immense burden on healthcare systems of heavily infected countries like Ethiopia(Federal Ministry of Health (FMOH), 2007).

HIV and *Mycobacterium tuberculosis* have a synergistic interaction; each accentuates progression of the other(Sharma SK and Mohan A, 2005). HIV infection, by impairing cell-mediated immunity, appears to be the highest known risk factor for the reactivation of tuberculosis. Recent evidence from Europe and USA suggests that HIV-infected people may also be more susceptible to new tuberculous infection and may rapidly develop the overt disease(HarriesAD, 1994).

Colby postulated that tubercle bacilli could be excreted through the kidneys and that the organisms could be demonstrated in the urine of active TB patients who have no symptoms pertaining to the urinary tract(Colby FH, 1961). This hypothesis was confirmed by studies carried out in HIV-positive cases(Aceti A et al., 1999). These studies showed that urine could be used as an adjunct specimen due to the convenience and non-invasive nature of collection.

Even though Ethiopia harbors the majority of TB in HIV cases; no such study has yet been undertaken in this country. Therefore, we carried out this study in order to evaluate the utility of urine as a clinical specimen for diagnosing PTB in people living with HIV, using culture, smear and PCR methods.

## 1.1. Literature Review

### 1.1.1. Etiological Agent

The German scientist Robert Koch first detected the causative agent of tuberculosis in 1892. The genus *Mycobacterium* includes members of the *Mycobacterium tuberculosis* complex, which contain medically important species (Shinnick TM and Good, 1994). TB is an infectious disease caused by *M. tuberculosis* complex (MTC) bacteria, which has an endemic character and worldwide distribution. The MTC comprises closely related species responsible for strictly human and zoonotic tuberculosis. The complex consists of seven species and subspecies including *M. tuberculosis*, *M. canetti*, *M. africanum*, *M. pinnipedii*, *M. microti*, *M. caprae* and *M. bovis*. Despite the different species tropisms, the MTC is characterized by 99.9% or greater similarity at the nucleotide level and possess identical 16S rRNA sequence (Dye C et al., 2005). There is little or no exchange of chromosomal DNA between cells from MTC, making this group of bacteria highly clonal. In a strictly clonal population, any mutation present in an ancestral strain will be present in all descendants and can be used to identify clonal complexes (Smith NH et al., 2006).

### 1.1.2. Virulence Factors

*Mycobacterium tuberculosis* and *Mycobacterium leprae*, the causative agents of tuberculosis and leprosy, respectively, produce large quantities of LAM, a highly immunogenic, cell wall associated glycolipid. This molecule has been previously reported to be a potent inhibitor of gamma interferon mediated activation of murine macrophages. Studies of the mechanism by which this mycobacterial glycolipid down-regulates macrophage effector functions provide evidence that LAM acts at several levels and that it can (i) scavenge potentially cytotoxic oxygen free radicals, (ii) inhibit protein kinase C activity, and (iii) block the transcriptional activation of gamma interferon-inducible genes in human macrophage-like cell lines (Chan et al., 1991). *Mycobacterium tuberculosis* uses the ESX-1/Snm system [early secreted antigen 6 kilodaltons (ESAT-6) system 1/secretion in mycobacteria] to deliver virulence factors into host macrophages during infection. Despite its essential role in virulence, the mechanism of ESX-1 secretion is

unclear(Champion et al., 2006). The pathogenicity of mycobacteria such as *Mycobacterium tuberculosis* is closely associated with their capacity to survive within host macrophages. A crucial virulence factor for intracellular mycobacterial survival is protein kinase G (PknG), a eukaryotic-like serine/threonine protein kinase expressed by pathogenic mycobacteria that blocks the intracellular degradation of *mycobacteria* in lysosomes(Scherr et al., 2007).

### 1.1.3. Pathogenesis and Pathology

MTB infection occurs mainly at the lung through the respiratory route. Following its penetration of the mucosal barrier, the bacteria are associated with intraepithelial leukocytes and subsequently conveyed to the draining lymph nodes. Then it spread from the site of initial infection in the lung through the lymphatic or blood to other parts of the body(Munoz S et al., 2003). The mycobacterium containing phagosomes are hampered in maturation and fail to fuse with lysosomes, which enable the cells to kill the bacillus(Fratti RA et al., 2000). Tuberculosis patients relapse if treatment is not continued for 6 months, because chemotherapy fails to convert the patients' response from the necrotizing pattern characteristic of disease to the nonnecrotizing bactericidal function required for optimal immunity(Rook and Hernandez-Pando, 1996)

Tuberculosis can involve a delay between infection and clinical disease ranging from several weeks to several decades. Active disease may arise almost immediately after infection in about 5% of exposed individuals. Most of the others infected individuals develop latent infections in which the tubercle bacilli persist in vivo without causing any clinical symptoms(Kaufmann SH, 2001). The consequences of inhaling or ingesting tubercle bacilli depend on both the virulence of the organism and the resistance of the host. At one extreme, organisms with little virulence for the particular host disappear completely, leaving no anatomic trace behind. At the opposite extreme, the bacilli flourish with in macrophages and disseminate widely, and cause death within a few months(Kaufmann SH, 2002). Generally, four potential outcomes of *Mycobacterium tuberculosis* infection can occur according to the fate of the microorganism inside the macrophages: the bacterium can be immediately eliminated, becomes dormant indefinitely inside the host, causes primary tuberculosis or reactivates many years after the primary infection(Giacomini E et al., 2001).

#### 1.1.4. Clinical Feature

Pulmonary TB patients usually have weight loss and productive cough for more than three weeks. Symptoms such as chest pain, dyspnea, fever, night sweating, anorexia and haemoptysis are common among TB patients(Nataraj G Kurup S et al., 2002). Clinical features of HIV associated pulmonary tuberculosis in adults are frequently atypical, particularly in the late stage of HIV infection, with non cavitory disease, lower lobe infiltrates, hilar lymphadenopathy and pleural effusion. More typical post primary tuberculosis with upperlobe infiltrates and cavitations is seen in the earlier stages of HIV infection. Extrapulmonary tuberculosis is reported more frequently, despite the difficulties in diagnosing it (Raviglione et al., 1992).

#### 1.1.5. Epidemiology of TB-HIV

Tuberculosis and human immune deficiency virus infections are two major public health problems in many parts of the world. The prevalence of TB-HIV Co-infection is higher worldwide and 90% of these co-infected cases live in developing nations(Friedland G et al., 2007). In persons infected with TB only, the lifetime risk of developing TB ranges between 10% and 20%(Sutherland I, 1976). However in persons who have been co-infected with TB and HIV, the annual risk can exceed 10% (Girardi E et al., 2000). An estimated 1.37 million HIV positive TB patients were diagnosed globally in 2007, and around 80% of them live in sub-Saharan Africa(Lawn SD and Churchyard G, 2009). In some areas of this region, TB and HIV co infection rates have reached 60% to 70%(Corbett EL et al., 2003). Pulmonary tuberculosis is the most frequent type of tuberculosis in patients with HIV infection, occurring in 42-80% of cases in Africa(Kelly P and et al, 1990) and 77% of cases in Brazil (Bethlem N, 1989). In contrast to western countries, where *Pneumocystis jiroveci pneumonia* was the commonest AIDS-defining illness(Mocroft A et al., 1997), in developing countries TB is the most common life-threatening opportunistic infection (OI) in patients with HIV/AIDS with about 25 to 65% patients with HIV/AIDS having tuberculosis of any organ(Kumarasamy N et al., 1995). Of the 5.1 million HIV-infected people in India, about half of them are co-infected with *M. tuberculosis*; approximately 200,000 of these co-infected persons will develop active TB each year in association with HIV infection(Khatri GR and Frieden TR, 2002). By the end of 2000, about 11.5 million people were co-infected with HIV and *M. tuberculosis*, globally; 70 % of co-infected people were in sub-Saharan Africa, 20% in South-East

Asia, and 4% in Latin America and the Caribbean. TB accounts for about 13% of all HIV-related deaths worldwide (Corbett EL et al., 2003).

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 [16]. In Ethiopia, one study noted higher prevalence of PTB (7.5%) among HIV positive pre-A RT patients (Wondimeneh Y et al., 2012)

#### 1.1.6. HIV/TB Co infection Immunology

Alveolar macrophages are presumably the first group of cells infected with *M. tuberculosis* and are the primary immune cells within the airways. Following the entry of *M. tuberculosis* into the parenchyma, monocytes migrate to the lungs and differentiate into different macrophage types within the granuloma. All of these macrophage types may be susceptible to HIV infection, as well as *M. tuberculosis* infection. HIV envelope phenotyping has suggested that HIV infects activated (HLA-DR) alveolar macrophages (CD14 CD36), as well as lymphocytes (CD26), in airways (Hoshino Y et al., 2004) of coinfecting individuals. Since HIV has been shown to infect macrophages in vivo, HIV is likely to disrupt the function of *M. tuberculosis* -infected macrophages leading to granuloma dysfunction and increased bacterial growth and dissemination (Patel N. R. et al., 2009).

Exogenous HIV Nef protein added to *M. tuberculosis* -infected macrophages inhibits ASK1/p38 mitogen-activated protein kinase signaling, which leads to a decrease in TNF release and TNF-dependent apoptosis (K. Kumawat et al., 2010), suggesting that infectious virus is not necessary for inducing this functional change within a macrophage. This is important because HIV is a retrovirus with an error-prone reverse transcriptase that causes numerous site mutations that render most viral buds noninfectious. Since phagolysosome fusion is inhibited in *M. tuberculosis* -infected alveolar macrophages from HIV individuals (Mwandumba, 2004), apoptosis may be used as a last resort of infected macrophages. This allows other activated macrophages to engulf the nearby apoptotic bodies, which may lead to killing of the mycobacteria and enhanced induction of T cell responses. *M. tuberculosis*-induced apoptosis in macrophages is complex and may not always be beneficial to the host (Hoshino Y et al., 2007).



Some evidence suggests that an increase in apoptosis occurs in alveolar macrophages from AIDS patients with pulmonary TB compared to that in individuals with only pulmonary TB. An increase in apoptosis may be beneficial to the pathogens because it would allow them to exit macrophages capable of killing. This may also lead to increased dissemination of *M. tuberculosis* and HIV.(Diedrich and Flynn, 2011)

#### 1.1.7. Risk Factors

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

#### 1.1.8. Diagnosis of TB in HIV-infected individual

In 2007, a WHO International Expert Committee issued new guidelines to improve the diagnosis of TB in HIV infected individuals(WHO, 2007). The feasibility, accuracy and operational performance of these guidelines were tested in various settings and were found to be acceptable(Koole O et al., 2011). It was recommended that screening for TB should include asking questions about a combination of symptoms rather than only about chronic cough. The best performing rule was the presence of any one of current cough, fever, night sweats or weight loss. The overall sensitivity of this rule was 79%, increasing to 90% in clinical settings but the specificity was only 50%(Padmapriyadarsini C et al., 2011).

#### Radiographic

The spectrum of radiographic manifestation of pulmonary TB is dependent on the relative level of HIV-related immunodeficiency. During the early phase of HIV when individuals are not immunosuppressed, the radiographic pattern is similar to HIV uninfected individuals with more typical lesions upper lobe infiltrates with or without cavities. With advancing immunosuppression, extra pulmonary involvement, intrathoracic lymphadenopathy, lower lobe infiltrate and miliary TB become more common(Lawn SD et al., 2006).

## Culture

Culture on Löwenstein-Jensen (LJ) medium is still the gold standards for the diagnosis of active TB although many new molecular diagnostic methods have been developed. For resource limited countries, culture and Zeihl-Neelsen staining are used to confirm TB in patients with a clinical presumption of active disease. Six weeks or longer on solid media and 7-21days with liquid culture media will take the organism to grow. It is also important to test drug susceptibility(Rieder H. L et al., 2007).

## 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 bromcresol 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 Aerophilic (growth on the surface) and microaerophilic (growth below the surface)(Normung D. I. F, 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 S. et al., 2000). Even if the Biochemical tests have now been replaced with molecular techniques for the identification and classification of MTC it also important for confirmation.

## Tuberculin Skin Test (TST)

TST or purified protein derivative (PPD) test was suggested initially by Charles Mantoux (1874-1944) and he won the Noble Prize in medicine in 1909. In due course, TST has been widely used up to present time as a screening diagnostic method. The result of TST is considered positive when 48 to 72 hours after PPD intradermal injection, the skin induration diameter is  $\geq 10$  mm. Since in developing countries, TB is highly prevalent, bacillus Calmette-Guerin (BCG) vaccination of the infants at birth is still continued as a preventive public health measure, the false positive results may occur in those individuals who were previously vaccinated by BCG. In addition, the false negative results may also happen in children, elderly, AIDS patients and those who recently infected with TB bacilli(Jacobs S et al., 2011).

## Polymerase chain reaction (PCR)

PCR is a rapid and sensitive test which detects DNA of Mycobacterium TB in sputum sample as well as in other body fluids such as blood, pleural fluid, CSF and urine. According to a recent study in 2011, the sensitivity and specificity of commercial real-time PCR for evaluating respiratory and non-respiratory samples were studied and the authors found that the sensitivity and specificity of real-time PCR were 100% for respiratory samples and for non-respiratory samples were 85.7% and 97.3% respectively (Malbruny B et al., 2011). A study from Burkina Faso confirmed the utility of urine specimens for diagnosing PTB by nested PCR with a sensitivity of 64.3% in culture-negative PTB cases and 46.3% in extrapulmonary tuberculosis (EPTB) cases (Torrea G et al., 2005).

## GeneXpert-RIF

The Xpert® MTB/RIF simultaneously detects *M. tuberculosis* and rifampin (RIF) resistance by PCR amplification of five overlap -ping probes that are complementary to the entire 81 base pair RIF resistance-determining region of the *M. tuberculosis* rpoB gene, and subsequently probes this region for mutations that are associated with RIF resistance. The PCR amplification process is hemi nested, and the amplified target is detected in real time by six color fluorescent molecular beacons (Blakemore R et al., 2010).

WHO endorsed the use of GeneXpert-RIF for the rapid diagnosis of TB as well as rifampicin resistance among HIV-infected individuals with clinical suspicion of TB (World Health Organization and STOP TB department, 2011). GeneXpert is a TB-specific automated, cartridge-based nucleic acid amplification assay, having fully integrated and automated sample preparation, amplification and detection using real-time PCR, providing results within 100 minutes (Rachow A et al., 2011). The assay has sensitivities of 98%–100% for smear-positive pulmonary TB, 57%–78% for smear-negative pulmonary TB, and 53%–81% for extrapulmonary TB when testing a variety of clinical samples (Blakemore R et al., 2010, Hillemann D et al., 2011). Sensitivity of a single Xpert MTB/RIF test in smear-negative/culture-positive patients was 72.5% which increased to 90.2% when three samples were tested. Xpert MTB/RIF specificity was 99%. HIV co-infection substantially decreased the sensitivity of microscopy (to 47%), but did not significantly affect Xpert MTB/RIF performance (Van Rie A et al., 2010). Xpert MTB/RIF detected rifampicin resistance with 99.1% sensitivity and excluded resistance with 100%

specificity. Mean time to detection was <1day for Xpert MTB/RIF, 1day for microscopy, 17 days for liquid culture and >30 days for solid culture(Van Rie A et al., 2010, Rachow A et al., 2011)

### Interferon- $\gamma$ release assay (IGRA)

This test can be used to diagnose latent TB infection and is particularly useful in profoundly ill patients and those with severe malnutrition. There are two in vitro tests to detect latent tuberculosis: QuantiFERON-TB Gold (Cellestis,USA) and the T SPOT-TB test (Oxford Immunotec, USA). Both use an enzyme-linked immunospot assay to quantify the number of peripheral blood mononuclear cells producing IFN- $\gamma$  in response to tuberculosis-specific antigen stimulation (ESAT-6 and CFP10). Both assays give objective results, with sensitivity (as measured in patients with active tuberculosis) comparable to that of the tuberculin skin test, but are significantly more expensive. IFN- $\gamma$  assays do not differentiate between latent and active tuberculosis or between immune reconstitution inflammatory syndrome (IRIS) and failure.(Pai M et al., 2004)

#### 1.1.9. Drug Resistance

One research from South Africa showed, out of 221patients detected MDR tuberculosis 53 had XDR tuberculosis. Prevalence among 475 patients with culture confirmed tuberculosis was 39% for MDR and 6% for XDR tuberculosis. Only 55% (of 47) of patients with XDR tuberculosis had never been previously treated for tuberculosis; 67% (of 42) had a recent hospital admission. All 44 patients with XDR tuberculosis who were tested for HIV were co-infected. Rates of multidrug-resistant (MDR) tuberculosis among new cases of tuberculosis in sub-Saharan Africa have been low in the past, ranging from 0.8% to 2.6% in the last global drug resistance survey (1999–2002), compared with 7.8–14.2% in countries with the highest rates. However, the prevalence of drug resistance in the region seems to have risen since the last global drug resistance survey(Nunes EA et al., 2005). In KwaZulu Natal, South Africa, the rate of MDR tuberculosis in new patients was reported at 1.7% between 2000 and 2002;(Weyer K et al., 2003) the rate was 9% in a study integrating treatment for tuberculosis and HIV that we undertook from 2003 to 2006 in the same region(Gandhi N et al., 2006).

#### 1.1.10. Treatment and Prevention

The basic principles of treatment for HIV-associated TB are the same as for HIV uninfected individuals. Certain areas of uncertainty remain, including the regimen duration, dosage and

frequency of administration of anti-TB drugs, optimal timing of initiation of ART and optimal antiTB drug combination for patients on second line treatment. It is important to know if the patient is taking antiretroviral treatment at the time TB treatment is being initiated, since that will affect treatment options. If the patient is on antiretroviral treatment, Most Temporally, paradoxical reaction occurs within a few weeks of patients get started on isoniazid, pyrazinamide, and ethambutol, along starting antiretroviral treatment and coincides most closely with viral with either rifampin or rifabutin, depending on the HAART regimen. If you have rifamycin resistance, which is uncommon, you can use whichever antiretroviral regimen you prefer.” The last option is to give a non-rifampin based regimen which includes the injectable agent streptomycin for nine months. While there are no drug interactions, few clinicians and patients opt for this treatment due to the need for injections for nine months(WHO, 1997).

## 1.2. Statement of the problem

Approximately 5% of HIV-infected patients with pulmonary tuberculosis have positive results on acid fast staining of sputum, despite normal chest radiographs(Perlman DC et al., 1997). In 2010, there were 350,000 tuberculosis related deaths in HIV- infected people, most of them in developing countries. One of the most important reasons for this high number of deaths is the difficulty of diagnosing tuberculosis in the HIV population(Getahun H et al., 2011). HIV-mediated immunosuppressant impairs granuloma formation, resulting in both ineffective containment of Mycobacterium tuberculosis bacilli and diminished formation of pulmonary cavities. These effects manifest clinically as frequent extrapulmonary disease, atypical chest radiographic findings(Pitchenik AE and Rubinson HA, 1985), greater involvement of the lower lobes of the lung, and lower concentrations of bacteria in sputum(Colebunders R and Bastian I, 2000)

Since Koch’s discovery of tuberculous bacilli in 1882, microscopic detection of the bacilli in clinical specimens has remained the cornerstone of TB diagnosis in low and middle-income countries. Since 1996, the World Health Organization has promoted the DOTS strategy for tuberculosis control, one aspect of which is case detection through sputum acid fast bacillus (AFB) smear microscopy(WHO, 1997). However, the sensitivity for diagnosing pulmonary tuberculosis using direct, unconcentrated sputum smear methods ranges from 40 to 60% for a combination of three examinations(Krasnow I and Wayne LG, 1969) and is lower for those with HIV co infection(Lockman S et al., 2003).

Diagnosis of TB in HIV-infected patients is often difficult due to several reasons: Frequently negative sputum smears, atypical radiographic findings, higher prevalence of Extrapulmonary TB (EPTB) especially at inaccessible sites and resemblance to other opportunistic pulmonary infections. However, the diagnostic approach to suspect TB in a HIV infected individual is similar to that in immunocompetent patients except that invasive diagnostic procedures are more often required to establish the diagnosis(Sharma SK and Mohan A, 2004).

With advancing HIV related immunosuppression, the frequency of extrapulmonary and disseminated forms of TB disease increase(Reid MJ and Shah NS, 2009), sputum smear microscopy performance is reduced, and up to a third of patients are unable to produce sputum for diagnostic testing (Cox JA et al., 2010). Screening for TB in HIV patient population is difficult, however, the World Health Organization's (WHO) intensify case finding symptom screen has low specificity and misses approximately 10%–20% of cases (Getahun H et al., 2011).

The failure to control TB in HIV-endemic areas has underscored two major limitations to microscopic diagnosis: the low clinical sensitivity of the technique in HIV-infected individuals and the logistic difficulty of ensuring good access to quality microscopy in resource-limited settings(Apers L et al., 2003). The fraction of HIV-coinfected individuals with pulmonary TB that can be detected by microscopy varies widely with the degree of immunosuppression, the length of TB illness, and the local diagnostics setting, including the organization and strength of the TB control program and its laboratory infrastructure. In overworked, underfunded laboratories, especially in areas where HIV infection is prevalent, the proportion of cases detected by microscopy is often as low as 20%–35%(Lawson L et al., 2005).

In addition to decrease the sensitivity of diagnosis technique, acquiring a diagnostic sample remains a major hurdle in HIV-infected sputum scarce patients suspected of having active TB. Sputum induction, using ultrasonic nebulisation, may facilitate obtaining sputum, but this is often unavailable in hospitals in resource-poor settings and infection control is a concern. Tissue biopsies and aspirated samples may be obtained from extrapulmonary disease foci (e.g. bone marrow and liver, pleural and pericardial fluid) but specialized skill and equipment requirements limit the availability and affordability in resource-poor settings. Urine is easily obtainable from

sputum scarce patients but there are few data about the performance of newer diagnostic tests using urine (Flores LL et al., 2011).

Therefore, there is a clear need for new, accurate, and rapid TB diagnostics that have utility in patients who cannot produce sputum. The purpose of this study is to investigate urine samples to diagnosis tuberculosis in people living with HIV.

### *1.3. Significance of the study*

Routine TB screening offers the opportunity to diagnose and promptly treat TB disease, and to identify those without TB disease who may be eligible for TB preventive therapy. The use of TB preventive therapy can reduce TB incidence and is therefore of considerable benefit to patients(Stephen D. Lawn et al., 2006). For this reasons, the World Health Organization (WHO) recommends regular screening for active TB disease of all people living with HIV with new range of diagnostics that is now emerging; employing various different technologies and providing either treatment for active disease or isoniazid preventive therapy (IPT) to mitigate TB morbidity, mortality, and transmission(WHO, 2012). To achieve this objective, one area of renewed interest has focused on the potential for TB diagnosis to be made from analysis of urine samples. Urine is an attractive sample for diagnosis of TB in HIV seropositivity patients because:

- It is simple to obtain, even from very ill patients who may not be able to produce sputum.
- Urine sampling does not generate hazardous infectious aerosols.
- Urine is relatively clean and easy to handle in the laboratory.
- Urine may be cultured and tested by polymerase chain reaction
- Urine can be tested for specific mycobacterial antigens such as LAM(Tuuminen, 2012)

## CHAPTER TWO

### OBJECTIVES

#### 2.1. General Objectives

- To evaluate urine as an adjunct specimen for the diagnosis of pulmonary tuberculosis by using different diagnostic methods in people living with HIV.

#### 2.2. Specific objectives

- To compare isolation rate of *Mycobacterium tuberculosis* from urine and sputum collected from pulmonary tuberculosis suspected patients living with HIV.
- To evaluate the detection rate of different diagnostic methods in the diagnosis of active pulmonary tuberculosis in people living with HIV using urine sample.
- To characterize and identify species of *Mycobacterium* from isolates of cultures positives
- To determine effect of CD4<sup>+</sup> T cell count on the presence of *Mycobacterium tuberculosis* in urine
- To identify the risk factors of pulmonary tuberculosis in people living with HIV.



## CHAPTER THREE

### METHOD AND MATERIALS

#### 3.1. Study area

Addis Ababa is the capital city of Ethiopia covering an area of 540 sq. km. 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, 27 public health centres, and 130 public health stations, 700 different levels private clinics are found in Addis Ababa city Administrative region. The study was conducted in Zewuditu memorial Hospital, Polis referral Hospital and Teklehaymanot Health center.

#### 3.2. Study design, time and place

This was a Health institution based cross-sectional study conducted in Addis Ababa Ethiopia, between August 2013 and January 2015.

#### 3.3. Population

##### 3.3.1. Source population

All TB patients visited at outpatient department in Zewuditu memorial Hospital, Polis referral Hospital and Teklehaymanot Health center during the period of study.

##### 3.3.2. Target population

During the study period, all suspected pulmonary tuberculosis patients who are HIV positive and visited outpatient department in Zewuditu memorial Hospital, Polis referral Hospital and Teklehaymanot Health center.

#### 3.4. Sample size determination

Sample size was determined using the previous prevalence from the research done on the same topic with 95% confidence interval and 5% degree of precision. One study from Adama show that the higher prevalence of PTB among HIV positive pre-ART patients is 7.5% (Wondimeneh Y et al., 2012). Having this information, sample size was calculated:-

P= 7.5%

d= 5%

CI = 95%

$$n = \frac{z^2 \cdot p \cdot (1-p)}{d^2} = \frac{(1.96)^2 \cdot 0.075(1-0.075)}{(0.05)^2} = 107$$

Where; n= Sample size; Z= confidence interval;  $\alpha$ = level of significance; d= tolerable error; P= prevalence.

The minimum sample size with 10% contingency with unknown circumstance was 117

Demographic, clinical and other relevant data were obtained by attending physician and was transferred to the questionnaire prepared for this study by the principal investigator.

### 3.5. Exclusion and inclusion criteria

#### 3.5.1. Inclusion criteria

- Patients with age above 18 years
- All HIV positive patients with suspected pulmonary TB who consented to join in the study.

#### 3.5.2. Exclusion criteria

- Patients who are critically ill
- Patients who are on ionized prevention therapy
- Patients who are on anti-tuberculosis treatment and ART.
- Patients those who suspected having urogenital TB.

### 3.6. Sampling Technique

Convenience sampling technique was employed to select samples from source population. On average about 117 pulmonary TB/HIV patients admitted at outpatient department were identified by the physician. Based on this, six patients from Teklehaymanot, 70 patients from Polis Referral hospital and 41 patients from Zewuditu Memorial Hospital were sampled for the study during the time of data collections.

### 3.7. Specimen collection and transportation

After interviewed using semi-structured questionnaire and receiving signed informed consent from each study participant, for each patient, 50 milliliters of urine and 10ml sputum of each two morning-spot samples were collected from suspected cases of pulmonary tuberculosis in people living with HIV from January 2014 to July 2014. Urine and sputum samples were refrigerated after collection and transported to the Akililu Lema Institute of PhatobiologyTB laboratory in

cooler boxes at 2-8°C on the day of collection. Then processed within four hours of collection and pooled at the second days. The processed samples were stored at -20°C until testing is performed.

### 3.8. Samples Processing

Urine was first concentrated by using a 50 ml Falcon tube and centrifuged at 1520g for 15 min. The resulting sediment was decontaminated by the standard N-acetyl-L-cysteine and sodium hydroxide method, with a concentration of 4% NaOH. After 30 min, the suspension was centrifuged again and the sediment suspended in 1.5 ml sterile phosphate buffer (pH 6.8).

Sputum specimen was digested and decontaminated by the NALC-NaOH method and centrifuged at 3000 rpm for 15 min. Two drops of phenol red indicator was added to the sediment after the supernatant discarded and 2N HCl was added to neutralize. 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 culture medium and the left were stored for further molecular analysis.

### 3.9. Laboratory Methods

#### 3.9.1. Microscopy

A loopful of sputum and urine were spread on a clean glass slide uniformly in oval shape by smearing repeatedly in coil-like patterns, approximately 2-3cm in size. After smearing, the slide was air dried completely. Then, flame-fixed by passing through the flame carefully so that it would not washed off during staining. The smear was stained for presence of acid fast bacilli using Ziehl-Nelsen staining and examined under light microscope.

#### 3.9.2. CD4 T cell count

After blood samples collected into the EDTA test tubes, CD4+ T-cell count was done using a FACS Count and fluorescent labeled monoclonal antibodies (Becton Dickinson Immunocytometry System, San Jose, California, USA) and expressed as cells/mm<sup>3</sup>. The CD4+ count was done by laboratory technician in Addis Ababa selected Hospital and Health Center (Polic Referral Hospital, Zewuditu Memorial Hospitals and Teklehaymanot Health Center) within the same day of sample collection. Then I collected the result of CD4 count of the patients from their document.

### 3.9.3. Culture

After urine and sputum specimens processed, around 0.1ml suspension were directly inoculated into Lowenstein-Jensen [LJ] and incubate on a slant in the dark at 37°C for 8 weeks. Slants were examined weekly, and contaminated slants were discarded and re-inoculate with re-decontaminate specimen. Positive growth was confirmed with Ziehl-Nelseen (ZN) smear. Every new batch of LJ media was controlled using American Type Culture Collection (ATCC) H37Ra.

### 3.9.4. Molecular techniques, Genomic DNA Extraction

#### Directly from clinical specimen

The urine specimens taken from study participants were centrifuged at 3000g for 20 minutes, the supernatant was removed, and the pellet was re suspended in 2ml of NaOH 4%. After incubation for 15 minutes 40ml of phosphate buffered saline (PBS) was added and centrifuged at 3000g for 20 minutes. The pellets were retained, resuspended in 1ml of PBS, and aliquots of 100µl were stored at -80°C for analysis by PCR.

#### From culture

Bacterial isolates grown on Lowenstein - Jensen media was resuspended in 400µl of 1x TE buffer with pH 8.0. The mixture was incubated in a water bath at 80°C for one hour to kill the bacteria and cooled to room temperature. 50µl of lysozyme (10mg/ml) (Sigma, Saint Louis, USA) was added to lyse the bacteria and the mixture was incubated at 37°C in water bath for 1hr. In the lysozyme treated samples, 75µl of 10% SDS/proteinase K mix (Sigma, Saint Louis, USA) was added and incubation was continued for 10 minutes at 65°C. To remove inhibitors, 100µl of 5MNaCl and 100µl of cetyltrimethyl ammonium bromide (CTAB)/NaCl solution was added to the sample, vortexed and the mixture incubated for 10 minutes at 65°C.

DNA extraction was performed with 750µl of chloroform-isoamyl alcohol in the 24:1(volume/volume) ratio respectively. 450µl of isopropanol was added to the aqueous phase to precipitate and obtain the DNA pellet. After the addition of isopropanol, the pellet was placed in -20°C freezer for 30 minutes. The DNA was recovered by centrifugation at 12,000 rpm for 15 minutes. The harvested DNA pellet was washed with 1ml cold 70% ethanol to remove CTAB and NaCl. The ethanol washed pellet was treated with 10 mg /ml DNase free RNaseA (Sigma, Saint Louis, USA) and incubated for 1hrs at 37°C. Finally, the pellet was dried at room temperature,

re-dissolved in 1x TE buffer (pH 8.0) and stored at 4°C for immediate processing or at -20°C freezer until required for further analysis.

## PCR

### Region of Difference Based Deletion Type

Heat killed AFB positive samples were investigated by multiplex PCR based deletion typing for the presence or absence of RD4 and RD9 using the following primers:

RD9\_FlankF 5'- AACACGGTCACGTTGTCGTG-3'

RD9\_FlankR 5'- CAAACCAGCAGCTGTCGTTG-3'

RD9\_IntenalF 5'- TTGCTTCCCCGGTTCGTCTG-3'

The result was interpreted as *M.tuberculosis* (RD9 present) when a band of 396bp was observed comparing to commercially available ladder, divided by 100bp. The cycling parameters include initial denaturation at 95°C for 5 minutes and 40 cycles of denaturation at 94°C for 30 seconds, annealing at 65°C for 1 minute and extension at 72°C for 10 minutes(Parsons LM et al., 2002).

RD4intF 5'-ACACGCTGGCGAAGTATAGC-3'

RD4flankR 5'-AAGGCGAACAGATTCAGCAT-3'

RD4flankF 5'-CTCGTCGAAGGCCACTAAAG-3'

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

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

### Genus typing-Multiplex PCR

Multiplex PCR was run after RD9 deletion typing for those who not diagnosed and identified by deletion typing. Five primers (Table 2.5.3), with concentration of 10µM were used. DNA amplifications was done in thermocycler with 20 µl reaction volumes consisting: 2 µl of genomic DNA as a template, 10 µl HotstarTaqMasterMix (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. All members of the Mycobacteria genus had 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. For quality control we used *M. avium*, *M.bovis*, *M.intracellulacae* and *M. tuberculosis* (H37Rv) as positive controls for always give two bands.

Table 3.9.4: Primers, primer sequence and interpretation of mycobacterium genus and mycobacterium complex (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'		

### Agarose gel electrophoresis

Agarose gel electrophoresis was performed to verify the amplification of the desired gene of interest. 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 at 100volts and 50 ampere for 30 to 45 minutes. The gel was visualized in Multi-image UV light cabinet (EPi Chemi II DarkRoom).

### Spoligotyping

Spoligotyping was carried out using the commercially available kit from Ocimum Biosolutions, India, according to the manufacturer's instructions. Accordingly, the direct-repeat (DR) region was amplified with primers DRa 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. tube rculosis* 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.

### 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>).

### 3.9.5. Overall laboratory procedures

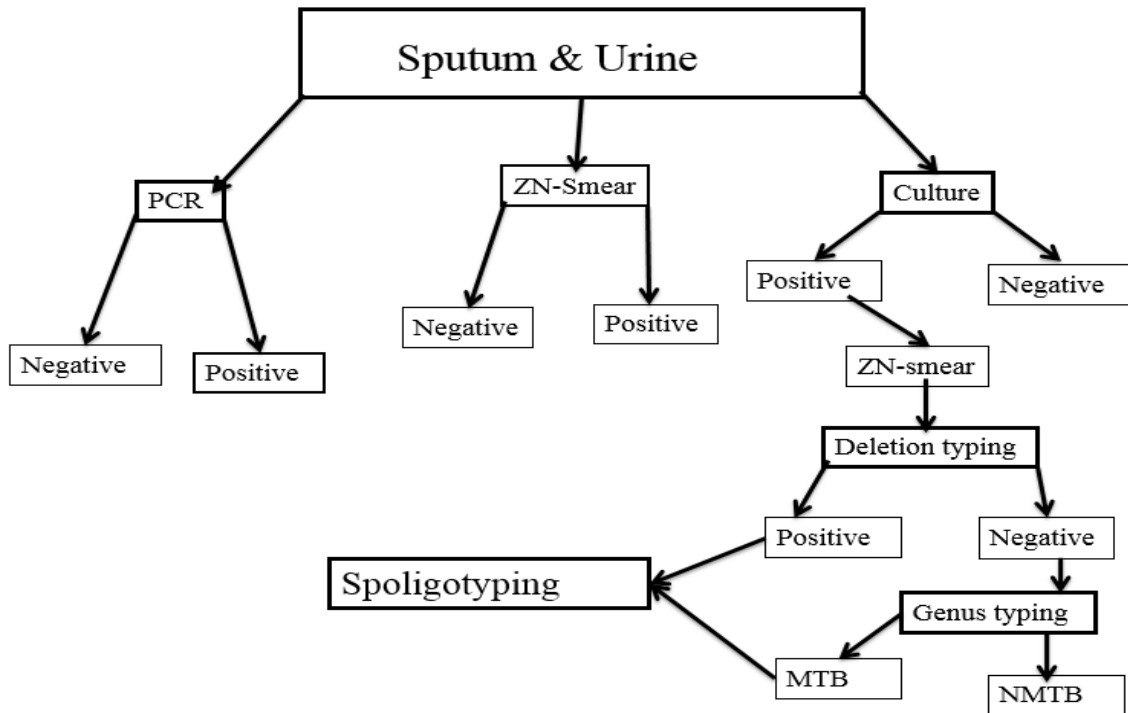


Fig.3.9.5. Flow chart showing overall procedure of the lab

### 3.10. Quality Assessment

All laboratories performing TB diagnosis should ideally do a quality control in all aspects. Care was taken in preparing media by put at 37°C for 48 hours to check for contamination. Positive and negative controls were run together in all steps of DNA extraction, AFB-smear and PCR amplification procedures. The area of PCR mix was cleaned with “DNA away” before and after each mix. Also we used a separate mix room and amplified the mix in a different room to prevent contamination. Urine and sputum samples were processed separately to avoid cross contamination.

### 3.11. Statistical Analysis

Data were entered into Epidata and transported to SPSS-version16 for analysis. Descriptive analysis was performed and presented in the form of tables, figures and charts and the prevalence figures was calculated for the total study population. A p-value less than 0.05 are considered statistically significant. Epi Info-version 7 was used to calculate sensitivity and specificity of the different diagnostic technique used in this study.

### 3.12. Ethical consideration

The study was approved by Departmental Ethical Review and Research Committee (DERC) of department of Medical Microbiology, Immunology and Parasitology (DMIP), College of Health Science, Addis Ababa University and Akililu lemma institute of pathobiology. Then formal letter were written to selected Hospital and Health Center in Addis Ababa. After, clearly stating the purpose, merit and demerit of the study in local languages, written informed consent were obtained from all participants. The result were given to the physician All participants who were positive for pulmonary TB were treated with first line anti TB drugs through their physician. The right of the participants to participate on this study is voluntarily of her or him and any information obtained were kept confidential.

### 3.13. Dissemination of Results

The result of the study was disseminated to concerned body through abstracts, reports, presentation and publication.



## CHAPTER FOUR

### RESULTS

#### 4.1. Socio-demographic characteristic of the study participants

A total of 117 eligible study participants were included from January to July, 2014. One hundred seventeen participants gave their urine, blood and sputum properly for routine TB/HIVco-infection investigation. The participants' age ranges from 19-61 years with a mean of  $34.5 \pm 8.89$  and with male to female ratio of 0.63:1. Most of the participants [66(56.4%)] were in the age category of 28-37 years. The majority of the patients (67.5%) were married and 17.9% came from rural areas. Above 47.3% of patients were government employers, and 60.2% attended high school and above (Table 4.1).

Table 4.1: Socio-demographic characteristic of the study participants in Addis Ababa, Ethiopia (N=117)

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

## 4.2. Clinical Data

A total of 117 participants were interviewed for their related clinical background using questionnaire and their medical charts were also reviewed for further clinical data. As shown in Table 4.2, the most common symptoms observed among the suspected pulmonary TB patients was cough 117(100%) followed by night sweat 111 (94.9%). Of the participants present with cough 45/117(38.5%) had pulmonary TB whereas, 39.6% (44/111) of the participants who had night sweat were also pulmonary TB cases/patients. Statistical analysis showed that clinical symptom such as cough and chills had no significant association with pulmonary TB infection ( $p=.651$ ). However, nausea, difficult breathing, chest pain, and weight loss demonstrated significant association with pulmonary TB infection ( $p=.001$ ). Of the 45 pulmonary tuberculosis cases identified 12/45(26.7 %) were in the WHO HIV stage IV ( $p=.001$ ). Around 40% PTB cases identified had less than 18.5 BMI ( $p=.001$ ) (Table 4.2).

Table 4.2: Clinical variables in the group confirmed as positive for PTB with AFB smear examination, culture and PCR compared with non-PTB cases in Addis Ababa, Ethiopia

Clinical profile	Frequency	PTB positives (N=45)	PTB negatives (N=72)	P- value ( $X^2$ -test)
<b>Symptoms</b>				
Cough	117	45(100%)	72(100%)	-
Fever	105	45(100%)	72(100%)	.002
Nausea	46	39(86.7%)	7(9.7%)	.001
Night sweat	111	44(97.8%)	67(93%)	.001
Difficult breathing	91	39(86.7%)	52(72.2%)	.001
Chest pain	97	41(91.1%)	56(77.8%)	.004
Weight loss	89	34(75.6%)	55(76.4%)	.001
Fatigue	54	29(64.4%)	25(34.7%)	.001
chills	61	16(35.6%)	45(62.5%)	.651
<b>WHO stage of HIV</b>				
I	39	8(17.8%)	31(43%)	.315
II	26	14(31.1%)	12(16.7%)	.001
III	26	11(24.4%)	15(20.8%)	.003
IV	26	12(26.7%)	14(19.4%)	.001
<b>Body mass index</b>				
<18.5	38	18(40%)	20(27.8%)	.001
18.5-24.99	85	27(60%)	58(80.5%)	.067
>25	0			

### 4.3. Laboratory Results

#### **A) Findings of Urine Specimens Subjected to Smear, Culture and PCR**

Urine samples from HIV positive PTB 117-suspected patients were subjected to smear, culture, and PCR examinations. Of these, 17(14.5%) were culture-positive on L-J, 7/117(6%) were smear positive and 29/117(24.8%) were direct-PCR positive. Thus, the culture isolation rate was significantly higher (54.5%) if the pulmonary samples from the same patient were also smear- and culture-positive (Table 4.4). As expected the detection rate of PCR higher than the culture and smear which is significant ( $\chi^2=61.7$   $p=.001$ ). Sensitivity and specificity of PCR in urine was 87.9% and 100% respectively (Table 4.3).

#### **B) Findings of Sputum Specimens Subjected to Smear, Culture and PCR**

Out of the 117 sputum samples evaluated 11 were positive (9.4%) by ZN staining, the gold standard i.e. TB culture had 33(28.2%) positive and the direct-PCR gave 40(34.2%) positive. The results revealed that PCR method was superior to ZN staining method and culture TB. All culture and smear positive were also PCR positive (Table 4.4). However, PCR detect additional MTB in 7/84(8.3%) out of sputum culture negative. The probability of pulmonary tuberculosis suspected patient diagnosis by PCR is highly significant than the culture ( $\chi^2=93.6$   $p=001$ ), but it is very expensive for the diagnosis in Ethiopia economic contexts. Sensitivity and specificity of PCR in sputum was 100% and 91.7% respectively (Table 4.3)

Table 4.3: Mycobacterium detection rates of various in vitro diagnostic methods applied to urinary and pulmonary specimens from pulmonary tuberculosis suspected HIV infected patients in Addis Ababa, Ethiopia

Specimen type	sputum (n=117)	Urine (n=117)	Total
<b>L-J culture</b>			
Percentage of culture-positive	28.2(33/117)	14.5(17/117)	31.6(37/117)
Time-to-positivity (weeks)	63.6(4-6)	5(4-6)	
<b>Smear Microscopy</b>			
Percentage of smear-positive	9.4(11/117)	6(7/117)	13.7(16/117)
Sensitivity	33.3(11/33)	21.2(7/33)	
Specificity	100(84/84)	100(84/84)	
<b>PCR</b>			
Percentage of PCR positive	34.2(40/117)	24.8(29/117)	37.6(44/117)
Sensitivity	100(33/33)	87.9(29/33)	
Specificity	91.7(77/84)	100(84/84)	

DNA extracted pellet from urine was amplified with RD9 primer and produced band at 396 comparable with standard 100bp ladder and known positive control, Fig. 4.1



Figure 4.3: Selected slide showing gel picture of DNA amplified with RD9 primer. Lane 1= ladder (100bp), lane 2= positive control (H37Rv) lane 3= Qiagen H<sub>2</sub>O (-ve control), lane 4= *M. bovis* and lane 5 to lane 17 =urine sample(DNA extracted pellet from urine )

\*MTB - Mycobacterium tuberculosis

#### 4.3.1. Culture time to positivity

Of 17 culture positive from urine, culture positivity was proven in 1/17(5%) and 16/17(95%) specimens within 4-6 and 7-16 weeks of incubation period respectively while 21/33(63.6%) and 12/33(36.4%) in sputum specimens. Culture time to positivity was shorter in sputum specimen compare to urine specimen [1(4-7) vs 21(4-7),  $p < 0.0001$ ].

Chart 1. Comparison of culture time to positivity of sputum and urine from pulmonary tuberculosis patients



#### 4.4. Isolation rate of MTB from different clinical specimens of HIV patients

The total isolation rate of *M. tuberculosis* among HIV infected pulmonary TB suspected patients (n=117) determined by the combination of different diagnostic methods from both urine and sputum specimens were 45/117(38.5%). Of this, *M. tuberculosis* was detected in 30/117(25.6%) and 40/117(34.2%) of urine and sputum samples respectively by either of the diagnostic test used in our study. Of 40/117(34.2%) *M.tuberculosis* detected from sputum specimens, 33(82.5%) showed growth after 8 weeks of incubation and the rest were culture negative. However, ZN smear technique detected mycobacteria in only 11(27.5%). Direct-PCR detected MTB in 40/40 (100%) implying all culture and smear positive sputum specimen were PCR positive. PCR detect additional MTB in 7/84(8.3%) out of sputum culture negative. On the other hand, out of 30/117(25.6%) *M. tuberculosis* detected from urine specimens 17/30 (56.7%) showed growth after 8 weeks of incubation and the rest were culture negative. Seven (23.3%) urine specimens were positive by ZN smear whereas 29 (96.7%) were positive by direct-PCR . Out of six patients who can't produce sputum, 3(50%) was urine culture and direct-PCR positive (Table 4.4).

Table 4.4. Isolation rate of MTB among suspected PTB in HIV positive patients in Addis Ababa, Ethiopia.

Samples	Smear		Culture		PCR		Total	
	Positive	negative	Positive	Negative	Positive	Negative	Positive	Negative
Urine	7	110	17	100	29	88	30	87
Sputum	11	106	33	84	40	77	40	77
Both sputum and urine	16	101	37	80	44	73	45	72

#### 4.5. Comparison of urine and sputum in diagnosis of PTB

The comparison of both urine and sputum cultures showed that the sputum culture detected more mycobacterial isolates [33 (28.2%)] than urine culture [17 (14.5%)]. Of the 84 sputum, culture-negative cases four (4.8%) were urine culture-positive. However, out of 37 total cultured MTB detected from the two specimens, 13/37(35.1%) were from the same patients which was moderate agreement ( $\kappa=0.406$ ). When urine culture conducted in parallel of sputum culture increased the detection rate of MTB by 3.4 % [from 28.2% to 31.6%]. In the same manner of 16 MTB detected by smear from the two specimens, 3/16(18.7%) were from the same patient ( $\kappa=0.281$ ), but urine AFB detected 4/106(3.8%) from sputum AFB negative. This figure showed when the urine AFB conducted side by side with sputum, the detection rate of MTB in pulmonary tuberculosis patients increased by 4.3% [from 9.4% to 13.7%]. Also of 44 MTB detected by PCR from both specimens, 24/44(54.5%) were detected from the same patients and 4/77(5.2%) were detected from sputum PCR negative. Moreover the agreement was moderate ( $\kappa=0.591$ ) (Table 4.5)

Table 4.5. Comparison of urine and sputum specimen for the diagnosis of active PTB in people living with HIV in Addis Ababa, Ethiopia

Sample		Urine AFB			Kappa value
		Negative	Positive	Total	
Sputum AFB	Negative	102	4	106	0.281
	Positive	8	3	11	
	Total	110	7	117	
		<b>Urine Culture(n=117)</b>			0.406
Sputum culture (n=117)		Negative	Positive	<b>Total</b>	
	Negative	80	4	84	
	Positive	20	13	33	
	Total	100	17	117	
		<b>Urine PCR (n=117)</b>			0.591
Sputum PCR (n=117)		Negative	Positive	<b>Total</b>	
	Negative	73	4	77	
	Positive	16	24	40	
	Total	89	28	117	

#### 4.6. Evaluation of different methods in detecting MTB

##### 4.6.1. Comparison of AFB smear and PCR with Culture using urine specimen

A total of 117 patients were included from clinically suspected cases of pulmonary tuberculosis. Out of the 117 urine samples evaluated 7(6%) were positive by ZN staining, while the positivity increased to 17(28.2%) by culture and the PCR gave 29(24.9%) positive. The results revealed that direct-PCR method was superior to ZN staining and culture method. All ZN-AFB smear positive were confirmed by PCR that make good agreement (kappa=0.342). And the combination of PCR with culture for the diagnosis of suspected PTB in HIV infected patient had moderate agreement (kappa=0.466). In urine, PCR detected 16/17(%) of urine culture proven PTB cases in HIV infected patients. The diagnostic sensitivity, specificity, PPV and NPV of PCR was 95%, 100%, 61.3% and 98.9% respectively (Table 4.6.1).

Table 4.6.1 Comparison of diagnostic yield of different laboratory methods used for diagnosis of PTB from **urine** specimen in Addis Ababa, Ethiopia.

Lab. meth		Culture (N=117)		Sensitivity	Specificity	PPV	NPV	Kappa
AFB	Negative	100	10	41.2%	100%	87.5%	88.7%	0.528
	Positive	0	7					
PCR	Negative	87	1	95%	87%	61.3%	98.9%	0.466
	Positive	13	16					

#### 4.6.2. Comparison of AFB smear and PCR with Culture using sputum specimen

A total of 117 sputum specimen were collected and analysed by ZN staining, culture and PCR to detect the bacilli. Of this, 11(9.4%) were detected by ZN smears with detection rate of AFB 9.4% while PCR and culture were detects 33(28.2%) and 40(34.2%) respectively. The agreement between the PCR and culture techniques to detect PTB was almost perfect (kappa=0.861). However, when ZN smear combined with culture the diagnostic ability was 33(28.2%) with fair agreement (kappa=0.418). In sputum, PCR detect all 33(100%) of culture proven PTB cases. The diagnostic sensitivity, specificity, PPV and NPV of PCR in sputum was 100%, 91.7%, 82.5% and 100% respectively (Table 4.6.2).

Table 4.6.2. Comparison of diagnostic yield of different laboratory methods used for diagnosis of PTB in HIV infected from **sputum** specimen in Addis Ababa, Ethiopia,

Lab. method		Culture (N=117)		Sensitivity	Specificity	PPV	NPV	Kappa
AFB	Negative	84	22	33.3%	100%	100%	79.2%	0.418
	Positive	0	11					
PCR	Negative	77	0	100%	91.7%	82.5%	100%	0.861
	Positive	7	33					



## 4.7. Identification and Characterization of MTB

### 4.7.1. Region of Difference Based Species Identification and Genus Typing

After confirming the acid fastness of the isolates from the culture by Ziehl-Neelsen, further investigations were conducted to confirm the isolates whether they are MTC or not and differentiate of species among MTC by presence or absence of RD9. Accordingly all sputum culture proven isolates were confirmed as *M. tuberculosis* by RD9[31/33(87.9%)] except 2 isolates were deleted[L16 & L20](fig 4.7.1).



Figure 4.7.1: Gel picture showing Isolates of Mycobacterium characterized for species identification with RD9 Primer from sputum culture. Description; Lane1= 1kb ladder, Lane2= H37Rv (*M.tuberculosis* +ve control), Lane3=Qiagen H<sub>2</sub>O (-ve control), Lane4= *M. bovis* (+ve control). Lanes 5 to 23 clinical samples from sputum culture isolate contained DNA from mycobacteria withinMTC.

After confirming the acid fastness of the isolates from the urine culture by Ziehl-Neelsen, further investigations were conducted to confirm the isolates whether they are MTC or not and differentiate of species among MTC by presence or absence of RD9. Accordingly 16(94.1%) urine culture proven isolates were confirmed as *M. tuberculosis* by RD9. Only one isolate was deleted on RD9 primer [L6](Fig 4.7 2)

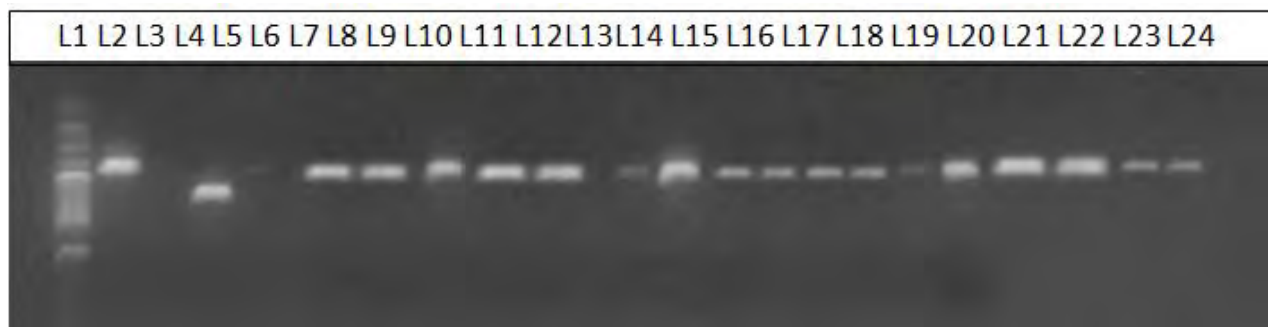


Figure 4.7.2. Gel picture showing Isolates of Mycobacterium characterized for species identification with RD9 Primer from urine culture. Description: Lane1= 1kb ladder, Lane2= H37Rv (*M.tuberculosis* +ve

control), Lane3=Qiagen H<sub>2</sub>O (-ve control), Lane4= *M. bovis* (+ve control). Lanes 5 to 15 urine samples from culture isolate contained DNA from mycobacteria within *M. tuberculosis* complex

Of the three culture isolates not confirmed on RD9 [*L6 and L16, L20* from urine and sputum respectively], Genus typing were performed and the result showed MTB complex in the 2 isolates of sputum but till one isolate from urine not confirmed, which was later affirmed as not among the member of MTC or not genus of *Mycobacterium* by genus typing (fig.4.4).

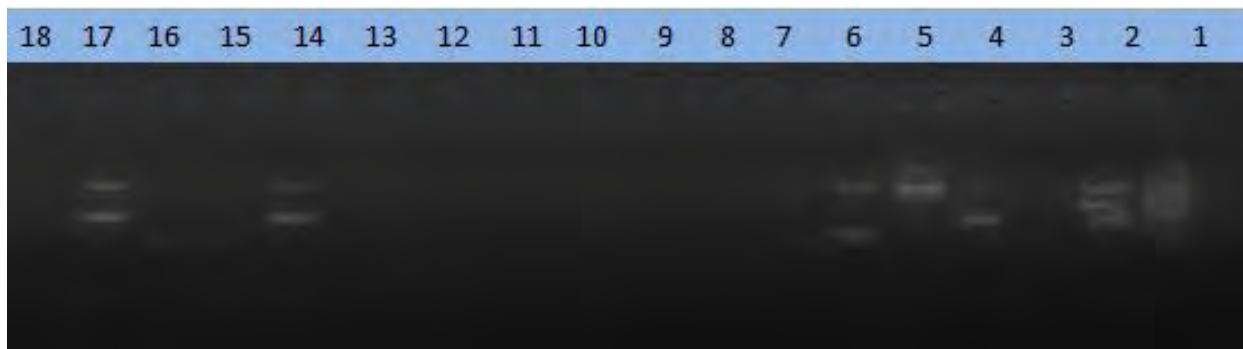


Figure 4.7.3: Gel picture showing Isolates of *Mycobacterium* characterized for genus identification with genus MycoF, MycoR, Intra genusF, *M.avium* R, TB1F, and TB1R primers. Description: Lane 1 = ladder (100bp), lane 2= H37Rv; lane 3=negative control; lane 4=*M. bovis*; lane 5=*M.intr*; lane 6=*M. avium*; lane 14 to 18 samples.

#### 4.7.2. Spoligotyping Result

Of 53 clinical isolates, 33/53(62.3%) were classified into one of 17 distinct spoligotype patterns shared international types (SIT) according to SpolDB4.0. The remaining 20/53(37.7%) isolates generate 17 different spoligotypes pattern that had not been previously reported to the SpolDB4.0. Among the distinct spoligotype pattern characterized, 4 patterns corresponding to cluster with 2-10 isolates per clusters were identified. The remaining 13 patterns represented by a unique (non-clustered) spoligotypes pattern were pseudo orphan which were represented as a single in the data base. Out of 20 isolates not found in spolDB4 that classified into 17 patterns 16 were represented by a unique pattern which were true orphan according to spolDB4.0, whereas the remaining 4 pattern consisted of cluster with 4 isolates each per cluster were identified.

Classification of the spoligotype pattern with web based SPOTCLUST data based showed different families “ill defined” (T), Central Asian (CAS), Family33, Beijing, H37Rv, Haarlem1 and EA14 were reported. Among these families both family33 and T family had equal proportion consisted 23/53(43.4%) isolates. T family consist T1, T3, T-ET and T4 with the clade accounted for 9/53(17%), 2/53(3.8%), 10/53(18.8%) and 2/53(3.8%) respectively. Other families present

were Beijing, 1/53 (2%) CAS, 2/53 (3.8%), H37Rv 1/53 (2%), Haarlem1 2/53 (3.8%) and EA14 1/53 (2%). This web also classified strains into different lineage: modern lineage, Euro-American 25/53 (47.2%), ancestor lineage, Indo-Oceanic 26/53 (49.1%) and 2/53 (3.8%) identified were unknown lineages (Table 4.5).

Figure 4.7.2. Spoligotype patterns of *M.tuberculosis* strains, family and lineage assignment of PTB from clinical isolates according to SpolDB4.0 and SPOTCLUST web based program

Strain	Webdings format	Spoligotype43	SIT	lineage	family	prob	no.ISO
001	oo	777777777777777777	Orphan	Indo-Oceanic	Family33	0.95	1.90%
003	oo	7777767777760731	336	Euro-American	T1 family	0.6	15.10%
004	o.oooooooo.ooo.oooooooooooooooooooooooooooo	577347777762671	Orphan	Indo-Oceanic	Family33	0.99	1.90%
005	o.oooooooo.ooo.oooooooooooooooooooooooooooo	577777777763771	1096	Indo-Oceanic	Family33	0.99	1.90%
006	oo	777747777763771	Orphan	Indo-Oceanic	Family33	0.99	7.50%
007	oo	777747777766771	Orphan	Indo-Oceanic	Family33	0.99	1.90%
008	oo	777000377760771	149	Euro-American	T3 family	0.99	18.90%
010	o.oo	47777774137771	415	Indo-Oceanic	Family33	1	1.90%
011	o.oooooooo.ooo.oooooooooooooooooooooooooooo	577347777763771	Orphan	Indo-Oceanic	Family33	0.99	1.90%
012	o.ooooo.o.ooo.oooo.oooooooooooooooooooooooooooo	575347477742771	Orphan	Indo-Oceanic	Family33	0.99	1.90%
013	oo	77777777777331	1149	Indo-Oceanic	Family33	1	1.90%
015	oooo.oo	75777777777761	Orphan	Indo-Oceanic	Family33	1	1.90%
018	oo	77774777773771	Orphan	Indo-Oceanic	Family33	0.99	1.90%
020	oo	777777777763771	54	Indo-Oceanic	Family33	0.99	1.90%
021	o.o.oooo.oooooooooooooooooooooooooooooooooooo	503777740003171	Orphan	Unknown	CAS family	0.99	1.90%
022	o.oooooooo.ooo.oooooooooooooooooooooooooooo	577347777761771	Orphan	Indo-Oceanic	T1 family	0.99	1.90%
023	oo	777777777767771	2731	Indo-Oceanic	Family33	0.99	3.80%
024	oo	777777777762771	1690	Indo-Oceanic	Family33	0.99	1.90%
025	oo	77774777777771	Orphan	Indo-Oceanic	Family33	1	1.90%
027	ooo.oooo.oooooooooooooooooooooooooooooooooooo	703777740001671	Orphan	Unknown	CAS family	0.99	1.90%
029	oo	77777777777771	523	Indo-Oceanic	Family33	1	5.70%
031	oo	777777477760771	451	Euro-American	H37Rv fam	0.74	1.90%
032	oo	77700777777771	Orphan	Indo-Oceanic	Family33	1	1.90%
033	oooooooooooo.o.oooo.oooooooooooooooooooooooooooo	777640007760771	249	Euro-American	T4 family	0.99	1.90%
034	oo	000001777400171	Orphan	Indo-Oceanic	T3 family	0.73	1.90%
035	oo	777760007760731	2246	Euro-American	T4 family	0.99	1.90%
036	oo	000000000003771	1	East Asian (Beijing fam)	Beijing fam	0.99	1.90%
037	ooooo.oooo.oooooooooooooooooooooooooooooooooooo	770000777740171	1123	Euro-American	T3 family	0.99	1.90%
038	oo	77777774020771	47	Euro-American	Haarlem1 fa	0.99	1.90%
039	oo	77773774020731	727	Euro-American	Haarlem1 fa	0.99	1.90%
040	oo	777737774413771	564	Indo-Oceanic	EA14 family	0.98	1.90%

☐= Present, - deleted, Prob=probability, no.iso=number of isolates, SpolDB4.0= fourth international spoligotyping database; SIT= Spoligo International Typing, CAS=Central Asian; T=Tuscany, EAI=East-African Indian

#### 4.7.3. Distribution of *M.tuberculosis* strain in urine and Sputum isolates

With regard to the comparison of the Spoligotyping pattern of *M.tuberculosis* isolated from the urine and sputum specimens of the same patients, 7 out of 13 (53.8%) differed in their family. Out of 7 MTB detected from urine specimen two belong to Haarlem1, one belongs to EAI1 and four belong to T3-ETH family while isolates of sputum specimen, 3 belong to T1 family, 2 were belong to family33 and 2 isolate belong to CAS family (Table 4.7.3).

Table 4.7.3. Distribution of *M. tuberculosis* strain in urine and sputum specimen

Sample	No. Isolate tested	T family	Family33	CAS family	Beijing family	EAI family	H37Rv family	Haarlem family
Urine	20	7	10	0	0	1	0	2
Sputum	33	16	13	2	1	0	1	0
Total	53	23	23	2	1	1	1	2

#### 4.8. Detection rate of MTB in urine depend on their CD4<sup>+</sup> T cell count

Of 30 pulmonary tuberculosis patients examined, 24(80%) had low urine mycobacterial burden (*most of them culture/smear negative*), 6(20%) had a high urine mycobacterial burden (*more than 80% were culture/smear-positive*). The mean CD4<sup>+</sup> lymphocyte count of the study participant was 201.97 ±150.7cells// μ l. Patients with higher levels of urine mycobacterial had lower CD4 cell counts, out of 9 patients had CD4<sup>+</sup> lymphocyte count less than 50 Cells/mm<sup>3</sup>, 5/9(55.6%) were smear positive, 7/9(77.8%) was culture positive. Statistical analyses showed that low CD4 strong association with the detection of MTB in urine (p=0.001) (Table 4.8).

Table 4.8. The correlation and detection rate of MTB in **urine** depend on their CD4 count

CD4	Particip ants	Smear	Cultur e	PCR	Lowbacterial burden(n=24)**	Highbacterial burden(n=6)***
<50cells/mm <sup>3</sup>	9	5	7	9	5(20.8),p= .045	4(66.7),p= .001
50-99cells/mm <sup>3</sup>	10	1	5	10	9(37.5)	1(16.7)
100-149cells/mm <sup>3</sup>	5	1	2	5	4(16.7)	1(16.7)
150-199cells/mm <sup>3</sup>	1	0	1	0	1(4.2)	0
>200 cells/mm <sup>3</sup>	5	0	2	5	5(20.8)	0
Total	30	7	17	29	24(80)	6(20)

#### 4.9. Risk factor for TB

A total of 117 pulmonary tuberculosis suspected patients was studied to identify the risks factor associated with TB. The risk factor for the TB we concentrated on smoking, alcohol, diabetic, TB case in family, raw milk in take and livestock in home were 30.8%, 23%, 4.3%, 10.3%, 19.6% and 5.1% respectively. A higher proportion of cases had a smoking history ( $P<0.001$ ) as well a larger proportion of them were living in a crowded house styles made of a mud wall compared to those who TB negative ( $P< 0.001$ )(Table 4.9).

Table 4.9: Risk factor of the study participants

<b>RISK FACTOR</b>	FREQUENCY		SMEAR (N=16)	CULTURE (N=37)	PCR (N=44)	OR
	(N=117)	%				
Smoking	36	30.8	11	13	16	4
Alcohol consumption	27	23	9	9	10	0.99
TB case in Family	12	10.3	6	8	8	1.105
Previous history of TB	16	13.7	2	3	5	0.98
DM	5	4.3	1	1	1	1
Raw milk in take	23	19.6	0	2	3	0.99
Livestock in home	6	5.1	0	1	1	1.21
<b>House style</b>						
Crowded	52	44.4	8	23	29	4.2
Ventilated	71	60.7	3	14	15	0.8

## CHAPTER FIVE

### DISCUSSION

Pulmonary tuberculosis is the commonest cause of death among human immunodeficiency virus infected persons, particularly among patients in developing countries where resource is very limited for diagnosis. Co-infection with HIV leads to challenge in diagnosis because of the poor performance of sputum smear microscopy as well as difficult sputum production in HIV-infected patients. Besides making difficult in diagnosis, the paucibacillary nature of TB in HIV infected patients also have a capable of result in misdiagnosis or in classifying patients as smear-negative. Therefore, both newer diagnostic tests and specimen are urgently required that are not only sensitive and specific but also easy to use in remote and resource-constrained settings. To our knowledge, this is the first hospital-based study from Ethiopia to evaluate the utility of urine as clinical specimen for the diagnosis of active pulmonary TB in HIV infected patients.

In our present study, of 117 eligible study participants included in study, 68 (55.3%) were among 28-37 age group, of which 66.7% of proven PTB cases were found among this age group. This finding is supported by most of the study conducted on HIV infected patients in the world (Daniel OJ et al., 2004, Friedland G et al., 2007, Kelly P and et al, 1990) which PTB/HIV was report high among this productive age. This might be explained by the fact that obviously, this age group show high interest for sexual intercourse which is the main way of AIDS/HIV transmission. Statistical analysis showed that clinical symptom such as cough and chills had no significant association with pulmonary TB infection in people living with HIV ( $p=.651$ ). This finding was consistence with study conducted to evaluate WHO guideline to improve the diagnosis of tuberculosis in ambulatory HIV-positive adults(Koole O et al., 2011).

In this study, the prevalence of pulmonary TB among HIV infected patients was 45(38.5%) which were comparable with study carried out in Nigeria(Affusim et al., 2012) where the prevalence was 37%. However, our finding was far up from research conducted in different parts of Ethiopia (Ali et al., 2012, Wondimeneh Y et al., 2012, Moges et al., 2012). This difference might be due to the diagnostic method and the additional specimen (urine) that we used in our study. The detection rate of MTB in patients those who can't produce sputum (cough without expectoration) was 50%. This finding completely differ from the study done in India which state no urine sample from a

sputum-scarce patient was smear- or L-J culture-positive(Singh and Bhargava, 2010), but there was no local data to compare our result in term of urine utility for the diagnosis of PTB.

Under resource constrain, the prevalence of pulmonary tuberculosis is determined by the ability of ZN-smear detection. In our finding, the ability of ZN smear detection of MTB in pulmonary tuberculosis was 16(13.7%) which is very difficult to categorize the patients and treat at early. However, this finding was lower than the research conducted in Nigeria(Lawson L et al., 2005) where the detection was 26%. This difference could be due to geographical difference, disease burden and additional specimen we used. The frequency of smear negative pulmonary tuberculosis in HIV infected patients is the major problem for the diagnosis as well as for treatment of tuberculosis. Under such condition one option is to undertake culture. Out of 117 suspected pulmonary tuberculosis cases included in our study, culture detected 37(31.6%) case. This finding was not comparable with the research carried out in India(Gopinath and Singh, 2009) where the detection rate of culture were 56.8% and showed slight difference with the research conducted in Singapore(Chan et al., 2008) where the detection rate of culture were 27.1%. Now days the future hope for the diagnosis of pulmonary tuberculosis in immunosuppressed patient is PCR based molecular techniques. In the present finding, the detection rate of PTB cases by PCR in people living with HIV was 44(37.6%). Our finding showed in line with the research conducted in Brazil (Silva et al., 2012) and Iran (Heydari et al., 2014)where the detection rate was 41.2% and 35.8% respectively.

Information regarding use of urine as clinical specimen for the diagnosis of PTB in developing countries as well as developed countries is scarce, but it gates attention after the emergence of HIV and future hope for those who cannot produce sputum or smear negatives. Even though the excretion of MTB through urine reported early 1975, till no sufficient funding is available for surveillances. In the present study, comparison of culture showed that sputum culture alone detected more mycobacterial isolates, 28.2% than urine specimen alone, 14.5%. This finding was not comparable with the research done in India(Singh and Bhargava, 2010) where sputum culture and urine culture were detected 56.8% and 19.7% respectively. This difference might be due to study groups were suspected PTB rather than smear positive. but our finding similar with the research conducted in Bangalore(Challu et al., 1989) which stated MTB was recovered from 35 (14.8%) of urines of PTB patients. However, of the 84 sputum culture-negative cases, four (4.8%)

were urine culture-positive. This finding is slightly comparable with the study done in India(Singh and Bhargava, 2010) where urine culture was positive in 8.6% of sputum culture negatives. This figure may lead us to suggest that urine specimens along with the sputum specimens will increase the chance of diagnosing pulmonary TB, especially in sputum smear-negative/scarcely pulmonary TB cases. None of the previous studies had reported high culture positivity rate of *M. tuberculosis* from urine as reported in the present study. This difference might be due to the fact that our study participants were HIV infected patients. Use of urine culture besides of sputum culture for diagnosis of PTB in HIV infected patients increased the detection of MTB by 4(12.1%). The combination of both cultures for the diagnosis of pulmonary tuberculosis in HIV patients increased the chance of MTB detection by 3.4% [from 28.2 % to 31.6%]. This finding is in consistence with the research conducted in India(Singh and Bhargava, 2010) where the detection rate was increased by 4.9% [from 56.8% to 61.7%]. Besides increasing the chance of MTB detection, urine specimen minimizes the use of more invasive techniques for collecting samples, such as BAL and gastric lavage and reduces the aerosol. While of 37 cultured MTB detected from sputum and urine specimens, 13(35.1%) were from the same patients which is moderate agreement ( $\kappa=0.37$ ) between the two specimens. The results showed that more than a quarter (35.1%) of the smear and/or culture-positive PTB cases also excreted *M. tuberculosis* in their urine specimens. This finding is slightly higher than the study conducted in India (26.1%)(Singh and Bhargava, 2010). This difference might be due to their study participants were not immunosuppressed patients.

Excretion of *M. tuberculosis* through urine was reported as early as in 1975 and confirmed by some recent studies using modern diagnostic tools(Bentz RR et al., 1975, Torrea G et al., 2005, Rebollo MJ et al., 2006, Heydari et al., 2014). Even if some studies tried to demonstrate the utility of urine as a clinical specimen in diagnosing of pulmonary TB in both HIV-positive and HIV negative cases to overcome the challenge of poor performance of sputum smear, there is major limitation on their study that bacteriological confirmation of the urine specimens and true positivity rate of PCR was not done. In this study, bacteriologically confirmed pulmonary TB among HIV infected patients from sputum was 33(28.2%), of which 22(66.7%) were urine PCR positive while 13(39.4%) were also urine culture positive. Our study showed when the easily available urine specimen supported by PCR and culture can identify 72.5% of PTB. This finding may lead as the excretion rate of MTB through urine was high in immunosuppressed patients. Out



of 16 MTB detected by Smear from two specimens, 18.7% were from the same patient and the remaining (81.3%) detected from different patients which indicated 4 (25%) of patients could be missed by using only sputum specimen for the diagnosis of PTB in HIV infected patients. Hence urine ZN smear can increase the chance of diagnosis in people living with HIV by 25%. To our knowledge, there is no publication or any data which compare urine and sputum in ZN-smear that is why we couldn't give any suggestion.

The data presented in our study could provide information about urine as clinical specimen which is responsible for diagnosis of active pulmonary tuberculosis in people living with HIV. With advancing HIV related immunosuppression, the frequency of extrapulmonary and disseminated forms of TB disease increase, i.e. sputum smear microscopy performance is reduced, and some patients are unable to produce sputum for diagnostic testing that is challenging and often delayed which also raise the burden of undiagnosed TB in HIV-infected hospitalized patients; this is the reason why we focused on easily available and aerosol free specimen, urine. In sputum-scarce, the usual alternative specimens are BAL and gastric lavage fluids(Singh and Bhargava, 2010), but collection of these specimens is often painful and awkward which make these specimens add insignificant advantage. Even in those patients whose pulmonary samples were negative by all bacteriological methods, the urine PCR was positive in 4.8% of patients. This finding is not comparable with the study done in India(Singh and Bhargava, 2010) where urine PCR was positive in 31.4% of patients those who pulmonary samples were negative by all bacteriological methods.

The ability of PCR for MTB detection showed differences when applied on urine and sputum specimen. As the result, when PCR applied on sputum, it can detect 36.4% over urine but missed 9.1% alone. However, in those patients whose pulmonary samples were negative by all bacteriological methods, the urine PCR was positive in 8.9% of patients. This finding far up with the research conducted in Iran(Heydari et al., 2014), where out of the 29 patients with negative sputum cultures, 10 (34.5%) patients were urine-PCR positive. This difference might be due to the primer used because most of the literatures put as DNA fragment in urine are very short. PCR was more sensitive and specific in sputum than urine. Of 115 urine specimen negative for AFB, PCR detected as positive 23 making detection rate (20%) over ZN staining in urine and in the same manner 29 were PCR positive in sputum, the detection rate of MTB by 27.4% over AFB from

sputum specimen in HIV patients. Moreover the combination of both specimen by direct PCR detected MTB in 44(35.8%) which indicate that PCR detect additional five MTB of culture negative. Even though we attempted to apply the most sensitive diagnostic method to the most easily available clinical sample, i.e., urine, with an improved detection rate, urine samples were tested along with pulmonary specimens and when genus- and species-specific PCR methods were applied to these samples, we were able to detect an additional 8.9% of PTB cases. This finding is highly significant and encouraging where urine culture also improved the mycobacterial isolation rate. Therefore, performing urine culture in parallel with direct PCR was the future hope for diagnosis of pulmonary tuberculosis in immunosuppressed patients.

Early diagnosis and treatment of PTB is decisive to reduce morbidity and mortality due to TB. Different researchers evaluated the performance of PCR to the conventional culture method in the diagnosis of TB from sputum and urine. However, the difference in the primer used and nature of the sample determines its sensitivity and specificity. In our finding, all culture positive (37) were also positive by PCR with sensitivity, specificity, PPV and NPV of 100%, 98%, 57% and 100% respectively. In our study, data on agreement between smear and PCR assay with culture were available that showed direct PCR analysis is more effective in detecting *M.tuberculosis*. ZN smear was detect only 16(40%) bacilli that all confirmed by culture as well as PCR. This finding was consistent with the research conducted in India (Challu et al., 1989, Singh and Bhargava, 2010). The sensitivity and specificity of ZN smear and PCR were 40% and 100%, and 97.5% and 94% respectively. The agreement between ZN smear and PCR methods with culture were 0.78 perfect and 0.56 with good agreement respectively. This finding showed slight difference with the research done in Iran(Heydari et al., 2014)

Focusing on urine, ZN smear detected 8 bacilli that make the detection rate of AFB 6.5% while PCR detected 28 bacilli in urine specimen. The agreement between AFB and PCR for the diagnosis of active pulmonary tuberculosis in people living with HIV was moderate agreement (kappa=0.342). However, by detecting 40% of urine culture proven MTB agents in HIV infected patients, ZN smear make good agreement(kappa=0.528) with culture method. Therefore, the diagnostic sensitivity, specificity, PPV and NPV of ZN-staining was 35%, 99%, 87.5% and 88.7% respectively. Also the efficiency of polymerase chain reaction (PCR) directly from urine for the diagnosis of active pulmonary tuberculosis performed with the sensitivity, specificity, PPV and

NPV 95%, 88.3%, 61.3% and 98.9% respectively. This finding is consistency with research done in South Africa(Ghaleb et al., 2013)

Our study had also revealed the agreement between the diagnostic methods in detection of MTB from sputum specimen. ZN smear was detect 11 MTB making the detection rate of AFB 9.4% while PCR detected bacilli in 40(34.2%). Our finding is supported by the research done in Nigeria(10.1% and 33.9% respectively) (Affusim et al., 2012). In the same manner with the urine specimen, when AFB combined with PCR, the detection rate of bacilli by two techniques in the diagnosis of PTB was 40(34.2%) with fair agreement ( $\kappa=0.339$ ). However, when combined with culture method, the diagnostic ability of the two techniques was 33(28.2%) which was moderate agreement as in urine specimen. Hence, ZN smears detected 33.3% MTB of culture proven PTB cases whereas PCR detected all of culture proven PTB cases. The diagnostic sensitivity, specificity, PPV and NPV of ZN-staining was 33.3%, 100%, 100% and 79.2% respectively. This finding slightly in line with the research conducted in Saudi Arabia showed that ZN smear examination has a sensitivity of 25% and a specificity of 100%(Ghaleb et al., 2013). However, the efficiency of polymerase chain reaction (PCR) directly from sputum for the diagnosis of active pulmonary tuberculosis performed with the sensitivity, specificity, PPV and NPV 100%, 91.7%, 82.5% and 100% respectively. Our finding was not comparable with the study carried out in Brazil(Silva et al., 2012) on HIV positive patient where sensitivity, specificity, PPV and NPV were 59%, 33%, 87% and 100%. This difference might be due to we used different primers for the amplification.

In our study, the majority of the isolates (86.8%) belonged to two major families: Family33(43.4%) and T family(43.4%). Our finding slightly differs from study conducted in Addis Ababa(Mihret et al., 2013). The T family, which is the most frequent spoligotype in this study, had been reported in previous studies in Ethiopia as well as elsewhere in the world(Debebe T et al., 2013). In our finding CAS family accounted only 5% with unknown lineage and orphan. This finding differs from the study conducted in Addis Ababa(Mihret et al., 2013) where CAS genotypes (21.9%) are the second predominant. The other families were EAI14, H37Rv family, Haarlem1 family and Beijing account 1, 2,2 and 1 isolates, respectively. Even though the clustering of isolates is an indicator of recent transmission, in our finding the problem was high in 28-37 age group (66.7%)

which suggested an increased likelihood of recent TB among this age group, which may be linked to a higher prevalence of HIV infection in reproductive age (Giri et al., 2013).

Distribution of the predominant clades of *M. tuberculosis* strains shows slight variation among sputum culture isolates and urine culture isolates population. With regard to the comparison of the Spoligotyping pattern of *M. tuberculosis* isolated from the urine and sputum specimens, of 13 *M. tuberculosis* detected from the same patients, 7 (53.8%) were different in their family. Out of 7 MTB detected from urine specimen two belong to Haarlem1 family. Of the remaining isolates, one belongs to EAI1 and four belong to T3-ETH while isolates of sputum specimen, 3 belong to T1 family, 2 belong to family33 and 2 isolate belong to CAS family. To our knowledge, there is no publication on epidemiology of PTB isolates from urine that is why we couldn't give any suggestion.

Mycobacterium tuberculosis isolation rate in urine was strongly associated with baseline CD4 cell count. A low CD4 lymphocyte count was strongly associated with the presence of active TB in HIV infected individual (Taha et al., 2011). The isolation rates among those whose CD4 cell count less than 50 cells/ $\mu$ l were 71.4%, 41.2%, and 24.1% by smear, culture and PCR respectively. However, the detection of MTB in patients whose CD4 cell count greater than 200 cells/ $\mu$ l was 0%, 11.8% and 37.9% in smear, culture and PCR respectively. In diagnosis of pulmonary tuberculosis, culture time to positivity rate of MTB in sputum specimen was shorter than the urine specimen. This is reflective of the lower bacillary load seen in urine specimen compared to sputum specimen. This load of bacillary is affected by CD4 T cell of the patient which inversely proportion between urine and sputum specimens. As CD4 T cell count decreases the load of bacillary in urine specimen slightly increased (Chan et al., 1991). The association of CD4 count with AFB method in diagnosis of pulmonary tuberculosis in HIV infected patient was negatively correlated (-0.372) with detection rate of MTB in urine specimens. Our finding support the idea of low CD4 lymphocyte count was strongly associated with the presence of active TB in HIV infected individual. Our study had also revealed a significant statistical association between WHO clinical stage and MTB detection rate in HIV infected patients. The detection rate of MTB in HIV patient was increase with the WHO clinical stage. Thus, those patients with advanced WHO clinical stage had higher likelihood of MTB detection rate as it has been seen in CD4+ lymphocyte count. Study from Jimma consistent with our finding (Kassu A et al., 2007b).

Our Study has shown that cigarette smoking increased the risk of TB in HIV infected individuals. Persons having the habit of smoking were 4 times more likely to develop a PTB than non smoking (95%CI 1.1–7.9). This finding is supported by the research conducted in India(Lönnroth et al., 2009). In the present study, alcohol consumption habitual, chronic disease like diabetes, raw milk in take and previous contact with a case of TB were not significantly associated with detection of MTB in HIV infected individual. This finding is not in agreement with research conducted in south Africa(Lawn et al., 2005), which stated all WHO screening has significant association with the detection of MTB in HIV infected patient. Patients those who living in crowded house was two times more likely to be develop PTB than in those who live in ventilated house (95% CI, 0.8–4.2). Our finding in line with the systematic review research done in Europe(Faustini et al., 2006)

## CHAPTER SIX

### CONCLUSION AND RECOMMENDATION

Diagnosis of PTB in HIV infected individual remains a great problem in Ethiopia as well as in other countries where TB and HIV is endemic. This study has been undertaken in HIV-positive patients in order to gain a better understanding into MTB excrete through urine in immunosuppressed patients. The present finding showed that urine culture and PCR can detected 66.7% and 39.4% respectively of bacteriologically confirmed PTB from sputum specimen. This figure indicate that MTB excrete through urine in immunosuppressed patients were high which showed, if available, urine PCR could be run in parallel in diagnosis of PTB in HIV infected individual to solving the problem related to diagnosis of pulmonary tuberculosis in immunosuppressed individuals. Accordingly, urine specimen in diagnosis of PTB, being not medically least important, is neglected by health and health related organizations and researchers. Surprisingly, when PCR applied on the most easily available clinical sample, i.e., urine, we were able to detect an additional 8.9% of PTB cases. These findings are highly significant and encouraging when urine culture also improved the mycobacterial isolation rate and alerts relevant bodies to know how its valuable as adjunct specimen under the scarcity of respiratory specimen in the diagnosis of active pulmonary tuberculosis in people living with HIV.

The following recommendations are forwarded based on the findings of our study:

- ✓ Even though burden of bacillus found more in sputum specimen and more culture positivity than urine specimen, urine should not be neglected for the diagnosis of pulmonary tuberculosis in HIV infected patients.
- ✓ In consideration of ease of urine sample preparation for culture and PCR, performing urine culture and PCR, when possible, could be used as a diagnostic aid for PTB cases when sputum samples is problematic.
- ✓ In addition to sputum culture, urine culture should be done to get more accurate analysis of PTB among HIV patients.

The limitation of this study is the difficult to differentiate extra pulmonary and pulmonary tuberculosis because of study participants are suspected pulmonary tuberculosis and also asymptomatic urogenital TB can affect the result.

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## ANNEX

### Annex 1: Participant Information sheet

Date -----

**Title:** Urine as an adjunct specimen for the diagnosis of active pulmonary tuberculosis in people living with HIV.

**Background:** Tuberculosis is the opportunistic infections which increases the mortality in human immunodeficiency virus infected individual. The diagnosis of pulmonary tuberculosis (PTB) is usually established by examination of three Zeihl-Neelsen stained smears but in HIV infected person show negative smear which negative results do not preclude active TB. In order to overcome this problem other attractive potential specimen that replaces the conventional method should be addressed. Since tubercle bacilli or their nucleic acids are also expected to be excreted through the kidneys, we will be interest to assess spot urine as a supplementary specimen for diagnosing PTB.

**Objective of the study:** The aim of this study is to evaluate urine as an adjunct specimen for the diagnosis of active pulmonary tuberculosis in people living with HIV. The sensitivity and specificity of PCR and ZN smear will be compared against culture method.

**Organizations:** The study will be conducted by Addis Ababa University, school of Graduate studies. Laboratory procedure will be carried out at Akililu Lemma Institute of Patho-Biology.

**Procedure:** Expert (in the area) Nurses will collect urine and sputum for routine microscopic diagnosis. Laboratory method to be used includes; ZN smear, culture, Spoligotyping, and PCR DNA extraction.

**Participation:** The procedure will be carried out after getting your willingness to participate. All volunteer patients with pulmonary problem, fulfilling inclusion criteria, will be included.

**Risks associated with sample collection:** No pain during specimen collection and to reduce the aerosol, you will be administered to use capped tube.

**Benefit:** As different study shows diagnosis of PTB in HIV patients is very difficult and end up with high mortality. You will be benefited from the study; because it will be part of your diagnosis and might be a key to your current and/or your future problem if it will come up with positive result.

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

**Confidentiality:** From medical ethics point of view and research ethics, every part of your personal information will be kept confidentially. Information to be collected and variables expressing your identity will be coded secretly. The only responsible person to link your variables (important for your follow up and treatment) with the code number is the principal investigator. However, other researchers can see your clinical information, which is without your identity. Your result and information will be used only for the mentioned purpose.

**Sharing the Result:** Eventually, the result, devoid of your identity, will be 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 will 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. Alemu Chemed, Address: Addis Ababa University, College of Health Science, department of Medical Microbiology, Immunology and Parasitology, Mobile: +251910337785, E-mail – chemedashuma1@gmail.com
2. Tamirat Abebe, Address: Addis Ababa University, College of Health Science, department of Medical Microbiology, Immunology and Parasitology, Mobile: +251911447227, E-mail tabebezeleke@yahoo.com
3. Dr. Gobena Ameni (PhD, Associate Professor), Address: Aklilu Lemma Institute of patho-biology (ALIPB), Addis Ababa University, Ethiopia, mobile: - +251-911413073, E-mail: - gobenachimdi2009@yahoo.co.uk

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 “urine as an adjunct specimen for the diagnosis of active pulmonary tuberculosis in people living with HIV at selected Hospital Addis Ababa, Ethiopia.” Finally, she/he told me that this will be 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
- 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 will be confirmed by my signature below.

The information sheet was given/ explained to me by:-----, signature -----, phone ----

Name of participant: -----, signature -----, phone ----

Name of physician: -----, 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: \_\_\_\_\_  
DD MM YYYY

Date of interview: \_\_\_\_/\_\_\_\_/\_\_\_\_

**To be Filled by the Interviewer**

<b>201.</b>	Sex	Male <input type="checkbox"/>	Female <input type="checkbox"/>
<b>202.</b>	Age	18-29 <input type="checkbox"/>	30-39 <input type="checkbox"/>
		40-49 <input type="checkbox"/>	50 and above <input type="checkbox"/>
<b>203.</b>	Marital status	Single <input type="checkbox"/>	Married <input type="checkbox"/>
		Divorced <input type="checkbox"/>	Widowed <input type="checkbox"/>
<b>204.</b>	Educational status	Non educated <input type="checkbox"/>	Elementary school <input type="checkbox"/>
		High school <input type="checkbox"/>	Certificate and above <input type="checkbox"/>
<b>205.</b>	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"/>	
<b>206.</b>	Housing style	1) Crowded <input type="checkbox"/>	
		2) Ventilated <input type="checkbox"/>	
<b>207.</b>	Place of residence	1) Urban <input type="checkbox"/>	
		2) Rural <input type="checkbox"/>	
<b>208.</b>	Body mass index(BMI), kg/m <sup>2</sup>	-----	
<b>210.</b>	Symptoms		
	Cough	Yes <input type="checkbox"/>	No <input type="checkbox"/>
	Fever	Yes <input type="checkbox"/>	No <input type="checkbox"/>
	Nausea or vomiting	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"/>
	Fatigue	Yes <input type="checkbox"/>	No <input type="checkbox"/>
	Chills	Yes <input type="checkbox"/>	No <input type="checkbox"/>

	Hematuria	Yes <input type="checkbox"/>	No <input type="checkbox"/>
	Burning micturition	Yes <input type="checkbox"/>	No <input type="checkbox"/>
	Sterile Pyuria	Yes <input type="checkbox"/>	No <input type="checkbox"/>
	Chronic cystitis	Yes <input type="checkbox"/>	No <input type="checkbox"/>
<b>211.</b>	Chest radiograph with any abnormality	Yes <input type="checkbox"/>	No <input type="checkbox"/>
<b>213.</b>	Previous history of TB	Yes <input type="checkbox"/>	No <input type="checkbox"/>
<b>214.</b>	Does the client take ionized prevention therapy(IPT)	Yes <input type="checkbox"/>	No <input type="checkbox"/>
<b>215.</b>	HIV test result	Positive <input type="checkbox"/>	Negative <input type="checkbox"/>
<b>216.</b>	WHO stage of HIV	1) I <input type="checkbox"/> 2) II <input type="checkbox"/>	3) III <input type="checkbox"/> 4) IV <input type="checkbox"/>
<b>217.</b>	Does the client on antiretroviral therapy (ART)	Yes <input type="checkbox"/>	No <input type="checkbox"/>
<b>218.</b>	If yes for how long?	< 1 year <input type="checkbox"/>	> 1 year <input type="checkbox"/>
<b>219.</b>	CD4+ Lymphocyte count	<50Cells/mm <sup>3</sup> <input type="checkbox"/> 50–99 Cells/mm <sup>3</sup> <input type="checkbox"/> 100–149 Cells/mm <sup>3</sup> <input type="checkbox"/> 150- 199 Cells/mm <sup>3</sup> <input type="checkbox"/> ≥200 Cells/mm <sup>3</sup> <input type="checkbox"/>	
<b>220.</b>	Sputum bacillary burden	Minimal (smear-negative) <input type="checkbox"/> Moderate (1+) <input type="checkbox"/> Most (2–3+) <input type="checkbox"/>	

DECLARATION

I, the undersigned, declare that this M.Sc. thesis is my original work, has not been presented for a degree in any other University and that all sources of materials used for the thesis have been duly acknowledged.

M.Sc candidate:

Alemu Chemedha Ifa

Signature

\_\_\_\_\_

Date and place of submission

\_\_\_\_\_

Supervisor

Adane Mihret, MVD, M.Sc, PhD

Signature

\_\_\_\_\_

Date and place of submission

\_\_\_\_\_

Addis Ababa, Ethiopia

Supervisor

Tamrat Abebe, M.Sc, PhD

Signature

\_\_\_\_\_

Date and place of submission

\_\_\_\_\_

Addis Ababa, Ethiopia