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DEPARTMENT OF MEDICAL LABORATORY SCIENCES

PREVALENCE OF SMEAR NEGATIVE PULMONARY TUBERCULOSIS AMONG PTB SUSPECTED PATIENTS AT ADAMA REFERRAL HOSPITAL, ADAMA, ETHIOPIA

By: Adugna Belew (MSc candidate)

A Thesis submitted to the Department of Medical Laboratory Science, school of allied health sciences, College of Health Sciences, Addis Ababa University in partial fulfillment of the requirements for the Degree of Masters in Clinical Laboratory Sciences (Diagnostic and Public Health Microbiology Specialty Track).

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Addis Ababa, Ethiopia

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This is to certify that the thesis prepared by Adugna belew, which is entitled with "**prevalence of smear negative pulmonary tuberculosis among PTB suspected patients at adama referral hospital, adama, ethiopia."** and submitted in partial fulfillment of the requirements for the degree of Masters of Clinical Laboratory Sciences (Diagnostic and Public Health Microbiology) complies with the regulations of the University and meets the accepted standards with respect to originality and quality.

Advisors: Signature Date

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Lists of Acronyms and Abbreviations

AFB	Acid-Fast Bacilli
AIDS	Acquired Immuno Deficiency Syndrome
BCG	Bacille Calmette-Guérin
BSL-2	Bio-Safety Level two
CXR	Chest Radiography
DOTS	Directly Observed Treatment, Short-course
HIV	Human immunodeficiency virus
IUATLD	International Union against Tuberculosis and Lung Disease
LJ	Lowenstein –Jensen
PTB	Pulmonary tuberculosis
SNPTB	Smear-negative pulmonary TB
SPPTB	Smear-positive pulmonary TB
TB	Tuberculosis
WHO	World Health Organization
ZN	Ziehl–Neelsen

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Operational definitions

Tuberculosis: A bacterial infection caused by *Mycobacterium tuberculosis*. The disease usually affects the lungs (pulmonary) but can spread to other parts of the body in serious cases (extrapulmonary).

Pulmonary tuberculosis: is a contagious bacterial infection that involves the lungs, but may spread to other organs.

Extrapulmonary case: A patient with tuberculosis of organs other than the lungs (e.g. pleura, lymph nodes, abdomen, genitourinary tract, skin, joints and bones, meninges).

Pulmonary tuberculosis suspects: are patients presenting with persisting cough for two weeks or more; productive cough with or without blood stained sputum, shortness of breath and chest pain; and loss of weight, intermittent fever, night sweats, loss of appetite, fatigue and malaise.

Case detection: The act of identifying active tuberculosis cases through sputum examination mainly among suspects attending a health facility for any reason.

Smear-positive pulmonary case: A patient with at least two initial sputum smear examinations (direct smear microscopy) AFB+; or one sputum examination AFB+ and radiographic abnormalities consistent with active pulmonary tuberculosis as determined by a clinician.

Smear-negative pulmonary case: A patient with pulmonary tuberculosis not meeting the above criteria for smear-positive disease. Diagnostic criteria should include: at least three sputum smear examinations negative for AFB; and radiographic abnormalities consistent with active pulmonary tuberculosis; and no response to a course of broad-spectrum antibiotics; and a decision by a clinician to treat with a full course of antituberculosis chemotherapy.

New case: A patient who has never had treatment for tuberculosis or who has taken antituberculosis drugs for less than one month.

Relapse case: A patient previously declared cured but with a new episode of bacteriologically positive (sputum smear or culture) tuberculosis.

SUMMARY

BACKGROUND: Tuberculosis is a communicable disease caused by infection with *Mycobacterium tuberculosis* complex group and infects almost one third part of the world population and kills around two million people worldwide each year. Much attention has recently been paid to the problem of smear-negative pulmonary tuberculosis. Quite appropriately, the discussion has focused on low-income countries, home to the vast majority of individuals with tuberculosis and HIV and where the ability to culture diagnostic specimens may be lacking. In particular, smear-negative pulmonary TB (SNPTB) has become an increasing important clinical and public health problem, especially in areas that are affected by the dual infection of tuberculosis and Human immunodeficiency Virus, such as sub-Saharan Africa including Ethiopia.

OBJECTIVE: The aim of the study is to determine the prevalence of smear negative pulmonary tuberculosis using standard culture methods and clinical algorithm among PTB suspected patients at Adama Referral Hospital, Adama, Ethiopia .

METHODS: A cross sectional study was conducted among suspected pulmonary tuberculosis patients (age >18 years old) visiting at Adama referral hospital in Eastern oromia Region from June 2013 to December 2013. The study subjects were all smear negative pulmonary tuberculosis patients and a consecutive sampling technique was used. A sputum sample was collected from patients who had smear negative result by Zihel Neelson method/technique. All smear negative pooled sputum samples was processed for culture using conventional Lowenstein-Jensen solid medium at the Adama regional laboratory. Descriptive statistics was computed. $P < 0.05$ will be statistically significance and multivariate logistic regression will be used for analysis of the result.

RESULTS: Of 232 AFB smear-negative patients, 130(56 %) were males. The mean age of the respondents was 40.2 ranging from 19 to 77 years old. Of 232 AFB smear-negative patients, 28 (12.1) were MTB culture positive. Among adults PTB suspected patients; smear-negative TB culture positive was less prevalent (10.7%) in age group 41-50 years. Majority (71.4%) of smear-negative TB culture positive was observed in male.

The majority of patients came with a combination of symptoms and the entire patient had chronic cough more than two weeks. Of the study participants, 116(50.0%) had abnormal chest x-ray finding.

The binary- logistic regression identified that Productive cough (OR (95%CI)= 0.82(0.81-0.91)), Haemoptysis (OR (95%CI)= 2.43(1.04-5.68)), night sweats (OR (95%CI)= 6.85(2.00-23.39)), Unexplained weight loss (OR (95%CI)= 3.44(1.53-7.72)), the presence of typical X-ray Findings (OR (95%CI)= 2.81(1.18-6.68)), and being HIV patients or positive(OR (95%CI)=4.92(2.1-11.5)) were independently associated with confirmed SNPTB. But in Multivariate regression analysis, Productive cough (OR (95%CI)= 0.82(0.81-0.91)), night sweats (OR (95%CI)= 8.27(2.27-30.1)), and being HIV patients or positive(OR (4.16(1.45-11.96)) were significantly associated with confirmed SNPTB.

CONCLUSION: The present study reveals that among smear negative PTB suspects, the prevalence of smear negative culture positive PTB was 12.1%, but 87.6% smear negative pulmonary tuberculosis cases were still etiologically unexplained by culture. Future studies on more affordable, rapid, and accurate tests for TB infection would also be necessary to timely provide specific treatment for patients in need, reduce mortality, and minimize TB transmission to the general population.

1. Introduction

1.1. Background

Tuberculosis is a communicable disease caused by infection with *Mycobacterium tuberculosis* complex group (*Mycobacterium tuberculosis*, *Mycobacterium bovis*, *Mycobacterium africanum and Mycobacterium microti*), with medical implications. It has always occurred disproportionately among disadvantaged populations such as the homeless, malnourished, and overcrowded. Tuberculosis is spread from person to person through the air by droplet of nuclei that contain M. tuberculosis complex group which causes the disease. Droplet nuclei are produced when persons with pulmonary or laryngeal tuberculosis cough, sneeze, speak, or sing. Droplet nuclei, containing two to three M. tuberculosis organisms, are so small that air currents normally present in any indoor space can keep them airborne for long periods of time (1).

Mycobacterium tuberculosis infects almost one third of the world population and kills around two million people worldwide each year. About 80% of the global TB burden occurs in low-income countries, where pulmonary disease and its transmission are most serious public health problems. Among bacterial pathogens of man, ***M. tuberculosis*** is best known for its slow growth rate and its acid-fast lipid-rich cell wall. Culture of mycobacteria is too slow for practical diagnosis, while their acid-fastness allows rapid detection in clinical specimens (2).

In spite of several initiatives and research to improve the performance of national tuberculosis control programs, pulmonary tuberculosis remains an important health problem which disproportionately affects less developed countries. During 2011, the worldwide number of cases and deaths were estimated at 8.7 and 1.4 million, respectively (World Health Organization 2012). Diagnosing tuberculosis in resource-constrained settings relies mainly on sputum smear examination, but approximately 35% of all patients with tuberculosis and 42% of pulmonary cases are smear-negative. In addition to the individual consequences of underdiagnosis, smear-negative pulmonary tuberculosis (SNPT) cases are thought to be responsible for approximately 20% of tuberculosis transmission (3).

In many countries with a high prevalence of TB, direct sputum smear microscopy remains the most cost effective tool for diagnosing patients with infectious tuberculosis and for monitoring their progress on treatment. Sputum microscopy is an essential component of the DOTS strategy recommended by the World Health Organization (WHO) and the International Union against Tuberculosis and Lung Disease (IUATLD). DOTS, relies on a network of laboratories to provide readily accessible and quality laboratory services to prevent the spread of infection in the community and prevent unnecessary treatment of cases that do not have TB (4).

So far, Sputum smear examination of acid-fast bacilli (AFB) is the cornerstone of tuberculosis (TB) diagnosis worldwide because of its simplicity, speed, low cost, and minimal requirement of equipment and technical skills. Sputum smear microscopy is done using the acid-fast staining procedure. Two procedures are commonly used for acid–fast staining: the carbolfuchsin methods, which include the Ziehl–Neelsen (ZN) and a fluorochrome procedure using auramine-O or auramine–rhodamine dyes. However it lacks sensitivity since a load of about 5,000 to 10,000 bacilli/mL of specimen is required to give a positive result after Ziehl-Neelsen staining. Before the HIV epidemic, AFB smear-negative TB was diagnosed in a smaller proportion of TB patients than it is today and was regarded as earlier or less severe disease. Patients diagnosed with smear-negative TB are less infectious and, prior to the HIV epidemic, had lower morbidity and mortality than smear-positive TB; for these reasons, smear-negative TB disease has been a lower priority for TB control efforts. In resource-limited countries, however, the proportion of pulmonary TB disease that is smear negative has increased over the past two decades, a shift largely attributed to the growing HIV epidemic. Moreover, in countries with a high prevalence of HIV, patients diagnosed with smear-negative TB have been shown to have higher mortality than patients with smear-positive TB, raising new questions and concerns about this form of TB disease (5).

In many sub-Saharan countries, the number of patients registered with SNPTB has increased. This may be due to several factors: co-incident HIV infection, poor diagnostic practices, false negative sputum smears, mistaking other respiratory infections or diseases for tuberculosis (6).

An increase in the annual number of notifications of all forms of tuberculosis (TB) has been seen in many low-income countries with high TB and HIV prevalence, and is attributed primarily to

the predisposing effect of HIV on TB. Associated with this increase in all forms of TB, the rate of increase in smear-negative TB has been greater than that of smear-positive TB in several low income countries (7).

Fluorescence microscopy increases the probability of detecting acid-fast bacilli, especially if the sputum contains few bacteria, and hence improves the sensitivity of microscopy in HIV-positive patients. The use of fluorescence microscopy in resource-constrained settings is limited by high costs because fluorescence microscopy is four to five times more expensive than light microscopy and the light bulbs must be replaced after 200 h of use. Other difficulties are the need for a reliable electricity supply and the presence of naturally fluorescent particles in sputum that can be confused with acid-fast bacilli. Advantages are that time needed to examine the smear is much lower and nearly15 times as many fields of view can be scanned in the same period (8).

Chest radiography is not always helpful in smear negative patients. The radiographic distinction between active and inactive tuberculosis can be difficult and appearance may be atypical due to other infections in HIV positive patients. In fact, substantial numbers of patients are treated for tuberculosis without definitive diagnostic criteria. With the advent of HIV associated tuberculosis with more frequent smear negative tuberculosis, the role of culture in TB control programs may need to be reassessed (9).

Almost 25% to 60% of patients with PTB have SNTB disease. The diagnosis of TB may be missed initially in 50% of these patients. This error may incur delays in treatment by 12.5 weeks until the return of positive culture results. The utility of rapid diagnostic tests in the early detection of smear-negative TB is, therefore, an important clinical consideration. It is also of considerable public health interest (10).

Much attention has recently been paid to the problem of smear-negative pulmonary tuberculosis. Quite appropriately, the discussion has focused on low-income countries, home to the vast majority of individuals with tuberculosis and HIV and where the ability to culture diagnostic specimens may be lacking. In particular, smear-negative pulmonary TB (SNPTB) has become an increasing important clinical and public health problem, especially in areas that are affected by the dual infection of TB and HIV, such as sub-Saharan Africa including Ethiopia (11).

1.2. Statement of the problem

According to WHO 2010 TB report, there were 9.4 million incident cases, 14 million prevalent cases, 1.3 million deaths among HIV-negative people and 0.38 million deaths among HIV-positive people. Most cases were in the South-East Asia, Africa and Western Pacific regions (35%, 30% and 20%) respectively. An estimated 11– 13% of incident cases were HIV-positive; the African Region accounted for approximately 80% of these cases (12).

With the sharp rise of PTB in countries which are worst affected by the HIV epidemics, the number of patients with suspected PTB who are sputum smear negative has increased. Out of the 5.7million cases of TB notified in 2008, approximately half of these were SNPTB cases which make it pertinent to consider the impact of SNPTB in our TB programmes. This observation reflects strong association between the two conditions but there is a possibility of misdiagnosis of other HIV-related respiratory infections as TB, probably due to limited use of the recommended guidelines by the clinicians or lack of other diagnostic modalities to exclude other opportunistic infections in resource-poor settings (13).

The global distribution of TB cases is skewed heavily toward low-income and emerging economies. The highest prevalence of cases is in Asia, where China, India, Bangladesh, Indonesia, and Pakistan collectively make up over 50% of the global burden. Africa, and more specifically sub-Saharan Africa, has the highest incidence rate of TB, with approximately 83 and 290 per 100,000, respectively. TB cases occur predominantly (approximately 6 million of the 8 million) in the economically most productive 15- to 49-year-old age group. Our understanding of TB epidemiology and the efficacy of control activities have been complicated by the emergence of drug-resistant bacilli and by the synergism of TB with HIV co-infection(14).

HIV has created an enormous impact on the epidemiologic and clinical features of TB worldwide, particularly in resource-poor countries. The epidemic has led to large increases in the frequency of SNPTB, which has made laboratory-confirmed diagnosis of PTB difficult thus resulting in poor treatment outcomes and excessive early mortality compared with smear-positive disease (15).

1.2.1. Tuberculosis in Ethiopia

Ethiopia ranks ninth among the world's 22 high-burden countries with TB and one of the top five in Africa, with regard to the prevalence of TB. According to WHO 2010 Global TB Report, the country had an estimated 44,398 TB cases in 2009, with an estimated incidence and prevalence rate of 300 and 470 cases per 100,000 populations respectively. But According to the 2011 WHO global TB report, the incidence and prevalence of TB for the year of 2010 burden decreases to 261 and 394 cases per 100,000 populations respectively. Similarly case detection was 50% for all forms of TB. Among all new TB cases 30% were smear positive, 35% smear negative, 34% extra pulmonary and ,<1% smear unknown cases. In addition among re-treatment cases 2,259 (64%) were relapse case, treatment after failure 381(11%), treatment after default 478(13%) and 56,040 had both TB and HIV co infection. According to the Ministry of Health hospital statistics data, TB is one of the leading cause of morbidity and the fourth cause of hospital admission and the second cause of hospital death in Ethiopia (16).

Ethiopia's National TB and Leprosy Control Program began to implement Directly Observed Treatment, Short-course (DOTS) strategy for TB control in 1991. DOTS is a proven, cost effective strategy recommended by World Health Organization for countries with limited resources. While treatment is integrated into general health services and DOTS geographical coverage is 95 percent, due to the limited health infrastructure in the country, only approximately 60 to 70 percent of the population has access to DOTS services. The DOTS detection rate remains low, at 34 percent, compared with WHO's target of 70 percent detection. The limited diagnostic capacity for TB in the country remains a challenge to improving case detection rate (17).

In fact, substantial numbers of patients are treated for tuberculosis without definitive diagnostic criteria. With the advent of HIV associated tuberculosis with more frequent smear negative tuberculosis, the role of culture in TB control programs may need to be reassessed. In countries where resources are limited like Ethiopia, and where the use of chest X-rays may be inadequate due to the cost as well as atypical presentation found in HIV infected patients, clinical and/or laboratory characteristics which are able to identify smear negative but culture positive PTB are required. Diagnosis of SNPT is a difficult task, and in Ethiopia, the majority of these cases has been treated only on the basis of clinical and chest radiographic findings. Without a standardized

clinical work up, the misdiagnosis rates have been estimated as high as 35% to 52%. Clinical prediction rules and affordable laboratory methodologies, including more efficient sputum examination and improved culture techniques, need to be developed and/or formally assessed in order to build an effective diagnostic approach for SNPT in less developed countries. This was the leading motivation and aim of our study to evaluate the diagnostic performance of available clinical, radiographic, and laboratory factors in diagnosing smear-negative pulmonary tuberculosis and its prevalence.

1.2.2. Significance of the study

SNPT represents a problem both in terms of under and over diagnosis cases. In 2011, 5.8 million pulmonary tuberculosis cases were reported, including 2 million SNPT cases (World Health Organization 2012). However, the interpretation of these global figures is not straightforward since the proportion of SNPT among pulmonary tuberculosis cases fluctuates widely over time and across regions. In the African region, at country level, this proportion varies even between 3 to 63% (3, 17).

The problem of false positives is also important. In a reference centre in Ethiopia, more than 80% of cases of smear negative tuberculosis were not confirmed by culture, pointing to a high rate of over-diagnosed cases. Another study showed that up to 60% of cases diagnosed as SNPT did not have the disease when re-evaluated by an expert panel (18). Lack of sensitivity of solid culture media is an issue, but clinical diagnosis also lacks specificity (19) and numerous patients are receiving an unnecessary and potentially harmful treatment.

The diagnosis of TB disease in developing countries like Ethiopia relies mainly on symptom screening, chest radiography (CXR), and AFB sputum smear, which have a poor sensitivity. Sputum culture is not routinely available for smear-negative patients, especially at district-level public health institutions. Moreover, current data on the performance of clinical and radiographic finding in predicting AFB smear-negative PTB are still limited and inconsistent.

Therefore determining the prevalence of smear-negative PTB and assessing the diagnostic performance of available clinical and radiographic finding in diagnosing of smear-negative Pulmonary tuberculosis among TB suspected patients attending Adama referral hospital will helps increasing detection, monitoring , prevention, and control of SNPTB.

2. Literature review

In particular, SNPTB has become an increasing clinical and epidemiological problem, especially in areas that are affected by the dual infection TB/HIV e.g. sub-Saharan Africa. For example, a study which was done in Zambia found out that 43% of 72 HIV-positive patients with culture-proven pulmonary TB in Lusaka, were smear-negative compared with 24% of 37 HIV-negative cases (p-value = 0.003) (20).

Study done by, Mesfin et al (2005) found that non-adherence to the national diagnostic algorithm is a common problem in hospitals, contributing to the over-diagnosis of SNPTB in the districts. Of the 101 SNPTB patients, only 3 were diagnosed as per the national diagnostic criteria. The diagnostic algorithm used for PTB screening in Ethiopia was reported to be less sensitive and specific among HIV-infected suspects (21).According to study done by van Cleeff et al, (2005), restricting CXR for the diagnosis of smear-negative TB among smear-negative suspects, the proportion of over-diagnosis is as high as 23% (22).

Another study done by Getahun et al, (2007) chest radiograph can be normal in up to 14% of HIV-infected patients with sputum-culture-positive TB (23) .Studies conducted in South Africa, a country with a high prevalence of HIV infection, where screening with amoxicillin was followed, in the absence of a clinical response, by a course of erythromycin (broad spectrum antibiotic), found that this does improve diagnostic accuracy among SNPTB suspects with abnormal CXRs (24).

A study conducted in Zimbabwe, evaluated the three symptoms: cough, drenching night sweat and weight loss and they noted that the sensitivity and specificity were similar for HIV-positive and HIV negative participants, while the presence of symptoms in HIV-positive participants had a higher positive predictive value and a lower negative predictive value (25).
Siddiqi et al, (2008) did a study in Cuba, Peru and Bolivia over two years with refresher courses in between so as to improve the diagnostic care for patients suspected of TB. Their findings were that clinical audit was most effective in improving standards of care in Cuba, but this had mixed results in Bolivia and limited success in Peru. Limited improvements were in some cases due to lack of coordination between the laboratories and health centres, lack of resources, political interference and perceived patients' beliefs (26).

According study done by Range. N et al in Tanzania, of the 637 patients remaining, 127 (19.9%) had three negative ZN microscopy smear slides at the recruitment centre. Of these, 34 (26.8%) had a positive culture in the reference laboratory. In the reference laboratory, fluorescence microscopy was performed before the culture was initiated. Of the 127 patients who had three negative smears at the recruitment center, 104 (81.9%) had also a negative smear at the reference laboratory. Of these, 13 (12.5%) were culture positive. Twenty three patients who had three negative smears at the recruitment center had a positive smear at the reference laboratory and of these, 21 (91%) were culture positive (27).

Systematic review of studies that used fluorescence microscopy showed that on average, in comparison with Ziehl-Neelsen microscopy, fluorescence microscopy showed a 10% increase in sensitivity and 9% incremental yield, and this improvement was not affected by HIV status (28).

Another study done at Dar-es-Salaam Tanzania shows that Of the 413 samples smear negative pulmonary tuberculosis analyzed, 30.8% (127/413) were MTB culture positive of whom 66 (51.9%) were correctly treated with anti-Tuberculosis drugs and 61 (48.1%) were missed and did not get anti-Tuberculosis drugs. Of the 286 subjects with sputum culture negative, 107 (37.4%) were incorrectly treated with anti-Tuberculosis drugs. The diagnostic algorithm for smear negative pulmonary tuberculosis had a sensitivity and specificity of 38.1% and 74.5% respectively. The positive predictive and the negative predictive diagnostic value were 52% and 62.5% respectively (29).

Another study done in Malawi using a combination of investigations, Of 352 smear-negative PTB suspects assessed**,** TB was confirmed in 39% of smear-negative PTB suspects about to start TB treatment under programme conditions. Diagnoses other than PTB were made in 78 cases (22%), and no diagnosis was made in 39% of patients; 89% of the patients assessed were HIV-positive (30).

Study done on 399 HIV patients in Vietnam 390 had initial AFB-negative smears and 22/390 patients had positive cultures. Symptom screening missed 54% (12/22) of smear-negative TB cases. No clinical symptom showed a significant association with having a positive AFB sputum culture (31).

Study done in Cambodia on 881 HIV patients (smear negative= 776, smear-positive = 105) shows that the Overall prevalence of culture-confirmed pulmonary tuberculosis (PTB) was 17% (150/881, smear-negative = 62/150). For those with any positive culture, a smear-negative case was four times more likely to be mycobacteria other than tuberculosis (MOTT) than *Mycobacterium tuberculosis (32)*.

Epidemiological study of smear-negative pulmonary tuberculosis done in the United States, from 1993 to 2008, 159 121 cases of culture confirmed pulmonary TB were reported in the United States, of which 58 786 (37%) were sputum smear negative. The annual proportion of smear-negative TB cases over the study period ranged from 35% in 1993 to a high of 41% in 1997 (33).

Another study done in Ethiopia by Desta.k et al shows that 247/297 (83.2%) patents with suspected pulmonary tuberculosis have had a negative smear results for acid fast bacilli. Abnormal chest x-ray findings were observed in 196 (79.4%) patients. 43/247 (17.4%) patients whose smears were negative for acid fast bacilli found to be positive for mycobacterial culture. The Mycobacterium species identified were M. tuberculosis (n = 40) (93%) and non-tuberculous mycobacteria (n = 3) (7%)(34).

3. Objectives

3.1. General objective

- ✓ The aim of the study is to determine the prevalence of smear negative pulmonary tuberculosis using standard culture methods and clinical algorithm among PTB suspected patients at Adama Referral Hospital, Adama, Ethiopia

3.2. Specific objectives

- ✓ To determine the prevalence of smear negative pulmonary tuberculosis by using culture.
- ✓ To evaluate the diagnostic performance of available clinical and radiographic finding in diagnosing AFB smear-negative pulmonary tuberculosis.

3.3. Hypothesis:

- ✓ The yield of clinical and radiological diagnosis of smear negative pulmonary tuberculosis is same with culture for diagnosis of smear negative pulmonary tuberculosis.

4. Materials and methods

4.1. Study site

The study was conducted in Adama referral hospital

4.2. Study design and period

A cross sectional study was conducted among suspected pulmonary tuberculosis patients visiting at Adama referral hospital in Eastern oromia Region from June 2013 to December 2013.

4.3. Source population

The source population was all patients who are suspected for TB at Adama referral hospital in Eastern oromia Region.

4.4. Study population

The study participants were all smear negative pulmonary tuberculosis patients at Adama referral hospital in Eastern oromia Region.

4.5. Sampling methods and Sample size determination

A consecutive sampling technique was used. The required sample size of the study population was determined using the formula for single population proportion. According to study done at **St. Peter's Tuberculosis Specialized Hospital, Prevalence of smear negative pulmonary tuberculosis** is 43/247 (17.4%) (34). so the sample size will be:

$$N = (Z\ \alpha/2)^2 * \frac{(1-p) * (p)}{(d)^2}$$

Where N = minimum sample size

Z α /2 = 1.96 at 95% Confidence Intervals (CI)

P = prevalence smear negative PTB

d= margin of error 0.05 at 95% CI

$N = (1.96)^2 * (1-0.174) * (0.174)$ divide by $(0.05)^2$,

N = 220, with 10 % contingency, N =242

4.6. Patient inclusion and exclusion criteria

4.6.1. Inclusion criteria:

Adult male and female patients aged greater than 18 years, presenting with chronic cough (≥2 weeks); who were three times sputum smear-negative for AFBs (ZN stain); and who gave a written informed consent to participate in the study.

4.6.2. Exclusion criteria:

Patients with known tuberculosis, on anti-TB for treatment or prophylaxis, have extra pulmonary; and confirmed smear positive pulmonary cases were excluded.

4.7. Study variables

4.7.1. Dependent variable: Prevalence of smear negative pulmonary tuberculosis

4.7.2. Independent variables: Age, occupation, sex, education, chronic cough, Night sweats, weight loss, Fever.

4.8. Sample collection

Sputum samples were collected from patients who had smear negative result by Zihel Neelson method/technique. During collection three consecutive sputum samples at least 5-10 ml collected in clean, sterile, leak-proof, screw caped wide-mouth, disposable containers (35). At the time of sample collection, a structured questionnaire was used to collect data such as Age, Sex, and other clinical presentation and history of patients.

4.9. Sample transportation

Sputum samples transported according to standard protocol that is the container sealed and packaged, labeled, and transported in water-tight container (Ice box) (35). During transportation, each cup containing sputum samples were covered by plastic separately inside ice box that was obtained from Adama regional TB laboratory.

4.10. Sample digestion and decontamination

The sputum sample analyze for *M.tuberculosis* was decontaminated by N-acetyl L-cysteine sodium hydro-oxide method (NALC-NaOH) methods as stated in the Annex part (35).

4.11. Direct smear preparation

Smear was prepared by taking a small portion of the purulent part (select the cheesy, necrotic) with an applicator stick, and was smeared on a microscope slide and air dried. Similarly after culturing the growth was confirmed for the presence of Acid Fast Bacilli using standard procedure proceeds according to the Zihel Nelseen method stated in the Annex part (35).

4.12. Inoculation and Incubation of Culture media

Primarily condense moisture observes at the bottom of culture medium slants were removed before inoculation. Inoculation for primary isolation and identification of *M.tuberculosis was* run at Bio-safety Level two (BSL-2) cabinet using pipettes. Each slope inoculates 100 µl of the centrifuged sediment and distributed over the surface.

All cultures were incubated at 35°-37°C until growth is observed or discarded as negative after eight weeks for primary isolation and identification. Inoculate media preferably be incubated in a slant position for at least 24 hours to ensure even distribution of inoculums. Thereafter, if incubator space is needed bottles placed upright. Tops should be tightened to minimize evaporation and drying of media (35).

4.13. Result Interpretation

4.13.1. Direct microscopy reporting

Smears were examined using a light/electrical microscope scanning 100 oil immersion fields before reporting a smear as negative or positive. Acids Fast Bacilli in specimens were red rods shaped, 1 to 10 micro meter long and 0.2 to 0.6 micrometer wide but they also appear coccoide or filamentous (long, slender, even branching) but back ground and other cells stained blue (35).

4.13.2. Culture reading

Egg based LJ was examined for growth twice a week for the first four weeks starting on day 3 post inoculation, thereafter once a week until the eighth week. All specimens showing growth in culture was confirmed as AFB by smear microscopy of the colonies and were reported immediately as “culture positive for *Mycobacterium* pending identification”. *M.tuberculosis* bacilli, in primary isolation, they hardly show any visible growth during the first week of culture. On egg-based media they produce characteristic non-pigmented colonies, with a general rough,

white creamy and dry appearance simulating breadcrumbs. Contaminated cultures and rapidly growing *Mycobacterium* (colonies yielded in less than 7 days) was removed and repeated sample processing from the sediment (35).

M.tuberculosis colonies were well developed within 3 to 4 weeks as white creamy appearance on LJ media and results reported immediately after detection and cultures were kept up to 8 weeks if no growth is detected at weekly examination (35).

4.14. Diagnostic algorithms for pulmonary tuberculosis(36)

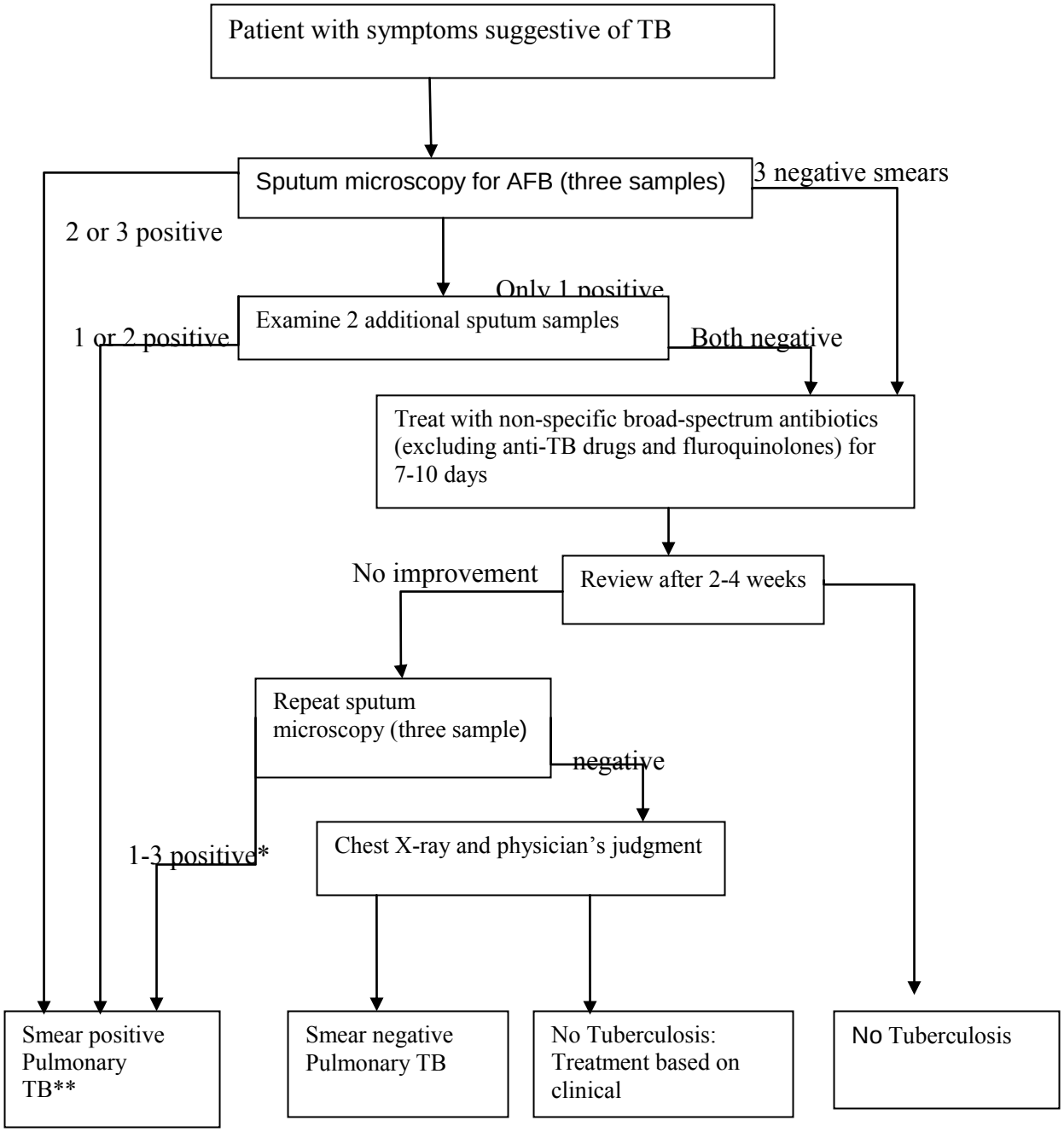

Figure 1: Diagnostic algorithms for pulmonary tuberculosis adopted from FMoHE,2008

* If initially all three smears are negative but after antibiotics ***only one*** repeated smear appears positive, it is advised to carry out two additional smears. If one or both are positive, proceed with

TB treatment. If both are negative, proceed with a chest Xray and evaluation for conditions other than TB.

** If the patient has never been treated before, register and treat as a new PTB smear positive patient. If the patient has been treated before, register for re-treatment regimen.

4.15. Quality control

Quality control for sample collection, transportation, digestion /decontamination, microscopy and culture was employed from Adama regional-TB laboratory procedures.

The sputum specimen was thick, mucoid and purulent, volume not less than 2 ml. All reagents, media (PH, colour, consistency) and other accessories prepared, used and stored in accordance with Standard Operation Procedure (SOP) used at adama regional laboratory TB laboratory.

4.16. Data management

Cross-checking and data cleaning was done and missing information was obtained by going back to the questionnaire and laboratory record logbook. All laboratory and clinical data will be recorded on a logbook during the study period and the data were also stored in a CD and USB device as a backup. The logbook and CD was stored in lockable shelves and only the investigator will have an access to the files. Dependent and Independent variable was entered into SPSS version 16

4.17. Statistical analysis

Data processing and statistical analysis were performed using the Statistical Package for Social Sciences (SPSS) software (Windows version 16.0).Descriptive statistics were computed. $P < 0.05$ was statistically significance. Finally multivariate analysis using logistic regression model was computed to know factors independently influence dependent variables smear negative pulmonary tuberculosis. Results were presented in the form of tables and graphs.

4.18. Ethical considerations

The research proposal was evaluated by the research and ethics committee of Department of Medical Laboratory Science and reviewed and cleared by Institution of Review Board (IRB) before the start of fieldwork.

Addis Ababa University/Health science collage, Department of Medical Laboratory Science was write official letter of co-operation to Adama referral hospital and Adama regional laboratory for the purpose of sample collection and laboratory testing. During data collection there were a high degree of confidentiality and informed consent also was obtained from each study subject, no name and other identifier on the questionnaire. At the end of the study, one copy of the study finding was submitted to Adama referral hospital and Adama regional laboratory and patients who smear negative but culture positive PTB are announce for the concerned body and treatment was given accordingly.

4.19. Dissemination of results

The findings of this study will be presented to School of Medical Laboratory Science and the result will be disseminated to the adama referral hospital. The finding will also disseminated to different organizations (governmental and non-governmental) that had contribution to improve and preventing the wide spread of pulmonary tuberculosis. Findings will be present in different seminars and workshops to disseminate and it may also be submitted to journal for possible publication.

5. Results

5.1. Socio –demographic characteristics

A total of 242 smear negative pulmonary tuberculosis patients were participated in this study. Of those, 5(2.1%) patients did not have final culture result because cultures were contaminated and 5 (2.1%) had MOTT results were excluded from further analysis. Of 232 AFB smear-negative patients, 130(56 %) were males. The mean age of the respondents was 40.2 ranging from 19 to 77 years old. Farming was the means of livelihood for most. Similarly 38.8 % (94) were illiterate. Of the respondents 59.1% (143) were Oromo and 54.4 %(131) were Muslim (Table 1).

Table 1: Socio –demographic characteristics of Smear Negative Pulmonary Tuberculosis among PTB Suspected Patients at Adama Referral Hospital, Adama, Ethiopia, February 2014.

Variable	Frequency	Percent (%)
SEX		
Male	**130**	**56**
Female	**102**	**44**
Occupation		
Farmer	**87**	**37.5**
Merchant	**73**	**31.5**
Governmental worker	**42**	**18.1**
Other	**30**	**12.9**
Living area (residence)		
Urban	**117**	**50.4**
Rural	**115**	**49.6**
Religious		

Christian	**94**	**40.5**
Muslim	**125**	**53.9**
Other	**13**	**5.6**
Age group		
19-30	**81**	**34.9**
31-40	**47**	**20.3**
41-50	**47**	**20.3**
>50	**57**	**24.6**
Ethnic group		
Oromo	**138**	**59.5**
Amhara	**52**	**22.4**
Tigray	**16**	**6.9**
Gurage	**24**	**10.3**
Other	**2**	**0.9**
Educational level		
Illiterate	**91**	**39.2**
Elementary	**70**	**30.2**
High school	**43**	**18.5**
College	**28**	**12.1**

5.2. Prevalence of smear negative pulmonary tuberculosis.

From A total of 242 smear negative pulmonary tuberculosis patients participated in this study, 5(2.1%) patients did not have final culture result because cultures were contaminated. Out of the remaining 237 patients, 28 (11.8%) were culture positive for MTB, 5 (2.1%) were MOTT results and 204 (86.1%) were culture negative for MTB at 8 weeks (Table 2). Among 242 AFB smear negative Patients, 5(2.1%) patients did not have final culture result due to contamination and 5 (2.1%) had MOTT results were excluded from further analysis. Of the 232 samples analyzed, 28 (12.1) were MTB culture positive.

Table 2: Culture results of Smear Negative Pulmonary Tuberculosis among PTB Suspected Patients at Adama Referral Hospital, Adama, Ethiopia, February 2014.

Variable	Frequency	Percent (%)
Positive	**28**	**11.8**
Negative	**204**	**86.1**
MOTT	**5**	**2.1**
Contaminated	**5**	**2.1**

5.3. Clinical characteristics and Chest X-ray finding of SNPTB patients

The majority of patients came with a combination of symptoms and the entire patient had chronic cough more than two weeks. The most frequently reported symptom production of sputum 198 (85.3%), fever 193 (83.2%), night sweet 137 (59.1 %), fatigue/tiredness 119(51.3 %), shortness of breath 118(50.9%) and unexplained weight loss 73(31.5 %). Of study participants, 44 (19.0%) had contact history with known positive PTB cases. of the study participants, 116(50.0%) had abnormal chest x-ray.

Among adults PTB suspected patients; smear-negative TB culture positive was less prevalent (10.7%) in age group 41-50 years. Majority (71.4%) of smear-negative TB culture positive was observed in male (Table 3).

Table 3: Prevalence of pulmonary tuberculosis (PTB) among sputum smear negative PTB suspect's patients, at Adama Referral Hospital, Adama, Ethiopia, February 2014.

Variable	Parameter	Culture positive/TB cases (n = 28)	Culture negative (n = 204)
Gender	**Males**	**20(71.4%)**	**110(53.9%) P = 0.08**
	Females	**8 (28.6%)**	**94 (37.0%)**
Age	**Mean (SD)**	**40.2(13.5)**	
Age groups	**19-30**	**9(32.1%)**	**72(35.3%)**
	31-40	**8(28.6%)**	**39(19.1%)**
	41-50	**3(10.7%)**	**44(21.6%)**
	>50	**8(28.6%)**	**49(24.0%)**

Using an unadjusted logistic regression model characteristics which predicts smear negative culture positive were determined. The binary- logistic regression identified that Productive cough (OR (95%CI)= 0.82(0.81-0.91)), Haemoptysis (OR (95%CI)= 2.43(1.04-5.68)), night sweats (OR (95%CI)= 6.85(2.00-23.39)), Unexplained weight loss (OR (95%CI)= 3.44(1.53-7.72)), the presence of typical X-ray Findings (OR (95%CI)= 2.81(1.18-6.68)), and being HIV patients or positive(OR (95%CI)=4.92(2.1-11.5)) were independently associated with confirmed SNPTB, as shown in Table 3. But sex, Fatigue/tiredness, Shortness of breath, Fever and Contact history not had any significant association with confirmed SNPTB ($p > 0.05$), as shown in Table 4.

Table 4: Unadjusted bi-variate logistic regression analysis of Socio-demographic, clinical and radiological variables in smear negative patients with and without pulmonary tuberculosis.

Variable	Total (n = 232)	PTB No (n = 28)	PTB (n = 204)	OR (95%CI)	P value
Male	130(56%)	20(71.4%)	110(53.9%)	2.14(0.9-5.07)	0.08
Productive cough	198(85.3%)	28(100%)	170(83.3%)	0.82(0.81-0.91)	0.02
Haemoptysis	48(20.7%)	10(35.7%)	38(18.6%)	2.43(1.04-5.68)	0.04
Night sweat	137(59.1 %)	25(89.3%)	112(54.9%)	6.85(2.00-23.39)	0.002
Fatigue/tiredness	119(51.3 %)	18(64.3%)	101(48.5%)	1.84(0.81-4.17)	0.15
Unexplained weight loss	73(31.5 %)	16(57.1%)	57(27.9%)	3.44(1.53-7.72)	0.003
Fever	193 (83.2%)	25(89.3%)	168(82.4%)	1.79(0.51-6.24)	0.36
Contact history	44 (19.0%)	7(25.0%)	37(18.1%)	1.52(0.6-3.8)	0.39
HIV infection +ve	39(16.8%)	12(42.9%)	27(13.2%)	4.92(2.1-11.5)	0.00
Abnormal CXR	116(50.0%)	8(28.6%)	108(52.9%)	2.81(1.18-6.68)	0.02

A multivariate regression model was developed from significant and clinically important covariates. Multivariate regression analysis found that Productive cough (OR (95%CI)= 0.82(0.81-0.91)), night sweats (OR (95%CI)= 8.27(2.27-30.1)), and being HIV patients or positive(OR (4.16(1.45-11.96)) were significantly associated with confirmed SNPTB, as shown in Table 5 .

Table 5: Variables significantly associated with smear negative pulmonary tuberculosis (multiple logistic regressions – final model)

Variable	Total (n = 232)	PTB No (n = 28)	PTB (n = 204)	AOR (95%CI)	P value
Productive cough	198(85.3%)	28(100%)	170(83.3%)	0.82(0.81-0.91)	0.02
Haemoptysis	48(20.7%)	10(35.7%)	38(18.6%)	2.64(0.94-5.68)	0.07
Night sweat	137(59.1 %)	25(89.3%)	112(54.9%)	8.27(2.27-30.1)	0.001
Unexplained weight loss	73(31.5 %)	16(57.1%)	57(27.9%)	1.38(0.5-3.77)	0.55
HIV infection +ve	39(16.8%)	12(42.9%)	27(13.2%)	4.16(1.45-11.96)	0.008
Abnormal CXR	116(50.0%)	8(28.6%)	108(52.9%)	0.41(0.16-1.07)	0.07

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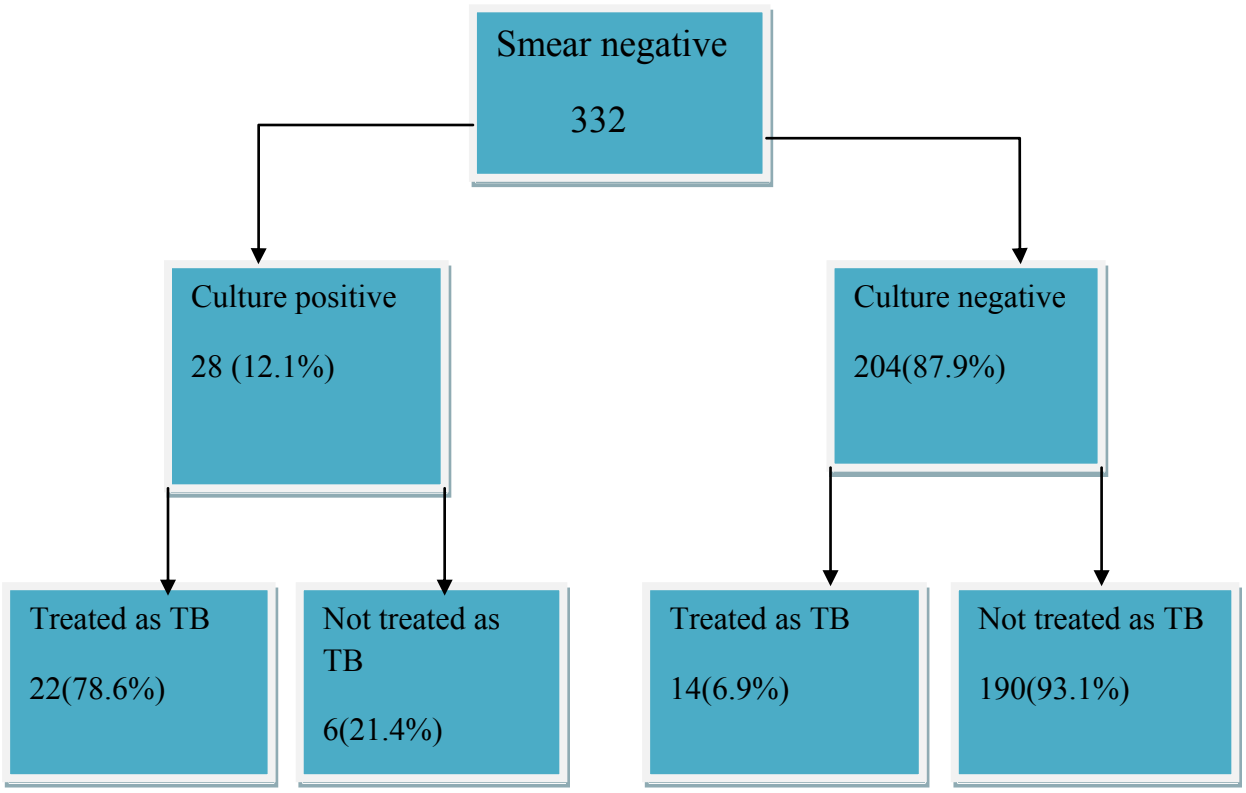

Figure 2: Study profile.*Treatment before culture results among sputum smear negative PTB suspect's patients, at Adama Referral Hospital, Adama, Ethiopia, February 2014.

6. Discussion

Despite the initial clinical suspicion of TB, when a patient's sputum smear results are negative for AFB, the diagnosis of TB may be missed. For those patients with a high clinical suspicion, clinicians must face the dilemma of empirically treating or waiting for up to 8 weeks for the final culture results. Clinicians may use criteria for smear- positive disease (*ie*, fever, productive cough, weight loss, night sweat) to predict risk in the patient with smear negative disease. But given the smaller mycobacterial burden present with smear-negative disease, these patients may have different clinical and radiographic findings (37).

Our study reveals that among smear negative PTB suspects, the prevalence of smear negative culture positive PTB was 12.1% (Table 1). Previous studies done among smear negative PTB patients documented comparable smear negative culture positive PTB prevalence among smear negative TB suspects; a study done United Kingdom showed a smear negative culture positive PTB prevalence of 15%(38), Ethiopia showed a smear negative culture positive PTB prevalence of 17.4% (34) and in Cambodia a prevalence of 9.9% was documented (32). Our finding was higher than in the previous studies done in Vietnam, (31)documented smear negative culture positive PTB prevalence of 5.6%, but lower than study done in Uganda (35.6%, 36.5%)(39,40) Peru (27.8%)(41) and in Tanzania (26.8%)(27). This may reflect the variations in the studied population and due to method variation.

The diagnosis of PTB in patients with negative smear by microscopy remains an important clinical problem in Low and Middle Income Country and often supplementary tests are used to improve the diagnostic accuracy for these patients (42). There is, however, no point of care diagnostics available with the sensitivity, specificity and timeliness needed for initial clinical management and most tests have limited benefit to the information obtained through a careful clinical history and examination. In our study, the clinical judgment of the clinician resulted in 79% of patients with SNPTB being correctly diagnosed with 93% specificity (figure 1).

A similar study done in Iran suggested that the clinical judgment of the clinician resulted in 73% of patients with SNPTB being correctly diagnosed with 95% specificity that consistent with our finding (38). But our finding was different from study done In South Africa, a setting with high HIV prevalence, an algorithm based on clinical judgment of the clinician and response to a trial

of antibiotics with amoxicillin and erythromycin in patients with negative smear microscopy had a sensitivity of 89% and specificity of 84% to identify patients with culture-positive TB (43).

In practice many patients with a diagnosis of SNPTB are not confirmed by culture, even if these are taken, in our study only 61.1% of cases of SNPTB are confirmed by culture that also similar in UK only 60% of cases notified are confirmed by culture and the proportion of cases with active TB with negative culture is poorly defined. By using this, clinicians would be able to identify patients with confirmed SNPTB. However, its relatively low specificity would result in an overall higher number of patients with unconfirmed sputum-negative patients being treated for TB.

Like other developing countries, the diagnosis of pulmonary tuberculosis in Ethiopia relies mainly on clinical screening, CXR, and sputum smear examination. While recommended by the World Health Organization (WHO) for diagnosis of AFB smear-negative PTB patients, sputum culture is still not routinely available at the district-level in Ethiopia (3). Some authors have suggested clinical symptoms can be significant predictors of smear-negative PTB (32), also in our study some clinical symptoms were significantly associated with smear-negative PTB in our population. Among available potential predictors, our data found that Productive cough (OR (95%CI)= 0.82(0.81-0.91)), night sweats (OR (95%CI)= 8.27(2.27-30.1)), and being HIV patients or positive(OR (4.16(1.45-11.96)) as being significant predictors for confirmed SNPTB patients having sputum AFB-negative smears (table 5).

In our study, we identify single symptom or combination of symptoms which could be a sensitive predictor for smear-negative, culture-confirmed PTB that consistent with study done in Iran that identify single symptom or combination of symptoms which could be a sensitive predictor for smear-negative, culture-confirmed PTB (38). But inconsistent with previous studies which failed to identify neither any single symptom nor combination of symptoms which could be a sensitive predictor for smear-negative, culture-confirmed PTB (31, 32,39). This inconsistent may reflect the variations in the studied population and due to method variation.

A similar study to our study in a setting of high TB prevalence suggested that smear-negative PTB cases were more likely to be confirmed by sputum culture as MOTT than in smear-positive PTB cases [32]. Our study found seven MOTT cases, which were distributed in the two groups of smear status; five in smear negative and two in smear-positive PTB. However, our sample size may not be powerful enough to evaluate the association between MOTT and AFB negative

smear status. Further studies with larger sample sizes should be conducted to investigate thoroughly this important issue. As MOTT and MTB have distinct prognoses and therapy (44), a better understanding on their diagnostic characteristics would be useful in developing clinical algorithms for their differential diagnosis, especially in low-resource settings.

7. Limitation of the study

- The Löwenstein-Jensen sputum culture technique used in our study may miss some PTB cases. Liquid-based culture technique such as the WHO approved MGIT should be deployed as a more rapid and accurate alternative for Löwenstein-Jensen technique where local resources allowed.
- The study was conducted at a single hospital and small sample size were used
- Some laboratory characteristics for diagnosis of smear negative pulmonary TB were not included.

8. Conclusions and recommendations

8.1. Conclusions

The present study reveals that among smear negative PTB suspects, the prevalence of smear negative culture positive PTB was 12.1%, but 87.6% smear negative pulmonary tuberculosis cases were still etiologically unexplained by culture.

Diagnosis of smear-negative PTB patients is a real challenge for clinicians where the sputum culture is not available. Our data showed the poor clinical performance of symptoms screening for AFB smear-negative PTB and confirmed the urgency of routinely applying sputum culture to improve the detection of not only smear-negative PTB but also TB disease in general. There is, however, no point of care diagnostics available with the sensitivity, specificity and timeliness needed for initial clinical management and most tests have limited benefit to the information obtained through a careful clinical history and examination. In our study, the clinical judgement of the clinician resulted in 79% of patients with SNPTB being correctly diagnosed with 93% specificity.

The current procedures of establishing AFB negative PTB are not sensitive enough to establish the diagnosis of active tuberculosis. They under-diagnose PTB and over treat people without PTB. The presence of a productive cough, night sweat and being HIV patients were found to be predictive of smear negative but culture positive PTB but poor diagnostic tool.

As no published data is available regarding MOTT in Ethiopia, the issue of differentiation between MOTT and MTB should be further investigated.

Future studies on more affordable, rapid, and accurate tests for TB infection would also be necessary to timely provide specific treatment for patients in need, reduce mortality, and minimize TB transmission to the general population.

8.2. Recommendations

Based on our results the following recommendations are forwarded:

- It is recommended that The Löwenstein-Jensen sputum culture and Liquid-based culture technique were used in combination to increase the detection rate of smear negative pulmonary tuberculosis.
- We recommended for future study on large sample size and including different laboratory factors which is very important for diagnosis smear negative pulmonary tuberculosis.
- We recommended that using sputum culture at the district level of the country to minimize under and over diagnosis of smear-negative tuberculosis and to increase the detection rate of smear negative pulmonary tuberculosis.
- . We recommended Future studies on more affordable, rapid, and accurate tests for TB infection would also be necessary to timely provide specific treatment for patients in need, reduce mortality, and minimize TB transmission to the general population.

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Annex I-Questionnaire

Questionnaire for investigation of prevalence of smear negative pulmonary tuberculosis among PTB suspected in adult patients at Adama Referral Hospital, Adama, Ethiopia.

Part 1. Patient Identification and demographic questions Date___/___/_____

101. Code No_____. Card No. _____

102. Hospital/ health centres No._____.

103.Address: wereda___ Keble _____ Tel ___

104.Age:_____

105. Sex: 1.M 2. F

106. Occupation? 1. Farmer 2. Merchant 3.governmental worker 4. Other_____(specify).

107. Living Area(**Residence)**: 1.Urban 2. Rural

108. Religious: 1. Christian 2. Muslim 3. Other _____(specify)

109. Educational level? **1.** Illiterate 2. Elementary **3.** High school **4.** College

110.Ethnic group: 1. Oromo 2. Amhara 3. Tigray 4. Gurage 5. Other ___ (specify).

Part 2. Clinical data (Symptom of TB,)

201.Chronic cough (>two weeks): 1. Yes 2. No

202.Production of sputum/cough: 1. Yes 2. No

203. haemoptysis(coughing up blood) : 1. Yes 2. No

204.Night sweats: 1. Yes 2. No

205.Fatigue/tiredness: 1. Yes 2. No

206.Shortness of breath: 1. Yes 2. No

207.Unexplained weight loss: 1. Yes 2. No

208.Fever: 1. Yes 2. No

209.Contact History with suspected Tb Patients: 1. Yes 2. No

210.If yes Q9 with whom 1.Family 2. Friends 3. Negioubrs 4.Other _____(specify)

211.chest X-ray finding

Normal : 1. Yes 2. No

Abnormal (state any)_____

212.Response to broad spectrum antibiotics if given for smear negative TB patients

1. Good response 2. No response

213.Antibiotic taken_____

Part 3 Laboratory request form (Laboratory data)

Name of Health Center/Hospital _____Date ___/____/_____

Patient's register number_____

Laboratory serial number _____ Date specimen received _____

301. Gross appearance of sputum

1. haemoptysis 2. purulent 3. muco-purulent 4. saliva

302. Direct microscopic examination of AFB result

Staining method: Ziehl-Neelsen

1. Not done 2. Negative 3. 1-9 AFB 4. 1+ 5. 2+ 6. 3+

Staining method

303. Culture results

Culture method _____

a. Not done b. Contaminated c. No growth d. 1+ c. 2+ d. 3+ e. 4+

Culture identification

Growth rate_____ Colony morphology _____

Culture identified as: Mycobacterium tuberculosis

: MOTT

Date _____ Signature _____

Annex II - Information sheet read to the respondents

My name is Adugna Belew, a master student of Addis Ababa University, Faculty of Medicine, Department of Medical Laboratory Science. The aim of the study is to determine the prevalence of smear negative pulmonary tuberculosis among PTB suspected patients at Adama Referral Hospital, Adama, Ethiopia.

The laboratory analysis will be conducted in adama regional laboratory, adama, Ethiopia. The study will be conducted through analysis of sputum sample that are negative for AFB by Ziehl Nelseon methods. The information you provide will be used to improve TB control and to design appropriate public health interventions for future. Your answers will not be released to anyone and will remain anonymous. Your name will not be written on the questionnaire or be kept in any other records.

Your participation is voluntary and you may choose to stop the interview at any time. Your participation or not do not have any influence for your service that you want to use. In addition in your participation in the study do not have any invasive procedure, only give three consecutive sputum samples as recommended by the health personnel and each questionnaire only take 10-15 minutes. At the end of the study the results of patients who smear negative but culture positive PTB are announce for the concerned body and treatment will be given accordingly. Thank you for your assistance.

Annex-III – Consent form prepared for study participants

I_____ here by giving my consent for giving three consecutive sputum samples as recommended by health personnel and to answer questions. I understand there is no serious invasive procedure at the beginning as well as at the end of the study. I understand this study will be used not only for me but also for other smear negative TB patients. All the information, which you are being, asked to provide in this questionnaire will be kept strictly confidential. And, will be used only for study purposes. The interview is voluntary and you have the right to stop the participation in the study at any time. However, your participation is important to full fill the study purpose.

Annex-IV: Laboratory test procedure

SPECIMEN COLLECTION

In tuberculosis diagnosis attention tends to be focused on the problems of microscopy, while an often overlooked problem is that of obtaining adequate specimens. Correct collection and transportation of specimens to the laboratory are important to ensure that results are accurate and reliable.

SPUTUM COLLECTION: *PROCEDURE*

- ✓ Give the patient confidence by explaining to him/her the reason for sputum collection.
- ✓ Instruct the patient to rinse his/her mouth with water before producing the specimen. This will help to remove food and any contaminating bacteria in the mouth.
- ✓ Instruct the patient to take two deep breaths, holding the breath for a few seconds after each inhalation and then exhaling slowly. Ask him/her to breathe in a third time and then forcefully blow the air out. Ask him/her to breathe in again and then cough. This should produce a specimen from deep in the lungs. Ask the patient to hold the sputum container close to the lips and to spit into it gently after a productive cough. Sputum is frequently thick and mucoid, but it may be fluid, with chunks of dead tissue from a lesion in the lung. The colour may be a dull white or a dull light green. Bloody specimens will be red or brown. Thin, clear saliva or nasopharyngeal discharge is not sputum and is of little diagnostic value for tuberculosis.
- ✓ If the sputum is insufficient encourage the patient to cough again until a satisfactory specimen is obtained. Remember that many patients cannot produce sputum from deep in the respiratory track in a few minutes. Give him/her sufficient time to produce an expectoration which s/he feels is produced by a deep cough.
- ✓ If there is no expectoration, consider the container used and dispose of it in the appropriate manner.
- ✓ Check that the container is securely closed and label the *container* (not the lid) clearly.
- ✓ Wash hands with soap and water.
- ✓ Give the patient a new sputum container and make sure that s/he understands that a specimen must be produced as soon as s/he wakes up in the morning.
- ✓ Demonstrate to the patient how the container should be securely closed.

- ✓ Instruct the patient to bring the specimen back to the health centre or laboratory.

SMEAR PREPARATION PROCEDURES

Sputum smear preparation

Smears should be prepared in manageable batches (maximum of 12 per batch). Labelling of smears should be done at the bench for incoming specimens using a permanent marker, eg. a diamond-point stylus or wax pencil. Avoid touching the surface of the slides.

The maximum chance of finding bacilli in unconcentrated specimens is in the solid or most dense particles of the sputum and the results of direct smear examination depend to a great extent on the choice of these particles.

Sputum smear preparation:PROCEDURE

1. Label a new, clean, unscratched slide at one end with the relevant patient number
2. Transfer an appropriate portion of the specimen to the slide by using an applicator stick (recommended) or bacteriological loop. Use blood-specked, opaque, greyish or yellowish cheesy mucus for smear preparation when it is present
3. Smear the specimen on the slide over an area approximately 2.0 by 1.0 cm. Make it thin enough to be able to read through it. *Do not make more than one smear per slide* (use the broken end of a wooden stick to smear the sputum)
4. Allow smears to air dry for 15 minutes. *Do not use heat for drying*
5. Fix smears to the slide by passing slides through a flame three or four times with the smear uppermost. Do not overheat. Allow to cool before staining
6. Discard the applicator stick in disinfectant or a biohazard receptacle, and use a new one for each specimen. Remove particles of adherent sputum from loops by moving it up and down through a flask containing sand and 70% alcohol. Flame the bacteriological loop thoroughly prior to re-use. The flame should be colourless or blue, because an orange or red flame is usually not hot enough

ACID-FAST STAINING PROCEDURES

Mycobacteria retains the primary stain even after exposure to decolorising acidalcohol, hence the term “acid-fast”. A counter-stain is employed to highlight the stained organisms for easier recognition. There are several methods of determining the acid-fast nature of mycobacteria. In

the carbolfuchsin (Ziehl-Neelsen) procedure, acid-fast organisms appear red against a blue background, while in the fluorochrome procedures (auramine-O, auramine-rhodamine), the acid-fast organisms appear as fluorescent rods, yellow to orange (the colour may vary with the filter system used) against a paler yellow or orange background.

Ziehl-Neelsen staining:PROCEDURE

1. Place the numbered slides on a staining rack in batches (maximum 12). Ensure that slides do not touch each other
2. Flood entire slide with Ziehl- Neelsen carbolfuchsin, which has been filtered prior to use, or cover each slide with a piece of filter paper if unfiltered carbolfuchsin is used
3. Heat the slide slowly until it is steaming. *Do not boil*. Maintain steaming for three to five minutes by using low or intermittent heat. *In no case must the stain boil dry*
4. If filter paper strips have been used, remove them with forceps. Rinse each slide individually in a gentle stream of running water until all free stain is washed away
5. Flood the slide with the decolorizing solution for a maximum of three minutes
6. Rinse the slide thoroughly with water. Drain excess water from the slide
7. Flood the slide with counter stain and Allow the smear to counter stain for 60 seconds
8. Rinse the slide thoroughly with water. Drain excess water from the slide. Allow smear to air dry. Do not blot

FLUOROCHROME STAINING

Fluorescence microscopy uses illumination from either a quartz-halogen lamp or a high-pressure mercury vapour lamp. The advantage of fluorescence microscopy is that a low magnification objective is used to scan smears, allowing a much larger area of the smear to be seen and resulting in more rapid examination. However, one drawback in using a low magnification is the greater probability that artifacts may be mistaken for acid-fast bacilli. It is therefore strongly recommended that suspect bacilli be confirmed at higher magnification, and that positive fluorochrome stains be confirmed by Ziehl-Neelsen microscopy.

Fluorochrome staining:PROCEDURE

1. Place the numbered smears on a staining rack in batches (maximum 12). Ensure that the slides do not touch each other
2. Flood entire smear with auramine O and allow staining for 15 minutes, ensuring that staining solution remains on smears. *Do not heat and do not use filter paper strips.*
3. Rinse with *distilled water* and drain. Tap water contains chlorine which may interfere with fluorescence
4. Decolorizes with 3% acid-alcohol for two minutes.
5. Rinse with distilled water and drain
6. Flood smears with either potassium permanganate or acridine orange and allow counter stain for two minutes. Time is critical with potassium permanganate because counterstaining for longer may quench the fluorescence of acid-fast bacilli
7. Rinse with distilled water and drain
8. Allow smears to air-dry. Do not blot. Read as soon as possible after staining

Precautions during staining

• Avoid under-de-colorization with acid-alcohol. Organisms that are truly acidfast are difficult to over-decolorize.

• Avoid making thick smears. This may interfere with proper de-colorization, and counter stains may hide the presence of acid-fast bacilli. Additionally, thick smears may flake, resulting in loss of smear material and possible transfer of material to other slides.

• Strong counter-staining may mask the presence of acid-fast bacilli.

• Smears that have been examined by fluorescence microscopy may be re-stained by Ziehl-Neelsen staining to confirm observations (recommended). However, once smears have been stained by Ziehl-Neelsen staining they cannot be used for fluorescence microscopy.

RECORDING AND REPORTING OF RESULTS

The microscopic observation must establish, first of all, if there are acidfast bacilli present in the smear and, if so, the approximate average number of these bacilli per microscopic field observed. It is recommended that a uniform pattern of reading is followed, observing 100 useful fields. A

useful microscopic field is regarded as one in which cellular elements of bronchial origin (leucocytes, mucuous fibres and ciliated cells) are observed. The fields in which there are no such elements should not be included in the reading.

Negative

Indicate the staining method. Report "negative for acid-fast bacilli" for all smears in which no acid-fast bacilli have been seen in 100 fields.

Positive

Indicate the staining method. The number of acid-fast bacilli found is an indication of the degree of infectivity of the patient as well as the severity of tuberculosis disease. Results should therefore be quantified. For *Ziehl-Neelsen stained* smears the following semi-quantitative method of reporting is recommended:

Table 6: Reporting method of direct microscopic examination of AFB

No of acid	fast bacilli Fields Report	No of acid
No AFB	per 100 immersion fields	No acid-fast bacilli observed *(No AFB per 100 fields)*
1-9 AFB	per 100 immersion fields	record exact figure *(1 to 9 AFB per 100 fields)*
10 to 99 AFB	per 100 immersion fields	1+ *(10 to 99 AFB per 100 fields)*
1 to 10 AFB	per field	2+ *(1 to 10 AFB per field in 50 fields*)
more than 10 AFB	per field	3+ *(More than 10 AFB per field in 20 fields)*

Quantification of fluorochrome smear results

When fluorochrome staining methods are used, smears are examined at much lower magnifications (typically 250x to 630x) than those commonly used for carbolfuchsin-stained smears (1 000x). Each field examined under fluorescence microscopy, therefore, has a larger area than that seen with bright field microscopy. Thus, a report based on a fluorochrome-stained smear examined at 250x may contain much larger numbers of bacilli than a similar report from the same specimen stained with carbolfuchsin and examined at 1 000x. To minimize confusion that conceivably could occur when different magnifications are used for smear examination and quantitative reporting of results, a method has been suggested whereby the number of acid-fast bacilli observed under fluorochrome staining could be divided by a "magnification factor" to yield an approximate number that might be observed if the same smear were examined under 1 000x after carbolfuchsin stain (see illustrations).

A simple table using the magnification factors enables reports to be comparable from laboratory to laboratory regardless of the stain or magnification used:

Table 7: Reporting method of fluorochrome smear results of pulmonary tuberculosis.

Carbol Fuchsin 1 000x	Report	Fluorechent microscopy magnification*		
		250x	450x	630x
0	No acid-fast bacilli seen	0	0	0
1-9 / 100 fields	Report exact count	Divide observed count by 10	Divide observed count by 4	Divide observed count by 2
10-99/100 fields	+1+2			
1-10/field				
>10/field	+3			

* To adjust for altered magnification of fluorescent microscope divide the number of organisms seen by the factor provided and refer to column 1 for range and column 2 for what to report.

CULTURE MEDIA

The definitive diagnosis of tuberculosis demands that *M. tuberculosis* be recovered on culture media and identified using differential *in vitro* tests. Many different media have been devised for

cultivating tubercle bacilli and three main groups can be identified, *viz* egg-based media, agar-based media and liquid media.

The ideal medium for isolation of tubercle bacilli should (a) be economical and simple to prepare from readily available ingredients, (b) inhibit the growth of contaminants, (c) support luxuriant growth of small numbers of bacilli and (d) permit preliminary differentiation of isolates on the basis of colony morphology.

For the culture of sputum specimens, egg-based media should be the first choice, since they meet all these requirements. There is increasing evidence that liquid media may give better results with other specimens. However, cost prevents their routine use with sputum specimens. It is recommended that all sputum specimens submitted for culture also undergo microscopic examination as outlined in the Technical Series on Microscopy.

INOCULATION AND INCUBATION PROCEDURES

Inoculation procedures

Condensed moisture is frequently observed at the bottom of culture medium slants. This should be removed before inoculation is attempted.

A common fault in inoculation is the use of too small an inoculum. Either loops (wire or disposable) or pipettes can be used for primary cultivation, although plastic Pasteur pipettes are recommended. Each slope should be inoculated with 0.2-0.4ml (2-4 drops or 2-4 loopfuls) of the centrifuged sediment, distributed over the surface. Fluid media can accommodate up to 1ml used for each specimen.

Two slopes of LJ medium should be inoculated per specimen.

Incubation of cultures

All cultures should be incubated at 35°-37°C until growth is observed or discarded as negative after eight weeks.

Inoculated media should preferably be incubated in a slanted position for at least 24 hours to ensure even distribution of inoculum. Thereafter, if incubator space is needed, bottles could be placed upright. Tops should be tightened to minimize evaporation and drying of media.

CULTURE EXAMINATION AND IDENTIFICATION

Examination schedule

All cultures should be examined 72 hours after inoculation to check that liquid has completely evaporated, to tighten caps in order to prevent drying out of media and to detect contaminants. Thereafter, cultures are examined weekly:

• After one week to detect rapidly growing mycobacteria which may be mistaken for *M. tuberculosis*

• After three to four weeks to detect positive cultures of *M. tuberculosis* as well as other slow-growing mycobacteria which may be either harmless saprophytes or potential pathogens

It is useful to label containers with cultures with the dates necessary for examination and to place containers in the incubator in chronological order. Should contaminated cultures be found during the examination, those where the surface has been completely contaminated or where medium has been liquefied or discoloured should be sterilised and discarded. Certain contaminating organisms produce acid from constituents of the medium and the lowering of pH unbinds some of the malachite green from the egg (indicated by the medium changing to dark green). Tubercle bacilli will not grow under these conditions and cultures should be discarded. Cultures with partial contamination should be retained until the eighth week. Late contamination does not exclude the presence of *M. tuberculosis*; it is therefore advisable to prepare a smear from the surface of the medium. Should microscopy indicate the presence of acid-fast bacilli, an attempt could be made to re-decontaminate and re-inoculate the culture.

Reading of cultures

Typical colonies of *M. tuberculosis* are rough, crumbly, waxy, non-pigmented (cream coloured) and slow- growers, ie. only appearing three weeks after inoculation.

For preliminary identification of tubercle bacilli the following characteristics apply:

• Tubercle bacilli do not grow in primary culture in less than one week and usually take three to four weeks to give visible growth

• The colonies are buff coloured (never yellow) and rough, having the appearance of bread crumbs or cauliflower

• They do not emulsify in the saline used for making smears but give a granular suspension

• Microscopically they are frequently arranged in serpentine cords of varying length or show district linear clumping. Individual cells are between 3μm and 4μm in length

RECORDING AND REPORTING OF LABORATORY RESULTS

Culture reports should be qualitative (ie. positive or negative) as well as quantitative (ie. number of colonies isolated). The *average number* of colonies on all the bottles/tubes per specimen should be reported. The following scheme is recommended.

Table 8: Reporting method of Culture results of pulmonary tuberculosis.

Reading	Report
No growth	Negative
1-19 colonies	Positive (number of colonies)
20-100 colonies	Positive (1+)
100-200 colonies	Positive (2 +)
200-500 colonies (almost confluent growth)	Positive (3 +)
>500 colonies (confluent growth)	Positive (4 +)
Contaminated	Contaminated

Waste management and other safety precautions

Used pipettes are collected inside the BSC in appropriate containers, metal or thermo resistant plastic bins, containing disinfectant (see SOP # 23). Test tubes with bacterial suspensions, if screw-capped tightly, can be sprayed with disinfectant and later be autoclaved as well as the pipettes. More or less open test tubes with suspensions in racks need to be tightly boxed before transfer to the autoclave. When tubes of solid cultures are discarded in solid containers (instead of autoclavable plastic bags), water with disinfectant should be added to the bottom of containers before autoclave. Otherwise steam may not be reach cultures and tubercle bacilli may be alive after a standard autoclave cycle. Gloves and other waste may be collected in an autoclavable plastic bag, which has to be closed and autoclaved.

Declaration

I the undersigned, declare that this thesis is my original work, has never been presented in this or any other university, and that all resources and materials used herein, have been duly acknowledged.

Name: **Adugna Belew (BSc, MSc candidate)**

Signature _____Date_____

Place: Addis Ababa University, Ethiopia

Date of submission:

This thesis has been submitted with my approval as a University advisor.

Name: Mr. Kassue Desta (**MSc, PhD fellow, Assistant professor**) Signature _____

Dr.Ibrahim Ali (**MSc, PhD, Assistant professor**) Signature _____