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COMPARISON OF COMBINATION OF BLEACH AND LIGHT EMITTING DIODE FLUORESCENT MICROSCOPY AGAINST MYCOBACTERIAL CULTURE FOR THE DIAGNOSIS OF TUBERCULOSIS AT ST.PETER'S TUBERCULOSIS SPECIALIZED HOSPITAL, ADDIS ABABA, ETHIOPIA

BY

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Comparison of combination of bleach and light emitting diode fluorescent microscopy against Mycobacterial culture for the diagnosis of tuberculosis at st. Peter's tuberculosis specialized hospital, Addis Ababa, Ethiopia

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List of abbreviations

AAU	Addis Ababa University
AFB	Acid-Fast Bacilli
AHRI	Armauer Hansen Research Institute
AIDS	Acquired Immune Deficiency Syndrome
AO	Auramine-O
BSL	Biosafety Level Laboratory
CDC	Center for Disease Control and Prevention
CSA	Central Statistical Authority
DOTS	Directly Observed Therapy Short course
DST	Drug Sensitivity Testing
EHNRI	Ethiopian Health and Nutrition Research Institute
FM	Fluorescent microscopy
FMOH	Federal Ministry of Health
HBCs	High-Burden Countries
HIV	Human Immunodeficiency Virus
IRB	Institutional Review Board
IUATLD	International Union Against Tuberculosis and Lung Disease
LED	Light Emitting Diode
LJ	Lowenstein-Jensen

MDG	Millennium Development Goals
MDR	Multi Drug Resistant
MGIT	Mycobacteria Growth Indicator Tube
MTBC	Mycobacterium Tuberculosis Complex
NaOCl	Sodium Hypochlorite
NTM	Non Tuberculosis Mycobacteria
OPD	Out Patient Department
PBS	Phosphate Buffer Solution
PTB	Pulmonary Tuberculosis
QC	Quality Control
SOPs	Standard Operating Procedures
TB	Tuberculosis
UV	Ultra Violet
WHO	World Health Organization
XDR	Extensively Drug Resistant
ZN	Ziehl-Neelsen

Abstract

Conventional light microscopy using Ziehl-Neelsen (ZN) stained smears prepared directly from sputum specimens is the most widely available test for diagnosis of tuberculosis (TB) in resource-limited settings. However, a major shortcoming of conventional microscopy is its low sensitivity compared with culture. A cross-sectional study in the outpatient departments of St. peter's TB specialized hospital in Addis Ababa, Ethiopia from May, 2010 to December, 2010 was conducted to compare the diagnostic value of the combination of bleach and light emitting diode fluorescent microscopy (LED-FM) against mycobacterial culture in the diagnosis of pulmonary tuberculosis (PTB) Adult patients with chronic cough, weight loss, sweats , fever, hemoptysis, chest pain with radiographic findings without treatment or on anti-TB chemotherapy were included in the study. Three consecutive sputum specimens from patients who fulfilled entry criteria were processed. Direct smear were prepared and stained using auramine phenol and Ziehl Neelsen techniques. Concentrated slides using bleach were stained by auramine-phenol method with culture on MGIT media as the gold standard. Capilia TB Assay method were used to identify M. tuberculosis.

Three hundrades and fifty three sputa were processed. The sensitivity, specificity, positive and negative predictive values achieved with direct fluorescent microscopy compared to culture were 59.7%, 98.3%, 94.4% and 83.6% respectively. The correlation between direct fluorescent microscopy and culture as gold standard method showed statistical significance ($\chi 2 = 159.78$, $P < 0.001$).Concentration of sputum with bleach significantly increased auramine/phenol smear sensitivity by 72.8%, a 13.1% incremental yield ($\chi 2 = 197.8$, $P < 0.0001$). A comparison of direct ZN and FM smear results showed that, direct FM sensitivity was significantly higher than direct ZN microscopy (59.7% vs 41.2%, difference 18.5%, $\chi 2 = 159.3$, CI 30.2 – 249.8, $P<0.0001$) but FM specificity was slightly lower (98.3% vs 100%). Sputum processing with bleach increased FM smear sensitivity by 31.6%, from 41.2% to 72.8%, which was remained significantly higher as compared with direct ZN microscopy ($\chi 2 = 204.17$, 95% CI = 37.64 – 209.2 $P<0.0001$) but FM specificity slightly decreased than direct ZN, from 97% to 100%. In this study it was shown that LED FM has correspondence with culture with decreasing number of bacilli. Low scanty results in fluorescence microscopy correlated more with culture than low scanty results found with the Ziehl-Neelsen technique. Symptoms like fever, cough and chest

pain were the common clinical findings among patient with suspected of having tuberculosis in our study. Weight loss was highly associated with development of PTB in both direct and concentrated flourochrome stained methods. Abnormal X-ray finding and positivity with both direct and concentrated fluorescent microscopy were highly associated. Therefore, the Auramine fluorochrome stain positive cases do not necessarily require to do X-ray examination as it saves time and money. According to the present study, combination of bleach and LED based fluorescent microscopy is sensitive and specific, easy to perform, inexpensive, rapid, diagnostic test for pulmonary tuberculosis.

Keywords/Phrases: *Light Emitting Diode Fluorescent Microscopy (LED-FM), Ziehl-Neelsen (ZN), Auramine O/Phenol, Sodium Hypochlorite (Household bleach). Mycobacterium tuberculosis. Mycobacteria Growth Indicator Tube(MGIT)*

CHAPTER 1: INTRODUCTION

1.1. Back ground

Tuberculosis (TB) is the leading cause of death from a curable infectious disease. It is a bacterial disease caused by *Mycobacterium tuberculosis*, and occasionally by other species of *Mycobacterium tuberculosis* complex, that includes *Mycobacterium bovis, Mycobacterium africanum, Mycobacterium canetti* and rarely by *Mycobacterium microti*. These organisms are also known as tubercle bacilli or Acid-Fast Bacilli (AFB) (Kumar *et al.,* 2007). Transmission occurs through airborne spread of infectious droplets. The source of infection is a person with TB of the lungs who is coughing (Horna-Campos *et al*., 2007).

The identification of infectious cases is a crucial first step for tuberculosis (TB) control programs worldwide. It relies exclusively on the detection of Acid fast bacilli (AFB) in sputum by smear microscopy, which continues to be the mainstay of diagnostic laboratories since its introduction in the late 19th century (Perkins, 2000).The target of a 70% case detection rate and 85% treatment success (WHO, 2002) are not likely to be achieved with the existing methods of smear microscopy. The greatest difficulty in diagnosing tuberculosis and other mycobacterial infections by sputum microscopy is lack of sensitivity (Matu *et al.,* 2007).

Efforts to maximize the yield and sensitivity of smear microscopy have led to changes in specimen collection, processing, and microscopy techniques (Mase *et al*., 2007; Steingart *et al.,* 2006; 2007). For developing countries with a large number of cases and financial constraints, evaluation of rapid and inexpensive diagnostic methods like demonstration of acid-fast bacilli (AFB) in fluorescent stained smears has great importance.

The development of fluorescent microscopy (FM) using auramine O staining has been shown to have a significant improvement over Ziehl-Neelsen (ZN) staining for use with light microscopy. The most important advantage of fluorescence microscopy is that it uses a lower power objective lens (typically 20-40×) than conventional microscopy (typically 100×), enabling the microscopist to assess the same area of a slide in less time. Fluorescent microscopy has been shown in numerous studies to be at least 10% more sensitive than traditional light microscopy with no

significant compromise in specificity (Steingart *et al.,* 2006; Ebersole, 1992). Thus, fluorescent stains are of paramount importance in confirming not only the presence of mycobacteria in a given specimen but also in providing an estimated quantification of organisms and has been proposed by some experts for use in countries with a high prevalence of TB-HIV co-infection (Toman, 2004; Kivihya-Ndugga *et al*., 2003).

The gold standard for Tuberculosis diagnosis is the cultivation of *M. tuberculosis*. It can be performed on a variety of specimens, such as sputum and bronchial washings, and also other non-pulmonary samples. It is much more sensitive than microscopy and it allows the recovery of the bacteria for other studies, such as drug susceptibility testing and genotyping (Schirm, 1995). Despite routine use throughout the developed world, TB culture remains unavailable in most high-burden countries, largely due to expense and infrastructure requirements (Apers *et al*., 2003; Hudson *et al*., 2000). Modern liquid media and accurate growth detection systems improve the sensitivity and greatly shorten the time needed for growth to be seen. The mycobacteria growth indicator tube (MGIT) is one of the most studied new culture methods (Huang *et al*., 2004). The mean time for detection of growth of mycobacteria in MGIT is short and ranged from 8 days to 16 days, including in HIV-infected tuberculosis patients, as compared with 20 days to 26 days in conventional culture (Lowenstein-Jensen) media (Diraa *et al*., 2003).

1.2. Epidemiology of Tuberculosis

1.2.1. Global Epidemiology

In 2009, there were estimated 9.4 million incident cases equivalents to 137 cases per 100,000 population of TB globally. The absolute number of cases continues to increase slightly from year to year. There were an increase from 9.27 million incident cases of TB in 2007, 9.24 million cases in 2006, 8.3 million cases in 2000 and 6.6 million cases in 1990. From the estimated 9.4 million new cases 3.3 million cases were women. Most of the estimated number of cases in 2009 occurred in Asia (55%) and Africa (30%); smaller proportions of cases occurred in the Eastern Mediterranean Region (7%), the European Region (4%) and the Region of the Americas (3%). The 22 HBCs that have received particular attention at the global level since 2000 account for 81% of all estimated cases worldwide (Figure 1).The five countries with the largest number of incident cases in 2009 were India (1.6–2.4 million), China (1.1–1.5 million), South Africa (0.40–0.59 million), Nigeria (0.37– 0.55 million) and Indonesia (0.35–0.52 million). India alone accounts for an estimated one fifth (21%) of all TB cases worldwide, and China and India combined account for 35% of TB cases worldwide. There were an estimated 14 million prevalent cases of TB in 2009 equivalent to 200 cases per 100,000 population (WHO, 2010a).

TB is a leading cause of death in HIV infected persons and HIV infection is the most potent risk factor for developing active TB disease from a latent TB infection. Of the 9.4 million incident cases in 2009, an estimated 1.0–1.2 million (11–13%) were HIV-positive, with a best estimate of 1.1 million (12%). Of these HIV-positive cases, 80% were in the African region and 13% were in the South-East Asia region. In 2009, an estimated 1.3 million deaths occurred among HIV negative cases of TB, including 0.38 million deaths among women. This is equivalent to 20 deaths per 100,000 population. In addition, there were an estimated 0.4 million deaths among incident TB cases that were HIV-positive (WHO, 2010a).WHO estimates that there were 440,000 cases of multi-drug resistant TB (MDR-TB) in 2008 and it was estimated that in 2009, 3.3% of all new TB cases had MDR-TB. Twenty seven countries (15 in the European Region) account for 86% of all MDR-TB cases (Figure 2).The four countries that had the largest number of estimated cases of MDR-TB in absolute terms in 2008 were China (100,000; range, 79,000–120,000), India (99,000; range, 79,000–120,000), the Russian Federation (38,000; range,30,000–45,000) and South Africa (13,000; range 10,000–16,000).

By July 2010, 58 countries and territories had reported at least one case of extensively drug-resistant TB (XDR-TB). Co-infection with HIV and MDR-TB led to reduced survival time and high mortality rates among patients. Recently, in Tugela Ferry, South Africa, an outbreak of XDR-TB predominantly affected people living with HIV and there were 52 deaths among 53 patients within an average of 3 weeks of being diagnosed (WHO, 2010b).

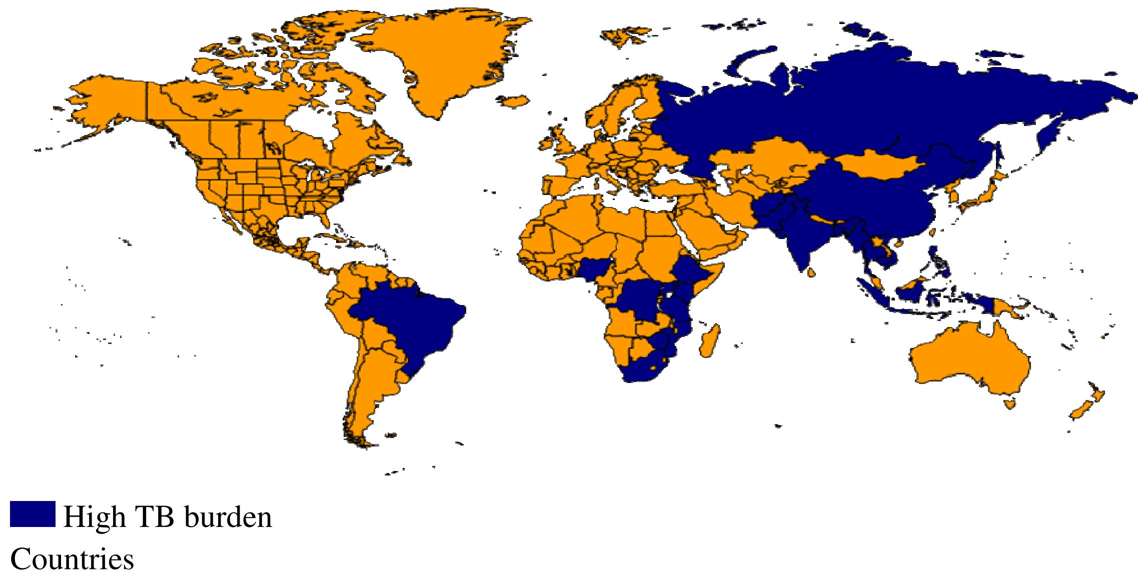

Figure.1: The 22 high TB burden countries adopted from: www. Global Health Facts.org, 2010.

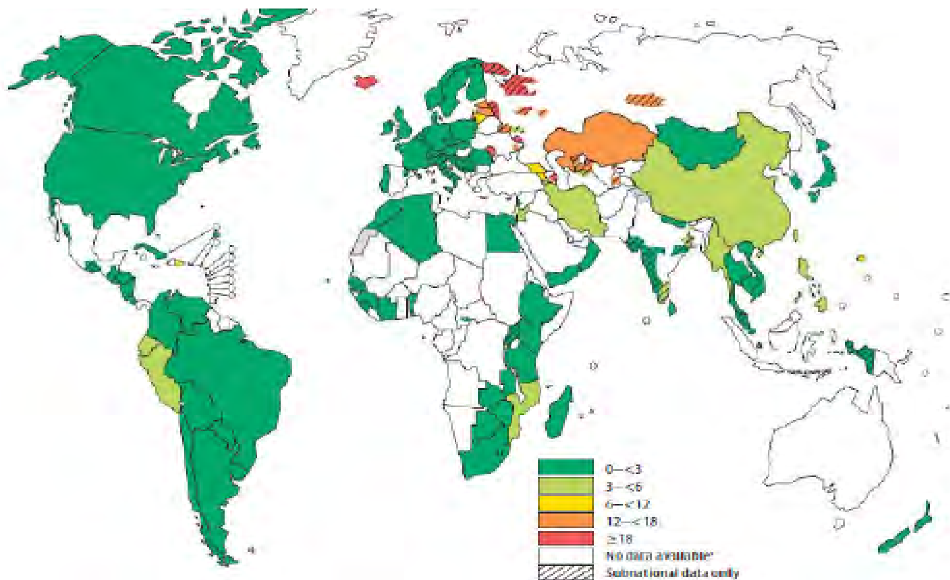

Figure.2: Multidrug and extensively drug-resistant TB (M/XDR) distribution: adopted from; 2010 global response on surveillance (WHO).

1.2.2. National perspectives

Ethiopia stands 7th in the list of the world's 22 high-burden countries for tuberculosis (TB) and third in Africa behind Nigeria and South Africa. According to the WHO estimate from a 2009 report, the prevalence of TB (including in HIV-positive) was estimated to be 572 and Mortality from tuberculosis (excluding HIV positive) was estimated to be 64 per 100,000 populations and the incidence of TB of all forms and smear positive TB stand at 359 and 163 per 100,000 populations per year respectively (WHO, 2009a).

Based on the Federal Ministry of Health (FMOH) hospital data, Tuberculosis is the leading cause of morbidity, the second cause of death and the third cause of Hospital admissions (After deliveries and malaria). According to the national 2009 Tuberculosis and Leprosy annual report, the total numbers of all forms of TB case reported were 156,928. The HIV epidemic has worsened the TB situation in Ethiopia and about 10,000 (15%) of registered and HIV-tested TB patients were found to be HIV positive (FMOH, 2009/10).

The emergence of Multidrug Resistant (MDR) TB is also another challenge faced by the country already overburdened by high prevalence of the disease. According to WHO 2010 report, Ethiopia stands 15th out of the 27 high-priority countries in the world and 3rd in Africa following South Africa and Nigeria with 5200 MDR TB cases estimated to have occurred in 2008. According to DST survey conducted nationwide in 2005 (EHNRI/FMOH), the prevalence of MDR-TB was 1.6% in newly diagnosed TB and 11.8% among previously treated TB cases. Currently, In order to validate WHO TB-related estimates for Ethiopia, the government of Ethiopia has been started to conduct a National TB Prevalence Study which will be finalized by the end of 2011.

1.3. Statement of the Problem

Tuberculosis (TB) is a global public health emergency, fueled by the spread of human immunodeficiency virus (HIV)/Acquired Immune Deficiency Syndrome (AIDS) and the emergence of drug-resistant strains of *Mycobacterium tuberculosis.* The World Health Organization has estimated the TB burden for Ethiopia, using a model based on a number of variables and parameters and uses their estimates to calculate incidence, prevalence and detection rates with an adjustment for the ongoing HIV epidemic.

In order to decrease the burden of TB, the Government of Ethiopia has exerted efforts since the 1960s to control TB using different approaches. The DOTS strategy has been incorporated in the TB Prevention and Control Program since 1992, The TB and Leprosy program was fully integrated into the general health services and TB/HIV collaborative activities started in 2004 and MDR-TB case management started in St Peter's TB specialized Hospital in Addis Ababa in February 2009 with a plan to scale up to 4-6 regional sites in the near future. Despite the above mentioned efforts, TB case detection is about half of the World Health Organization (WHO) global target, standing at 36% according to FMOH administrative report for the year 2009/10.

Sputum smear microscopy is the principal method of diagnosing pulmonary tuberculosis (PTB) in resource poor settings like Ethiopia, however the sensitivity of microscopy is influenced by numerous factors. Staff shortages combined with increasing numbers of requests for sputum microscopy are creating unmanageable workloads with negative effects on TB case finding and the poor sensitivity of light microscopy even under optimal conditions further aggravates this situation. (Kivihya-Ndugga *et al*., 2003; Sohn *et al*., 2009).The detection rate remains low as compared with WHO target of 70 % case detection. The limited diagnostic capacity for TB in the country remains a challenge to improving case detection rates. Therefore, there is a need of highly sensitive, specific and feasible technique for combating TB in Ethiopia and to achieve the target set for the Millennium Development Goals (MDG) by 2015.

This work is therefore, tried to compare Direct and concentrated Flurochrome auramine O phenol stained smears for the detection of *Mycobacterium tuberculosis* in comparison to culture of sputum samples from patients suspected to have pulmonary tuberculosis.

1.4. Review of the literature

Although much work is currently being conducted in order to develop new diagnostics, in most resource-limited countries, sputum smear microscopy remains the primary means for the diagnosis of TB. Given the known limitations of smear microscopy, considerable research has been conducted to identify methods that can increase the sensitivity and optimize its yield (Steingart *et al.,* 2007).

The acid-fast smear is less sensitive than culture which detects only about half of culture-confirmed cases in most settings. A single smear of a respiratory specimen has a reported sensitivity of between only 22 and 43%, and when multiple specimens are examined, the detection rate improves and as many as 96% of patient with PTB may be detected by acid fast smear examination .The sensitivity of sputum microscopy become lower where fewer bacilli are excreted in the sputum, as in children and human immunodeficiency virus (HIV) co-infected patients (Matu *et al.,* 2007). This has led to a need for more sensitive methods for sputum microscopy of PTB. This test must also be feasible in hospitals with limited resources and basic laboratory equipment (Gebre *et al.,* 1995; Bruchfeld *et al.,* 2000).

Smear from a non processed sputum specimen can be positive if the specimen contains >100,000 AFB/mL, but the sensitivity of this method can be increased to detect 10,000 and even 1,000 AFB/mL, if the specimen is concentrated through appropriate processing method and centrifugation, and the smear is examined by means of fluorescent microscopy (Matu *et al.,* 2007; Angeby *et al.,* 2004).

The gold standard for TB diagnosis is the cultivation of *M. tuberculosis*. It can be performed on a variety of specimens, such as sputum and bronchial washings, and also other non pulmonary samples. It can detect 100 bacilli/mL of sputum in comparison with 5,000–10,000 bacilli/mL needed for microscopy (Schirm, 1995). It also provides material for further identification of drug susceptibility testing and genotyping. *Mycobacterial* culture can be performed on conventional egg based solid medium such as Lowenstein-Jensen medium and agar based ones, such as Middle brook 7H10 or 7H11 and liquid media such as Middlebrook 7H9 broth.

The major constraints of culturing *mycobacteria* in conventional media results are often not available up to 6–10 weeks after sample presentation. Identification of *M. tuberculosis* is done by performing several further biochemical tests however; it is laborious and requiring additional time to obtain the results. It is therefore possible that treatment may be withheld from TB cases until the results are available. During this time patients may infect other individuals in the community while their condition also deteriorates (Kent; Kubica, 1985; Metchock *et al*., 1999).

The introduction of the BACTEC radiometric system (BACTEC TB-460; Becton Dickinson, Sparks, MD, USA) in the 1980s was a breakthrough since it allowed the detection of *M. tuberculosis* in a few days compared with weeks in the conventional culture media (Roberts *et al.,* 1983). However the use of radioactive material, cumbersome manual loading and unloading, potential hazard of needle stick injury, risk of cross contamination, and lack of computerized data management Precluded its use on a routine basis, except in reference laboratories predominantly in developed countries. Recently, the BACTEC Mycobacteria Growth Indicator Tube System (BACTEC 960/MGIT), a newly developed non-radiometric, fully automated, continuously monitoring system, was introduced as an alternative to the radiometric BACTEC 460 for growth and detection of mycobacteria (Hanna *et al*., 1995).

The Mycobacteria growth indicator tube (MGIT) system is based on a glass tube containing a modified Middle-brook 7H9 broth together with fluorescence quenching-based oxygen sensor embedded at the bottom of the tube. When inoculated with *M. tuberculosis*, consumption of the dissolved oxygen produces fluorescence when illuminated by a UV lamp. The MGIT system has been thoroughly evaluated in clinical settings for the detection and recovery of mycobacteria (Cruciani *et al*., 2004).

Study conducted by (Badak *et al*., 1996) compared the MGIT BACTEC TB-460 and LJ culture medium in 1,441 clinical specimens. Out of 178 isolates recovered, 30 (17%) were *M. tuberculosis* with the MGIT system recovering 28 (93%) compared with 25 (83%) recovered with the LJ medium. In another multicentre study, (Pfyffer *et al*., 1997) analyzed 1,500 clinical specimens detecting a total of 180 mycobacterial species comprising 113 *M. tuberculosis* complex isolates. The combination of MGIT and BACTEC detected 171 (95%)

of all isolates with a time to detection of *M. tuberculosis* of 9.9 days compared with 9.7 days with BACTEC and 20.2 days with solid medium proving that MGIT was a valuable alternative to the radiometric system.

More recently the MGIT system has been fully automated and turned into the BACTEC MGIT 960 system, which is a non radiometric, noninvasive system with the tubes incubated in a compact system that reads them automatically. In a multicentre study the BACTEC MGIT 960 system was compared with the radiometric BACTEC TB-460 system and LJ medium. Analyzing 2,576 specimens, the best yield was obtained with BACTEC TB-460 (201 isolates), compared with 190 isolates with BACTEC MGIT 960 and 168 isolates with LJ medium (Tortoli *et al.,* 1999).

In another study (Idigoras *et al*., 2000), compared the BACTEC MGIT 960 system for sensitivity and time to detection of mycobacteria with solid medium. Sensitivity of each media compared with all media combined for growth was 93%, 76% and 79% for MGIT 960, Middle-brook 7H11 and LJ.

Broth base culture systems are faster and more sensitive than egg-based media for detecting *Mycobacterium tuberculosis* complex (MTBC) and potentially pathogenic non-tuberculous mycobacteria (NTM) and recommended for use in high-burden settings of developing countries by the WHO, are being implemented in a phased manner, and integrated into a country-specific comprehensive plan for laboratory capacity strengthening (WHO, 2007).

A series of recent systematic reviews have demonstrated that microscopy can be optimized using at least three different approaches: chemical and physical processing (e.g. treatment with bleach or centrifugation), fluorescent microscopy (FM), and examination of two (rather than three) sputum specimens (Mase *et al*., 2007; Steingart *et al.,* 2006 and 2007). Fluorescent microscopy has several advantages over light microscopy using ZN staining and is widely used in most developed nations. First, the fluorochrome staining procedure used with FM is simpler than that of ZN staining. Second, it has been estimated, using meta-analysis, that FM has approximately 10% greater sensitivity for detecting acid fast bacilli in patient specimens. Third, and possibly most important, since FM can be examined at lower magnification than ZN (20 – 40 vs 100x); slides are read more quickly and efficiently with

FM. It has estimated that using FM may take up to 75% less time than ZN (Steingart *et al.,* 2006). This advantage would be of tremendous benefit for over burdened laboratory systems in many low resource settings. The fluorescent dye most widely used is auramine O, alone or in combination with rhodamine B. Established texts (Betty *et al*., 2002; Chapin and Lauderdale, 2003) and other sources state that auramine O, which is excited by blue (450–500 nm) light and emits across the region from around 500 nm to 700 nm (green–yellow–red), stains mycolic acids, and thus the mycobacterial cell wall. Consequently, microscopists search for bacillus-shaped objects that fluoresce homogeneously.

In fluorochroming, a direct chemical interaction occurs between the fluorescent dye and a component of the bacterial cell; interaction is the same as occurs with the stains used in light microscopy. But, a significant difference in the sensitivity of the two methods has been reported (Kumar *et al.,* 1998).

Light emitting diode (LED) technology has been developed over recent years to allow the benefits of fluorescent microscopy without the associated costs. In 2009, the evidence base for LED microscopy was assessed by the World Health Organization (WHO) following standards appropriate for evaluating both the accuracy and patient/public health impact of new TB diagnostics. Results showed equivalent accuracy of LED microscopy to international reference standards, improved sensitivity over conventional ZN microscopy, and qualitative, operational and cost advantages of LED relative to both conventional fluorescent and ZN microscopy (WHO, 2010c).

Several studies have been performed to assess the usefulness of adding a chemical reagent, such as sodium hypochlorite, to liquefy and then concentrate the sputum by further centrifugation or sedimentation to increase sensitivity. In most of these studies, a statistically significant improvement in the proportion of positive smears or sensitivity was obtained; however, for several reasons, sodium hypochlorite, also known as the ‘‘bleach’’ method is not used routinely in many settings (Angeby *et al.,* 2004). Available solvents which can be used for concentration techniques includes; sodium hypochlorite (household bleach), sodium hydroxide, N-acetyl-L-cysteine-sodium hydroxide solution, and ammonium sulphate and sodium hydroxide solution (Colebunders and Bastian, 2000).

A systematic review of 83 studies showed that studies that used sputum processing with chemicals including bleach and centrifugation yielded a mean 18% increase in sensitivity and an incremental yield (positives with bleach minus positives with Ziehl-Neelsen stain only) of 9%. Studies using bleach and overnight sedimentation showed a 6% mean increase in incremental yield (Steingart *et al.,* 2006). Studies in settings with high HIV prevalence showed that the addition of household bleach to sputum, followed by overnight sedimentation, increased the number of TB cases detected by up to 17 % (Gebre-Selassie, 2003; Yassin *et al.,* 2003).

In study conducted by (Laifangbam *et al.,* 2009) in India, showed that fluorochrome stain is more efficient over ZN stain in detecting TB bacilli in sputum, especially the paucibacillary cases. In their study they showed that the sensitivity of fluorochrome (AO) stain was 97.22% when compared against the golden standard culture technique. Similar study conducted by (Jain *et al.,* 2003), showed that the sensitivity and specificity of fluorochrome (AO) stain was 86.6% and 90% respectively when compared against the golden standard culture technique. Furthermore, they indicated that the fluorescing bacilli are easily identifiable and cause less eye-strain compare with conventional ZN-staining smears.

In study conducted by (Bahador *et al.,* 2006), on a comparison of direct and concentrated Fluorochrome-stained smears for the detection of *Mycobacterium tuberculosis* in clinical respiratory specimens , showed that the concentrated Fluorochrome-stained smears is more sensitive than direct microscopic examination. Another similar study on lymph aspirates by (Annam *et al.,* 2009), on comparison of the modified fluorescent method and conventional Ziehel-Neelsen (ZN) method in the detection of acid fast bacilli (AFB) indicated that the modified fluorescent method is more sensitive than the conventional ZN method. A study from Kenya showed superior sensitivity of fluorescence microscopy in comparison with bright field microscopy for low density smears (Githui *et al*., 1993), and fluorescence microscopy has proved at least as reliable as bright-field microscopy (Kubica, 1980).

CHAPTER TWO: OBJECTIVES

2.1. General objective

- To compare Direct and concentrated Fluorochrome auramine O stained smears for the detection of *Mycobacterium tuberculosis* in comparison to culture of sputum samples from patients suspected of having pulmonary tuberculosis.

2.2. Specific objectives

- To compare the performance of direct and concentrated LED Fluorescent microscopy with Ziehl-Neelsen techniques in the diagnosis of pulmonary tuberculosis.
- To evaluate the efficacy of fluorochrome stain in detecting paucibacillary pulmonary tuberculosis cases in comparison to Ziehl Neelsen stain.
- To correlate the clinical data with LED Fluorescent microscopy.
- To correlate the radiological data with LED Fluorescent microscopy.

CHAPTER THREE: MATERIALS AND METHODS

3.1. *Study area and population*

The study was conducted in St.Peter's TB specialized Hospital. It is owned by the government under the Federal Ministry of Health (FMOH) which is situated at central part of Addis Ababa, the capital city of Ethiopia. The hospital serves as a TB referral center for Addis Ababa and other regional states and also gives regular health services for inpatient and ambulatory patients. The hospital has Facilities for tuberculosis diagnosis, treatment and monitoring. Based on the 2007 Census conducted by the Central Statistical Agency of Ethiopia (CSA), Addis Ababa has a total population of 2,739,551 of whom 1,305,387 are men and 1,434,164 women and all of the populations are urban inhabitants (CSA, 2007).

3.2. *Study design and period*

A cross sectional study was conducted from May, 2010 to December, 2010.

3.3. *Study Subjects*

Patients who were suspected of having pulmonary tuberculosis and eligible subjects were those who agreed to participate in the study.

3.4. *Sample size and Technique*

Convenient sampling technique was used for this study and the sample size calculation was based on the average national TB prevalence in general population of 2005/06 .The crude prevalence of TB was taken as 29.8 % for convenience. The following single population proportion formula was applied to obtain the actual sample size with 10 % contingency and the total sample size for the whole study, therefore, was 353.

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

Where N = minimum sample size

$Z\alpha/2$ = 1.96 at 95% Confidence Intervals (CI)

P = National TB prevalence

d= margin of error 0.05 at 95% CI

3.5. ***Inclusion criteria***

Patients attending the St Peter's TB Specialized Hospital Out Patient Department(OPD), and having fever, night sweats, cough last for two or more weeks with sputum production , loss of appetite, loss of weight, chest pain, haemoptysis and/ or radiological evidence of tuberculosis were included in the study.

3.6. ***Exclusion criteria***

Those patients with known cases of carcinoma of lung, pediatric cases and patient unable to produce at least 10 ml of sputum were excluded from the study.

3.7. ***Clinical and Socio-demographic data collection***

Clinical and Socio-demographic data were collected from patients who were having fever, night sweats, cough last for two or more weeks with sputum, loss of appetite, loss of weight, chest pain, haemoptysis by the attending physician. After filling the consent form the patient were asked to give sputum samples which were sent to the laboratory for smear microscopy and culture.

3.7.1. ***Radiological examination***

The chest X-rays were taken for all suspected patient in anterior-posterior view by x-ray technician and were read and reported by the radiologist of St.Peter's TB specialized hospital. The X-ray was reported as normal, Upper lobe infiltrations (bi-lateral or uni-lateral right), Cavitation, Patchy, nodular shadows around the cavity.

3.8. Laboratory methods

3.8.1. *Sample collection*

Sputum specimens were collected from patients suspected of having pulmonary tuberculosis (PTB) attending St.Peter's Specialized TB Hospital, Addis Ababa. Three sputum samples were collected on two consecutive days from each patient - spot specimen on the first day, one early morning and one spot specimen on the second day for microscopy as per the current guidelines of tuberculosis control program and a volume of 10 ml morning sputum per patient for culture were collected in clean, sterile, leak-proof, wide-mouth containers.

3.8.2. *Direct smear preparation*

Two sets of direct smears were prepared from each sputum sample of the patient (spot-morning-spot) by taking a small portion of the purulent part of the sputum with an applicator stick, and smearing it on a microscope slide, dried in the air and fumed on a hot plate. One set for ZN and the other set for the auramine O phenol method (Smith wick, 1975).

3.8.3. *Concentrated smear preparation*

About l-2 mL of sputum were transferred to 50 mL screw-capped Falcon tubes and mixed with an equal volume of household bleach (5% NaOCl, Ghion Industrial-Chemical Sector PLC, Addis Ababa, Ethiopia). The mixtures were then incubated at room temperature for 10 min and vortexed at regular intervals. Then, equal amount of distilled water were added and centrifuged at 3000x*g* for 15 min. The supernatant were discarded and the pellets were suspended in a few drops of the remaining fluid. Fairly thick smears were prepared from the suspended sediment, air-dried and heat fixed (Wright., *et al* 1998).

3.8.4. *Direct ZN smears staining*

The prepared smears were placed on the staining rack and heat fixed then stained with 0.3% Carbol fuchsin, heated gently until steam rose, and left for 5 minutes, washed with gentle stream of water and flooded with 3% acid-alcohol for 1 minute, washed and flooded with 1% methylene blue for 1 minute (WHO,1998).

3.8.5. *Direct and Concentrated Flurochrome staining*

The prepared slides by both direct and concentrated methods were placed on a staining rack and heat fixed and stained with auramine-O phenol stain for 20 minutes, then rinsed briefly with gentle stream of water, flooded with 0.5% acid alcohol for 3 minutes then rinsed with water and counter stained with 1% Potassium Permanganate for 1 minute (Rieder *et al*., 1998; WHO, 1998).

3.8.6. *Examination and grading of fluorescent stained slides*

Fluorescent stained slides were examined by principal investigator using a light emitting diode (LED) fluorescent microscope (the Primo-star plus Transmitted-Light Microscope with an attached Epi-fluorescence illuminator optical microscope) with a 40× objective under a standard fluorescence UV filter viewing at least 40 fields. The tubercle bacilli were seen as yellow luminous organisms in a dark field and the results were graded and recorded in a defined manner as per the guidelines of International Union Against Tuberculosis and lung disease (IUATLD/WHO) scale. Smears were interpreted by the principal investigator and smear results were recorded before culture results were available (Appendix VI).

3.8.7. *Examination and grading of ZN stained slides*

ZN stained slide was examined for the presence of bacilli by using a 100x oil immersion objective, viewing at least 100 fields .AFB were seen as bright pink to red, beaded or barred forms where as the tissues cells and other organisms were stained blue. The results were graded and recorded in a defined manner as per the guidelines of International Union Against Tuberculosis and lung disease (IUATLD/WHO) scale. Smears were interpreted by the principal investigator, and smear results were recorded before culture results were available (Appendix VI).

3.8.8. *Laboratory procedure for sputum culture*

The sputum specimens were liquefied and decontaminated by modified Petroff's methods (Kent and Kubica, 1985). Equal amount of NaOH-NALC-sodium citrate solution were added to a volume equal to the quantity of Sputum and then vortex and incubated for 15 minutes. The tube then filled with sterile phosphate buffer solution (PBS) at pH 6.8 up to the top ring on the centrifuge tube (plastic tube has a ring for 50 ml mark) then concentrated by refrigerated

centrifuge at a speed of 3,000 x *g* for 15 minutes then allowed the tubes to sit for 5 minutes to settle aerosols. After centrifugation the supernatant was decanted and add a small quantity (1-2 ml) phosphate buffer (pH 6.8) and resuspended the sediment with the help of a vortex mixer. Then, the pellet was inoculated into a MGIT broth (contains 7 mL of modified Middlebrook 7H9 broth base supplemented with 0.8 ml of a mixture of polymyxin B, amphotericin B, nalidixic acid, trimethoprim, and azlocillin) then MGIT broths were incubated at 37°C in the BACTEC MGIT 960 system.

MGIT 960 system is an automated system for the growth and fast detection of mycobacteria with a capacity to incubate and continuously monitor 960 mycobacteria growth indicator tubes (MGIT) every 60 minutes for increase in fluorescence. Growth detection is based on the AFB metabolic Oxygen utilization and subsequent intensification of Oxygen quenched fluorescent dye contained in a tube of modified MGIT. A series of algorithms are used to determine presumptive positivity and alert the operator to the presence and location of positive tubes (Diraa *et al*., 2003).

The plates were examined when MGIT broths were flagged as positive and at the end of the incubation period. All broth cultures flagged by the MGIT 960 as positive were removed and a portion stained for the presence of AFB using the Ziehl–Neelsen method. Once confirmed as positive for AFB, the Capilia TB assay was performed for rapid identification of *M. tuberculosis* complex (Siddiqi *et al*., 2006). Specimens were handled with extreme care, adhering very closely to procedures and recommendations for controlling excessive media contamination. Culture tubes were inspected after 24 hours to identify contaminated tubes. Those identified contaminated MGIT tubes were removed the contents to a 50 mL falcon centrifuge tube and repeated the decontamination/digestion process with 4% NAOH solution and Inoculate into a fresh MGIT tube and placed in the MGIT instrument and followed for observation of growth.

3.8.9. *Capilia TB Assay*

The test was performed directly from instrument positive cultures after confirmed the presence of cording Acid fast bacilli (AFB) by using Ziehl–Neelsen method. Hundred microliters was taken by micro pipette from the MGIT tubes and dripped on on a broth culture placing area of the test plate. The colloidal gold labeled MPB64 antibody dissolve and formed an immune complex with MPB64 antigens in the specimen. This immune complex migrates through the developing area by capillary action and is captured by the anti-MPB64 antibody B (fixed

antibody B) fixed in the reading area. The resultant complex forms a purple red line of colloidal gold in the reading area. This indicates the existence of MPB64 antigens in the specimen, a protein secreted from the cells during the culture of an *M .tuberculosis* complex and it was reported that non-tuberculosis mycobacterium (NTM) produces no MPB64 antigen. The test provided results in 15 minutes and is highly sensitive (98.6%) and specific (97.9%) (Yamaguchi *et al*., 1986; Andersen, *et al*., 1991)

3.8.10. ***Safety Precautions***

Sputum processing, decontamination, inoculums preparation, inoculation to MGIT media, and slide preparations were performed in a suitable biosafety level III laboratories dedicated for mycobacterial work with negative air pressure and with an appropriate ventilation system. Accesses to the room were restricted when work was in progress, and proper protective gowns, gloves and respirator masks were used while handling specimens.

3.8.11. ***Quality control***

Internal Quality control was performed continuously to ensure precise and reliable operation by the BACTEC MGIT™ instrument tubes present in a row of MGIT drawer. When the QC failed for a row, any affected tubes in that row were automatically blocked by the instrument and were moved to a new, functioning row.

3.9. ***Data recording and analysis***

All laboratory and clinical data were recorded on a logbook during the study period. Each completed questionnaires were properly coded and key was prepared for each code. All data were double entered into an excel spreadsheet, cleaned, verified and then transferred for statistical calculation in STATA 10 (Stata Corporation, College Station, TX). The sensitivity, specificity, positive and negative predictive values including their 95% confidence intervals (CI) were calculated by using the sputum culture results as the "gold standard". Chi-square test was used to assess whether difference between values obtained are significant. All statistical tests were considered significant if the two sided P-value (p) was <0.05.

3.10. ***Ethical considerations***

The protocol was approved by the Department of Microbiology, Immunology and Parasitology (DMIP) and Institutional Review Board (IRB) Ethical Review Committees, College of Health Sciences, Addis Ababa University. Support letters had been obtained from the Federal Ministry of Health Bureau .The purpose of the study was clearly explained for each study participant. Written and oral consent was obtained from the subject prior to enrolment. The results of the study were communicated to the responsible physicians as soon as they had been confirmed of having tuberculosis and treatment were given free of charge to the patients according to Ethiopian national policy for tuberculosis management and all results were kept confidentially.

CHAPTER FOUR: RESULTS

4.1. Socio-demographic characteristics of the study population

In this study, a total of 353 pulmonary tuberculosis suspected patients were enrolled from May, 2010 to December, 2010 in St.Peter's TB specialized hospital. Socio-demographic characteristics such as age, sex and living area were obtained for all study subjects. Information regarding demographic characteristics of the 353 subjects were analyzed and summarized in Table 1. The patients were mainly young adults and the median age was found to be 29.5 years. The highest prevalence of tuberculosis suspected patients were observed in the age group of 25–34 years old. The minimum and maximum age was 15 years and 80 years respectively (range 15–80). There were 245 (69.4%) males, and 108 (30.6%) females with a ratio of 2.3:1. Relatively males were more often affected by tuberculosis than females (33.5% versus 29.6%). Among the 353 patients, 301 (85.3%) were living in urban while 52 (14.7%) were in rural area.

Table 1: ***Distribution of pulmonary tuberculosis suspected cases by age and sex*** (**n = 353**)

Age group (yrs)	Male n (%)	Female n (%)	Total n (%)
15-24	53 (15.0)	25 (7.08)	78 (22.0)
25-34	80 (23.3)	34 (9.63)	114 (32.3)
35-44	38 (10.8)	16 (4.53)	54 (15.3)
45-54	25 (7.1)	15 (4.25)	40 (11.3)
55-64	27 (7.7)	9 (2.55)	36 (10.2)
≥65	22 (6.2)	9 (2.55)	31 (8.8)
Total	**245 (69.4)**	**108 (30.6)**	**353 (100)**

4.2. Comparison of direct and concentrated fluorescent stained microscopy with culture

A total of 353 pulmonary tuberculosis suspected patients (1059 specimens) were enrolled; 72 (20.4%) were positive for direct fluorescent microscopy, 114 (32.3%) were culture positive. Of these, 68 (19.3%) were positive for culture and Fluorescent stained microscopy. Four (1.1%) specimens were fluorescent microscopy positive, but culture negative. The direct and concentrated fluorochrome stained smears results of 114 culture positive and 239 culture negative participants were compared (Table 2 and 3). The sensitivity, specificity, PPV and NPV achieved with direct fluorescent microscopy using culture as a gold standard were 59.7%, 98.3%, 94.4% and 83.6% respectively (Table 2**.** The correlation between direct fluorescent microscopy and culture as gold standard method showed statistical significance ($\chi 2$ = 159.78, $P < 0.001$).

Of the 353 pulmonary tuberculosis suspected patients 90 (25.4%) were positive for Fluorescent stained microscopy after concentrated with 5% house hold bleach. Out of these, 83 (23.5%) were positive for both culture and Fluorescent stained microscopy. Seven (1.98%) were fluorescent microscopy positive, but culture negative. Concentration of sputum with bleach significantly increased auramine smear sensitivity by 72.8%, a 13.1% incremental yield ($\chi 2$ = 197.8, $P <$ 0.0001). But specificity slightly decreases by 1.2% (P > 0.05), from 98.3% to 97.1% (Table **3**).The test efficiency of direct and concentrated fluorescent microscopy against culture results showed that 15.8 and 17% respectively. Calculated concentrated Flurochrome-stained smears Positive Predictive Value (PPV) and Negative Predictive Value (NPV) were 92.2% and 88.2%, respectively (Table 4).

Table 2: ***Test characteristics of the direct and concentrated fluorescent Microscopy against culture results***

	Culture results		
	Positive (%)	**Negative (%)**	**Total (%)**
Direct Fluorescent Microscopy			
Positive	68 (19.3)	4 (1.1)	72(20.4)
Negative	46 (13)	235(67)	281(80)
Total	**114 (32)**	**239 (68)**	**353(100)**
Concentrated Fluorescent Microscopy			
Positive	83 (23.5)	7 (1.98)	90(25.4)
Negative	31 (8.8)	232(66)	263(74.5)
Total	**114 (32)**	**239 (68)**	**353(100)**

Table 3: ***Sensitivity, Specificity, PPV, NPV and Test efficiency of direct and concentrated fluorescent microscopy against culture results.***

	DFM vs Culture (%)	**CFM vs Culture (%)**
Sensitivity	59.7	72.8
Specificity	98.3	97.1
PPV	94.4	92.2
NPV	83.6	88.2
TE	15.8	17.0

DFM = Direct Fluorescent Microscopy PPV= positive predictive value

NPV= Negative predictive value TE= Test efficiency

4.3. Comparison of direct ZN with direct and concentrated FM using culture as a gold standard

Table 4: Compares the patient-based yield of the various microscopy methods by culture result.

A comparison of the direct ZN and FM smear results showed that, direct FM sensitivity was significantly higher than direct ZN microscopy (59.7% vs 41.2%, difference 18.5%, $\chi 2 = 159.3$, CI = [30.2 – 249.8], $P < 0.0001$) but FM specificity was slightly lower (98.3% vs 100%, difference 1.7%). Sputum processing with bleach increased FM smear sensitivity by 31.6%, from 41.2% to 72.8%, which was remained significantly higher as compared with direct ZN microscopy ($\chi 2 = 204.17$, 95% CI = 37.64 – 209.2 $P < 0.0001$) but FM specificity slightly decreased than direct ZN, from 97% to 100%, a 3% decrease (Table 5).

Table 4: ***Comparison of patient results in Ziehl-Neelsen (ZN) and fluorescent microscopy (FM), by culture status (n = 353)***

Microscopy results		**Culture results**		
		Positive (n = 114)	**Negative (n = 239)**	**Total (n = 353)**
DZN	Positive (%)	47 (13.3)	-	47 (13.3)
	Negative (%)	67 (19)	239 (67.7)	306 (86.7)
DFM	Positive (%)	68 (19.3)	4 (1.1)	72 (20.4)
	Negative (%)	46 (13)	235 (66.6)	281 (79.6)
CFM	Positive (%)	83 (23.5)	7 (2)	90 (25.5)
	Negative (%)	31 (8.8)	232 (65.7)	263 (74.5)

DZN = Direct Ziehl-Neelsen DFM= Direct fluorescent microscopy

CFM= Concentrated fluorescence microscopy

Table 5: ***Comparison of direct ZN microscopy with direct and concentrated FM***

Percent	Direct ZN	Direct FM	Concentrated FM
Sensitivity	41.2	59.7	72.8
Specificity	100	98.3	97.1
PPV	100	94.4	92.2
NPV	78	83.6	88.2

PPV= Positive predictive value NPV= Negative predictive value

ZN= Ziehl-Neelsen FM = Fluorescent microscopy

4.4. ***Quantifications of AFB smears reported by the different techniques***

A total of 1,059 sputum specimens included in the study, 29.5% were salivary (contained no mucoid elements), 61% muco-purulent, 4.8% mucous and blood stained and 4.8% were not filled for sputum consistency. Table 6: Compares quantifications of AFB smears reported for the different techniques. Strongly positive (2+ and 3+) smears made up only 59.6% of DZN, but 50% of DFM and 68.9% of concentrated FM smears. Smears with 1+ made up 38.3%, 34.7% and 14.4% for DZN, DFM and CFM respectively. Smears with scanty AFB were rare with DZN (2.1%). However, smear with DFM and CFM showed (15.3%) and (16.7%) respectively.

Table 6: ***Distribution of quantified smear results by different technique***

	Percentage by technique		
Quantification grade	DZN (n = 47)	DFM (n = 72)	CFM (n = 90)
Scanty	1	11	15
+1	18	25	13
+2	12	20	25
+3	16	16	37

DFM= Direct fluorescence microscopy DZN= Direct Ziehl—Neelsen.

CFM=concentrated fluorescence microscopy

4.5. Clinical characteristics of the study subjects compared with direct and concentrated fluorescent Microscopy

Clinical signs and symptoms for tuberculosis were obtained from 98% of Pulmonary Tuberculosis (PTB) suspected patients. Cough was the most common presenting symptom of TB in this group. Among these 344 (97.4%) of the suspected cases had productive cough. Chest pain was present in 213(60.3%) followed by haemoptysis in 22 (6.2%). Constitutional symptoms like night sweat is present in 294 (83.3%), weight loss in 150 (42.5%) and fever present in 323 (91.5%) of the study subjects (Table 7). Weight loss was highly associated with development of PTB ($\chi2 = 49.8$, $P < 0.001$). Statistical analysis also revealed that there was a statistically significant association between haemoptysis and night sweat with direct fluorescent microscopy ($\chi2 = 9.16$, $P<0.01$) and ($\chi2 = 8.49$, $P < 0.014$) respectively. However, patients with fever and productive cough showed insignificant association between developing PTB and becoming positive with DFM ($\chi2 = 2.3$, $P < 0.319$) and ($\chi2 = 0.36$, $P< 0.84$) respectively.

After concentrated with bleach, weight loss were significantly more associated with development of PTB ($\chi2 = 61.5$, $P < 0.001$) and hemaptysis and night sweat were also showed to have a statistically significant association with development of pulmonary tuberculosis ($\chi2=7.5$, $P<0.024$) and ($\chi2 = 6.7$, $P< 0.035$) respectively. Fever and productive cough showed insignificant association with developing PTB even after concentrated with bleach ($\chi2 = 5.8$, $P < 0.055$) and ($\chi2 = 0.3$, $P < 0.861$) respectively.

Table 7: ***Clinical characteristics of the study subjects compared with direct and concentrated fluorescent microscopy*.**

Clinical Presentations	**Characteristics**	**DFM**		**χ2**	***P*-Value**	**CFM**		**χ2**	***P*-Value**
		Positive	**Negative**			**Positive**	**Negative**		
Fever	Yes	69	254	2.3	0.32	87	236	5.8	0.055
	No	2	21			1	22		
P. Cough	Yes	70	274	0.36	0.84	87	257	0.3	0.861
	No	1	1			1	2		
Chest Pain	Yes	65	148	34.3	0.001	82	131	49.8	0.001
	No	6	127			6	127		
Hemaptysis	Yes	10	12	9.16	0.01	11	11	7.5	0.024
	No	61	263			77	247		
Night Sweat	Yes	68	226	8.49	0.014	82	212	6.7	0.035
	No	50	3			6	47		
Weight loss	Yes	57	93	49.8	0.001	70	80	61.5	0.001
	No	15	188			20	183		

P.cough = productive cough DFM = Direct fluorescent microscopy CFM= Concentrated fluorescent microscopy

4.6. Comparison of chest radiography findings with direct and concentrated fluorescent stained slides

Chest radiography findings and results of direct and concentrated fluorescent microscopy were presented in Table 8 for all pulmonary TB suspected patients. From the total 353 pulmonary TB suspected patients, 142 (40.2%) of the cases had abnormal chest x-ray findings. Out of 72 (20.4%) direct fluorescent microscope positive slides, 69 (19.5%) of the cases had abnormal X-ray findings. From 90 (25.4%) concentrated fluorescent stained slides, 85 (24%) of the cases had abnormal X-ray findings. The most common reported chest x-ray findings were cavities, being present in 20% of patients with TB and 4% were with upper lobe infiltrations, 1.7% were patchy, and nodules was present in only 0.8% of all TB suspect and the rest (9.6 %) were diagnosed to have pneumonia.

The sensitivity, specificity, positive and negative predictive values were presented with results of direct and concentrated fluorescent microscopy in Table 9. The sensitivity, specificity, PPV and NPV of chest X-ray results were found to be 48.6%, 98.6%, 95.8% and 74% respectively. The association between DFM and X-ray finding were shown to have statistical significant ($\chi 2$ = 128.9, $P < 0.001$). Concentration of the sputum samples with 5% NaOCl increases the sensitivity from 48.6% to 59.9%, with incremental yield of 11.3% ($\chi 2$ =162, $P < 0.001$) with slight decrease in specificity (98.6% to 97.6%).The predictive value positive and negative were 94.4% and 78.3% respectively.

Table 8: ***Chest radiograph findings in pulmonary tuberculosis suspected patients (n = 353)***

Characteristics	**DFM**			**CFM**		
	Positive (%)	Negative (%)	Total (%)	Positive (%)	Negative (%)	Total (%)
X-ray findings						
Abnormal	69 (19.5)	3(0.9)	72 (20.4)	85 (24)	5 (1.4)	90 (25.4)
Normal	73(20.7)	208(58.9)	281(79.6)	57 (16.2)	206 (58.4)	263 (74.6)
Total	**142 (40.2)**	**211 (59.8)**	**353 (100)**	**142 (40.2)**	**211 (59.8)**	**353 (100)**

DFM = Direct fluorescent microscopy CFM= Concentrated fluorescent microscopy

Table 9: ***Sensitivity, Specificity, PPV and NPV of direct and concentrated fluorescent microscopy against X-ray findings.***

X-ray findings (%)	DFM	$\chi 2$	P-value	CFM	$\chi 2$	P-value
Sensitivity	48.6			59.9		
Specificity	98.6	128.9	0.001	97.6	162	0.001
PPV	95.8			94.4		
NPV	74.0			78.3		

DFM =Direct Fluorescent Microscopy CFM= Concentrated fluorescent microscopy

PPV= positive predictive value NPV= Negative predictive value

5. CHAPTER FIVE: DISCUSSION

In developing countries direct Sputum smear microscopy is simplest and available diagnostic method and has been the mainstay of tuberculosis diagnosis for the past 100 years and a key technique in the DOTS strategy. Bacilli-positive TB patients are the most potent sources of *M. tuberculosis* transmission in the community (American Thoracic Society, 2000; Beggs *et al*., 2003). Direct microscopic examination stained by ZN technique is the commonly used method for the diagnosis of TB (American Thoracic Society, 2000). Its sensitivity is, however, very low because ZN staining is unlikely to detect less than 5000 bacilli/mL of sample (Jenkins, 1994). Cultivation of the tubercle bacilli is the most sensitive method for the detection of *M. tuberculosis* and can detect 100 bacilli per ml of sputum (Kubica, 1980). Unfortunately, it can take several weeks to yield results and the routine implementation of the identification of *M. tuberculosis* by culture is very limited, especially in low-income countries. Therefore, there is a need of a simple and inexpensive method, which provides a valid alternative, to conventional method helpful in the diagnosis of TB. Treatment of sputum with sodium hypochlorite (NaOCl) and fluorochrome staining has been used to increase sensitivity of direct microscopic technique in many settings. However, no study has established the effect of NaOCl on LED-based fluorescent microscopy on the diagnosis of PTB.

We compared the performance of light-emitting diode (LED) based fluorescence microscopy techniques of both direct and bleach concentrated sputum sample in patients with symptoms of pulmonary TB by using MGIT 960 liquid culture system as the gold standard. In this study, the total sputum specimen studied were 353x3, from each clinically suspected pulmonary tuberculosis patients. These specimens were examined by direct and concentrated sputum microscopy by Auramine fluorochrome stain, Direct Ziehl-Neelsen stain and Culture.

The results from both direct and concentrated Fluorescent methods showed statistical significant. We found that the sensitivity of direct fluorescent microscopy was 59.7% ($\chi2 = 162.7$, CI: [30 – 249.8] $P < 0.001$). The sensitivity of Fluorescent microscopy increases from 59.7 to 72.8%, a 13.1% incremental yield ($\chi2 = 197.8$, $P < 0.0001$) after concentrated with 5% NaOCl. Results from this study also showed a high specificity of 98.3% regardless of NaOCl concentration. But specificity slightly decreases by 1.2%, after concentrated with 5% NaOCl solution. Our results

strongly suggest that the optimum detection of acid fast bacilli is achieved by the application of auramine/phenol fluorescent staining after digestion (liquefied) and concentrated samples.

Studies assessing fluorescent microscopy have shown more benefit with these techniques, one study conducted by (Bahador *et al.,* 2006), on a comparison of direct and concentrated Fluorochrome-stained smears for the detection of *Mycobacterium tuberculosis* in clinical respiratory specimens showed that the concentrated Fluorochrome-stained smears is more sensitive than direct microscopic examination. This is in agreement with our study. Another study determined the sensitivity of fluorescence microscopy after sputum treatment with bleach and the use of a polycarbonate membrane filter (Murray *et al*, 2003). In their study, they showed an increase in sensitivity after sputum treatment with auramine/phenol stain applied to a liquefied, concentrated sample is the most effective method for the demonstration of acid fast organism in sputum.

Bleach treatment would make microscopy safer and easier to perform and was shown in some study not to require high power centrifugation (Rickman and Moyer, 1980) with greatly increased yield, even after sedimentation. The major advantage of NaOCL method is helping to obtain the higher density of bacill per field and leaving free field for bacterial detection by reduction of debris, this facilitate the examination of the slides and the time required for microscopy. The only disadvantage is the possible increased risk of contamination for the laboratory personnel during the centrifugation, but if safety cabinets are available this risk can be reduced to an acceptable minimum and recently TB control program give considerable attention for bleach concentration technique and Fluorescent microscopy (Steingart *et al* 2006 and 2007). The use of sodium hypochlorite is ideal since it is cheap and available as household bleach, and also inactivates HIV and *M. tuberculosis* which could reduce the rate of nosocomial infections in laboratory workers (Rattan *et al.*, 1994). Although it required a slightly longer period of time for detection, sputum decontamination and concentration with 5% bleach procedure is simple and can easily be applied in a district laboratory with basic equipment and staff training. However, the technique is still not widely implemented for various reasons (Gebre *et al.*, 1995), and warnings concerning false positives and false negatives from bleach, and associated logistical challenges (Van Deun *et al.*, 2005).

In our study, we found that 4 (1.1%) specimens were direct fluorescent microscopy positive, but culture negative and seven (1.98%) were fluorescent microscopy positive after concentration of sputum samples but culture negative. This may be due to the following reasons; inorganic material that absorbs fluorochrome stains may on occasion be mistakenly identified as AFB (WHO, 1998). In addition, certain non tuberculosis organism may also be detected by fluorescent microscopy. False-negative results of the culture may be due to very low number of mycobacteria and/or the presence of inhibitors in the specimen (Klein *et al*., 1989; Pollock and Wieman, 1977).

We also compared the sensitivity, specificity, PPV and NPV of direct ZN versus direct and concentrated fluorochrome staining technique. Direct FM sensitivity was significantly higher than direct ZN microscopy (59.7% vs 41.2%, difference 18.5%, $\chi 2$ = 159.3, CI = [30.2 – 249.8], P<0.0001). Sputum processing with bleach further increased the sensitivity of FM smear to 72.8%, incremental yield of 31.6%, compared with direct ZN microscopy ($\chi 2$ = 204.17, 95% CI = [37.64 – 209.2], $P<0.0001$). Our results showed that the sensitivity of fluorochrome staining method remarkable when compared with the sensitivity of direct microscopy (ZN) in diagnosing pulmonary tuberculosis (Table 4 and 5)**.** This is in agreement with other studies (Prasanthi et *al*., 2005; Cheng *et al*., 2005; Sethi *et al*., 2003).

Study conducted by (Habeenzu *et al*., 1998) showed an increase of almost two fold in the sensitivity of AFB detection by flurochrome stain compared with conventional direct microscopy. The sensitivity of fluorochrome stain was also compared by Ulukanligil *et al*, 2000 and they found higher sensitivity in fluorochrome (85.3%) than ZN (67.6%) stain in HIV patients.

Many reports have showed that sensitivity of conventional microscopy ranged from 32% to 94%, and sensitivity of fluorescence microscopy ranged from 52% to 97%. A recent review confirmed that there is sufficient evidence of increased sensitivity of fluorescent microscopy compared with AFB conventional microscopy, without apparent loss of specificity (Steingart *et al*., 2006). On average, sensitivity in studies using culture as gold standard increased by 10%, while in those without the incremental yield over ZN was 9%. The average specificity of fluorescent microscopy was 98%, similar to that of conventional microscopy (Steingert *et al*., 2007). This is

also in agreement with our study. On comparison against ZN, the positive predictive value of the FM stain was lower than those of ZN even in direct microscopy and after concentration with bleach (Table 6).

The use of LED-based FM greatly improves the diagnostic value of the sputum smear especially in patients with a low density of bacilli that are likely to be missed on ZN stained smears. LED Fluorescence microscopy is recommended by the WHO to substitute the conventional FM since it is a useful, rapid, and reliable tool for the examination of specimens for AFB as compared with conventional fluorescent microscope and conventional ZN technique (WHO, 2010c). It should be seriously considered for use in laboratories that handle large numbers of specimens and in the area where there is large number TB and HIV infection.

Most recently, LED modules attached to standard light microscopes have been successfully applied in fluorescence-based screening of tuberculosis, pointing to the considerable reduction of related costs combined with increased safety and, as a consequence, to the potential for low-income countries to perform such advanced diagnostics of Tuberculosis in the near future. Since screening is done under lower power of magnification (200-400x), FM allows more-rapid screening of sputum smear specimens, particularly when high numbers of samples are screened per day. The tubercle bacilli stood out as bright objects against a dark background in fluorescence microscopy which makes them easily identifiable hence causing less eye-strain.

Fluorescence microscopy, while providing a more sensitive approach to TB microscopy, does have its limitations. A reliable source of electricity (AC power, 12V battery, etc) is needed for power, so in many isolated areas implementation of this technique would be difficult, at best. Initial costs are higher than for traditional ZN techniques, but costs would decrease with prolonged use. Fluorescence techniques do have an initial learning curve, as do all new methods, so there may be resistance to change on the part of microbiology staff who have been performing traditional Ziehl-Neelsen techniques for many years. In addition, fluorescence microscopy is not used to speciate mycobacteria, therefore in areas with high levels of atypical mycobacterial infections (non-TB), culture techniques should still be used to identify smear positive specimens accurately. Overall, these techniques do need a clean and dust-free environment, not only during use of equipment but also for storage of materials. Clear standard operating procedures (SOPs)

should be implemented as well, with which all laboratory personnel should be familiar (Armstrong, 2010).

In this study it was shown that LEDs FM has correspondence with culture with decreasing number of bacilli. Low scanty results in fluorescence microscopy correlated more with culture than low scanty results found with the Ziehl-Neelsen technique. Our result is comparable with study from Kenya (Githui *et al*., 1993). There was a significant increase in the average number of AFB seen per microscope field in the smears prepared after digestion and concentration by centrifugation. Smears which were graded l+ by the direct FM method increased to 2+ or 3+ after concentration with bleach (Table 6). The results of the smear microscopy were reported according to an internationally agreed (IUATLD/WHO) scale.

Symptoms like fever, cough and chest pain were the common clinical finding among patient with suspected of having tuberculosis in our study. Weight loss was highly associated with development of PTB in both direct and concentrated flourochrome stained methods. There was a statistically significant association between haemoptysis and night sweat with direct and concentrated fluorescent microscopy. However, in both direct and concentrated flourochrome stained methods patients with fever and productive cough showed insignificant association between developing PTB. There is no study specifically address the comparison of LED fluorescent microscopy with clinical signs and symptoms.

In our study, both direct and concentrated smears stained with Auramine fluorochrome stain were compared with chest X-ray. From the total 72 direct fluorescent microscope positive slides, 69 of the cases had abnormal X-ray finding and from 90 concentrated fluorescent stained positive slides, 85 of the cases had abnormal X-ray findings. However, 3 from DFM and 5 from the CFM were positive by FM, but had normal chest X-ray findings. These may be due to: FM may detect paucibacillary cases or other non-mycobacterial tuberculosis species, inorganic material that absorbs fluorochrome stains may be mistakenly identified as AFB and the reader's may fail to detect abnormal X-ray findings and interpret them correctly (Jain *et al.,* 2002). In a comparative study conducted by Khagi *et al*., 2009 between Auramine fluorochrome stain, Ziehl-Neelsen stain and Culture, showed that all the specimens positive by Auramine fluorochrome stain were also positive in chest X-ray report. This result is almost comparable with our findings.

In this study, in both direct and concentrated Auramine fluorochrome stained positive cases almost showed positive in X-ray findings. Therefore, the Auramine fluorochrome stain positive cases do not necessarily require to do X-ray examination as it saves time and money but some of the Auramine negative cases showed positive in culture.

6. Limitations of the study

1. The study area coverage is restricted to St. Peter's TB specialized hospital and it does not represent the whole city (Addis Ababa).
2. HIV testing was not included in the current investigation

7. Conclusions

- LED fluorescent microscope with auramine-phenol staining sensitivity in AFB detection was 59.7% when compared with gold standard culture technique. A combination of bleach (NaOCL) and LED fluorescent microscope with auramine/phenol staining improves detection of AFB by about 13.1%. Our results strongly suggest that the optimum detection of acid fast bacilli is achieved by the application of auramine-phenol fluorescent staining after digestion (liquefied) and concentrated samples.

- Fluorescent microscopy positive, but culture negative were also reported in both direct and concentrated methods and these may be due to the following reasons; inorganic material that absorbs fluorochrome stains. In addition, certain non tuberculosis organism may also be detected by fluorescent microscopy.

- Direct FM sensitivity was significantly higher than direct ZN microscopy 59.7% vs 41.2%, difference 18.5%. Sputum processing with bleach further increased the sensitivity of FM smear to 72.8%, incremental yield of 31.6%, compared with direct ZN microscopy.

- This study showed that LED FM has correspondence with culture with decreasing number of bacilli and correlated more with culture than low scanty results found with the Ziehl-Neelsen technique. There was a significant increase in the average number of AFB seen per microscope field in the smears prepared after concentration.

- Sign and symptoms like weight loss, haemoptysis and night sweat was highly associated with development of PTB in both direct and concentrated flourochrome stained methods. However, fever and productive cough showed insignificant association between developing PTB.

- Abnormal X-ray finding and positivity with both direct and concentrated fluorescent microscopy were highly associated. Therefore, the Auramine fluorochrome stain positive cases do not necessarily require to do X-ray examination, it saves time and money but some of the Auramine negative cases showed positive in culture.

8. Recommendations

- According to the present study, combination of bleach and LED based fluorescent microscopy is sensitive and specific, easy to perform, inexpensive, rapid, diagnostic test for pulmonary tuberculosis. This is especially true for laboratories in developing countries, where limited resources for usage of culture and molecular techniques for rapid detection of tuberculosis.

- LED fluorescent microscopy in tuberculosis endemic countries might be reasonably expected to improve tuberculosis case-finding by increasing sensitivity and expected decrease in time spent on microscopic examination. This would translate into quicker turnaround times for smear results and thereby potentially reduce patient drop-out from the diagnostic process.

- Additional study should be done for Extra Pulmonary tuberculosis, in HIV/AIDS patients, Pediatrics and smear negative cases.

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Appendix I-Questionnaire

This questionnaire form is intended to determine the effect of combination of Bleach and LED florescent Microscopy against Mycobcaterial culture for the diagnosis of tuberculosis in St. Peter's TB specialized hospital, Addis Ababa, Ethiopia. The study will be conducted through analysis of sputum samples from patients who are suspected of having pulmonary tuberculosis (PTB).

I. Patient Identification **Date___/___/_____**

1. Patient Name__________________ 2. Code No. _________

3. Hospital No________________ 4. Address______ (Tele.)_______

5. Age______________________ 6. Sex________________

7. Living Area: Urban________________Rural___________________

II. Clinical Data

8. Cough (≥ 2 Week) Yes ☐ No ☐

9. Productive cough Yes ☐ No ☐

10. Haemoptysis Yes ☐ No ☐

11. Night Sweat Yes ☐ No ☐

12. Fever Yes ☐ No ☐

13. Weight Loss Yes ☐ No ☐

14. Chest pain Yes ☐ No ☐

15. Chest X-ray finding

a. Normal ☐

b. Abnormal (state any) ____

III. Laboratory data

Date of sample collection _________day________ Month_________year

Time of sample collection 1st ___________ 2nd ____________ 3rd _________

Total no of sample received ______________

Results:

a) Completed ☐ b) Incomplete ☐ c) Excluded ☐

Action taken for the incomplete data___________________________ (please use additional blank paper if the space is not enough)

16**. Gross appearance of sputum**

Bloody ☐ Purulent ☐

Mucopurulent ☐ Saliva ☐

17**. AFB Results**

By principal investigator: ZN (Direct) positive 1+______2+_____3+____

FM (Direct) positive 1+______2+______3+______

FM (concentrated) positive 1+______2+______3+______

18**. Culture Result**

MGIT positive ☐ negative ☐

ZN stain from instrument positive Tube; positive ☐ negative ☐

19**. Capilia TB Neo Test (chromatographic test)**

Positive ☐ Negative ☐

***Date and signature of laboratory technician* ______________________________**

Comment:

____

____

Appendix II: Information sheet for study subjects (English version)

In this particular study we would like to use the sputum you give during study for research.

Principal Investigator: Nebiyu Gizaw

Addis Ababa University

Purpose: The purpose of this study is to compare a combination of bleach and fluorescent microscopy against mycobacterial culture for the diagnosis of tuberculosis in St Peter's TB specialized hospital from sputum samples.

Procedures to be carried on: you are invited to participate in the study after giving your consent by giving sputum samples for bacteriological analysis as the doctors find best for you and you will give 10 ml of sputum and have X-ray examination. We will do an investigation for *Mycobacterium Tuberculosis* at St. Peter's hospital laboratory. We will use the sputum only once for this particular study.

Risks associated with the study: There is no risk and serious invasive procedure at the beginning as well as at the end of the study and there is no additional time required from you to stay during study.

Benefits of the study: There will be no financial or other direct benefit to you. But the result of the study will play a role in the TB control program.

Compensations: There will be no compensation for using your sputum.

Confidentiality of your information: The results of the lab findings will be kept confidential and could only be accessed by the researcher and the responsible physician. There will be no personal information to be attached to your data.

Termination of the study: We will respect your decision if you later on change your mind. Your withdrawal of consent will not affect your right to receive medication.

Additional information: This study is approved by the department of microbiology, immunology and parasitology of the Medical Faculty, and IRB, Ethical Review Committees of Addis Ababa University.

Based on the above information I agree to participate in the research

Signature: ___________________ Date: __________________

If you have any question you can ask the following individuals

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Appendix III: Information sheet for study subjects (Ameharic version)

251-91-169 23 27

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Appendix IV. Consent form (English version)

Mr/Mrs/Miss____

My name is ________________________Having read/heard the information about the purpose of this study I would like to ask for your consent to participate in this study entitled "(*Comparison of Combination of Bleach and Fluorescent Microscopy against Mycobcaterial culture for the diagnosis of tuberculosis in Addis Ababa, Ethiopia*). I would like that you confirm your agreement by signing your name if you agree.

Signature of Study subject _____________________________Date_______________

Signature of the researcher _______________________________Date________________

Appendix V: Consent form for study subjects (Amharic Version)

[illegible]

[illegible]/[illegible]/[illegible] ____

[illegible] ____ [illegible]

[illegible]

[illegible] ______________________ [illegible] ____________

[illegible] ______________________ [illegible] ____________

[illegible] 1. ______________________ [illegible] ____________

2. ______________________ [illegible] ____________

Appendix VI: IUATLD/WHO recommended grading of sputum microscopy results

IUATLD/WHO scale (1000x field = HPF) Result	MICROSCOPY SYSTEM USED		
	Bright field (1000x magnification 1 length = 2 cm = 100 HPF	Conventional fluorescence (200-250x magnification 1 length=30 fields = 300 HPF	iLED fluorescence (400xmagnification; 1 length=40 fields = 200 HPF
Negative	Zero AFB/1 length	Zero AFB/1 length	Zero AFB/1 length
Scanty(actual count)	1-9 AFB/1 length or100 HPF	1-29 AFB/1 length	1-19 AFB/1 length
1+	10-99 AFB/1 length or100 HPF(1-9 AFB/10field	30-299 AFB/1 length	20-199 AFB/1 length
2+	1-10 AFB/1 HPF on average	10-100 AFB/1 Field on average	5-50 AFB/1 Field on average
3+	≥10AFB/1 Field on average	≥100 AFB/1 Field on average	>50AFB/1 Field on average

Appendix VII: ZIEHL-NEELSEN AND FLUOROCHROME STAINING REAGENTS PREPARATIONS

ZIEHL-NEELSEN STAINING REAGENTS PREPARATIONS

Carbol Fuchsin (3%)	**Quantity per liter**
Basic fuchsin	3.0 g
Denatured alcohol or methanol (95% ethanol) technical grade	100.0 ml
Dissolve basic fuchsin in ethanol	**Solution 1**

Phenol

Phenol crystals (technical grade)	50 g
Distilled water(purified water	850 ml
Dissolve phenol crystals in distilled water (gentle heat may be required)	Solution 2

Working solution

Combine 10 ml of solution 1 with 90ml of solution 2 and store in an amber bottle. Label bottle with name of reagent as well as preparation and expiry dates. Store at room temperature for six to twelve months and filter before use.

Decolorizing agent: 3% acid-alcohol

Concentrated hydrochloric acid (technical grade)	30ml

Alcohol ,95% ethanol (technical grade)	970 ml

Carefully add concentrated hydrochloric acid to 95% ethanol. Store in an amber bottle. Labelebottle with name of reagent and dates of preparation and expiry. Store at room temperature for six to twelve months.

Counterstain: Methylene blue (0.3%)

Methylene blue chloride	3.0 g
Distilled water	1000.0 ml

Dissolve methylene blue chloride in distilled water and store in an amber bottle. Label bottle with name of reagent and dates of preparation and expiry. Store at room temperature for six to twelve month.

FLUOROCHROME STAINING REAGENT PREPARATION

Auramine O (0.1%)

Auramine	1.0 g
95% ethanol (technical grade)	100 ml
Dissolve auramine in ethanol	Solution 1

Phenol

Phenol crystals(analytical grade)	30.0 g
Distilled water	870 ml
Dissolve phenol crystals in water	Solution 2

Mix solutions 1 and 2 and store in a tightly stoppered amber bottle away from heat and light. Label bottle with the name of the reagent and dates of preparation and expiry. Store at room

temperature for three months. Turbidity may develop on standing but this does not affect the staining reaction.

Decolorizing solution

Concentrated hydrochloric acid (37%)	5 ml
Denatured 95% ethanol (technical grade)	995 ml

Carefully add concentrated hydrochloric acid to the ethanol. Store in an amber bottle and labeled the bottle with name of reagent and dates of preparation and expiry. Store at room temperature for three months.

Counter stains

Potassium permanganate (0.5%)

Potassium permanganate (KMnO4) certified grade	5.0 g
Distilled water	1000ml

Dissolve potassium permanganate in distilled water in a tightly stoppered amber bottle. Label bottle with name of reagent and dates of preparation and expiry. Store at room temperature for three months.

Appendix VIII: PREPARATION OF REAGENTS FOR DIGESTION AND DECONTAMINATION OF SPUTUM

A. NaOH-NALC reagents

Preparation

• Prepare 4% NaOH solution by dissolving 4g NaOH pellets into 100 ml distilled/ deionized water. Sterilize by autoclaving. Concentration of NaOH may be varied (3- 6% NaOH solution at the beginning).

• Prepare 2.9% sodium citrate solution by dissolving 2.9 g sodium citrate (21120) in 100 ml distilled/deionized water. Sterilize by autoclaving.

Mixing

Prior to use, mix equal quantities of NaOH and sodium citrate solution. Prepare only as much volume as can be used in a day. Add NALC powder to achieve a final concentration of 0.5% (for 100 ml NaOH-Na citrate solution, add 0.5 g NALC powder). Mix well and use the same day. NALC activity is lost if left standing for more than 24 hours.

B. Sodium hydroxide solution

Prepare 4% NaOH solution by dissolving 4g of NaOH in 100 ml distilled/deionized water. Sterilize by autoclaving. This solution can be stored and used for decontamination of (nonmucoid) contaminated cultures and specimens.

C. Phosphate buffer (pH 6.8, 0.067 M)

• Dissolve 9.47g of anhydrous disodium phosphate (Na_2HPO_4) in 1000 ml (1 liter) distilled/deionized water, using a volumetric flask.

• Dissolve 9.07 g monopotassium phosphate (KH_2PO_4) in 1000 ml (1 liter) distilled/ deionized water, using a volumetric flask.

• Mix equal quantities of the two solutions. Check the pH. Adding more solution A will raise the pH; more solution B will lower the pH. The final pH should be 6.8.

• Sterilize by autoclaving.

Appendix IX: Reagents for MGIT 960 liquid Culture media

A. MGIT medium

The MGIT 960 tube contains 7.0 ml of modified 7H9 broth base. The approximate formula, per 1000 ml of purified water, contains:

- Modified Middlebrook 7H9 broth base
- 5.9 gm Casein peptone 1.25g

Adjusted and/or supplemented as required to meet the performance criteria. There is a fluorescent sensor embedded in silicone on the bottom of the tube. The tube is flushed with 10% CO_2 at the time of filling and then capped with polypropylene screw caps. Keep the caps closed until we are ready to make any addition to the medium.

B. MGIT growth supplement (enrichment)

MGIT growth supplement is provided for the BACTEC MGIT 960, 7 ml tube. For manual MGIT, a different enrichment (BBL MGIT OADC, 15 ml) is used. The enrichment was added to the MGIT medium prior to inoculation of a specimen. MGIT growth supplement contains 15 ml of the following approximate formula:

- Bovine Albumin -------------------50.0 gm
- Dextrose-----------------------------20.0 gm
- Catalase-------------------------------0.03 gm
- Oleic Acid--------------------------- 0.1 gm
- Polyoxyethyline state (POES) ---- 1.1 gm

MGIT growth supplement, or OADC enrichment, is a sterile product. Handled aseptically and were not used if turbid or if it appears to be contaminated and the growth supplement were in the biological safety cabinet to avoid contaminating the medium.

C. MGIT PANTA™

Each vial of MGIT PANTA (for MGIT 960) contains a lyophilized mixture of the antimicrobials with the concentrations, at the time of production, as follows:

- Polymyxin B-------------------6,000 units
- Amphotericin B---------------600 µg
- Nalidixic Acid------------------2,400 µg
- Trimethoprim---------------------- 600 µg
- Azlocillin--------------------------- 600 µg

2. Procedures

A. Reconstituting PANTA

Reconstitute MGIT PANTA with 15.0 ml MGIT growth supplement. Mix until completely dissolved. Add 0.8 ml of this enrichment to each MGIT tube. The enrichment with reconstituted PANTA should be added to the MGIT medium prior to inoculation of specimen in MGIT tube. Do not add PANTA/enrichment after the inoculation of specimen. Do not store MGIT tube after the addition of enrichment/ PANTA.

B. Inoculation of MGIT medium

• Label MGIT tubes with specimen number.

• Unscrew the cap and aseptically add 0.8 ml of MGIT growth supplement/PANTA to each MGIT tube. Use of an adjustable pipettor is recommended.

• Using a sterile pipette or a transfer pipette, add up to 0.5 ml of a well mixed processed/concentrated specimen to the appropriately labeled MGIT tube. Use separate pipette or pipette tip for each specimen.

• Immediately recap the tube tightly and mix by inverting the tube several times.

• Wipe tubes and caps with a mycobactericidal disinfectant and leave inoculated tubes at room temperature for 30 minutes.

• Work under the biologic safety cabinet for the specimen inoculation.

C. Inoculation of additional media

It is customary to use two different types of media for maximum recovery of mycobacteria. With the MGIT system, maximum recovery of mycobacteria may be achieved by using an additional solid medium, most commonly an egg-based medium such as LJ is used. The decision to use MGIT medium alone or in combination with an additional conventional solid medium should be made after reviewing each institution's own experience and requirements. Usually 0.1 to 0.25 ml of processed/concentrated specimen is inoculated onto solid medium.

D. Precautions

• One of the major sources of contamination in MGIT medium is environmental contaminants introduced during addition of growth supplement.

Appendix X. Declaration

I declare that (***Comparison of Combination of Bleach and LED Fluorescent Microscopy against Mycobcaterial culture for the diagnosis of tuberculosis at St. Peter's tuberculosis specialized hospital in Addis Ababa, Ethiopia***) is my own work, that it has not been submitted for any degree or examination in any other university, and that all the sources I have used or quoted have been indicated and acknowledged by complete references.

Name of the candidate: Nebiyu Gizaw

Signature -------------------------- Date -------/-------/---------- Place: Addis Ababa

This thesis has been submitted for examination with my approval as university advisor.

Name of the advisors:

Dr. Solomon G/selassie (MD, Msc)

Signature ------------------------- Date -------/-------/---------- Place: Addis Ababa

Kassu Desta (BSc, Msc)

Signature ------------------------- Date -------/-------/---------- Place: Addis Ababa

Adugna Abera (BSc, Msc)

Signature ------------------------- Date -------/-------/---------- Place: Addis Ababa