A Comparative Study of Direct LED Fluorescent Microscopy with Concentrated Ziehl-Neelsen Microscopy Techniques Against Culture for the Diagnosis of Pulmonary Tuberculosis At St. Merry Aksum General Hospital, Aksum, North Ethiopia

By Rigat Solomon(BSC)

A Thesis submitted to the collage of Health science of Jimma university , department of epidemiology in partiality fulfillment of the requirement for degree of master of public health

INVESTIGATOR:-

Rigat Solomon(EMPH, student ID SSMPH00812/04)

Address - phone n0 : 0912072825

- e-mail: rgiatsolomon@gmail.com

APRIL 2016

JIMMA University

A Comparative Study of Direct Fluorescent Microscopy with concentrated Ziehl-Neelsen Microscopy Techniques Against Culture for the Diagnosis of Pulmonary Tuberculosis At St. Merry Aksum General Hospital, Aksum, North Ethiopia

By: Rigat solomon (BSc)

Advisors:

1 Honok Asefa (BSc), collage Of Health science ,Department of Epidemiology , Jimma University.

2. Alemayehu Atomsa(BSc, MPH), collage Of Health science ,Department of Epidemiology , Jimma University.

A Thesis submitted to the collage of Health science of Jimma Universty , department of epidemiology in partial fulfillment of the requirements for degree of masters of public health

April 2016,

Jimma university

Abstract

Back ground:-*Conventional light microscopy using Ziehl-Neelsen stained smears from sputum specimens is widely available test for diagnosis of tuberculosis in resource-limited settings. However, a major shortcoming is its low sensitivity compared with culture.*

Objective:-*To compared efficacy of Direct LED fluorescence microscopy with concentrated Ziehl-Neelsen microscopy techniques against Mycobacterial culture of sputum sample*

Method:- The study design were *a cross-sectional , in the outpatient departments of St. Merry Aksum hospital, from September, 2015 to December, 2015 was conducted Three consecutive sputum specimens from patients who fulfilled entry criteria were processed. Direct smear were stained using auramine phenol and Concentrated slides using bleach were stained by Ziehl Neelsen techniques method with culture as the gold standard. Three hundrades and ninety four sputa were processed. The data were summarized and organized in tables and described with different descriptive measures. Associations were analyzed using* X^2 *– test and the kappa-test.*

Result:-*The sensitivity, specificity, positive and negative predictive values achieved with direct LED FM compared to culture were 71.59%, 98.03%, 91.3% and 92.3% respectively. Inter-test agreement between Direct LED FM and LJ culture systems determined by using the kappa-test were (Kappa (κ) = 0.754), and the observed agreement was found to be also statistically significant (p = 0.042).A comparison of concentrated ZN and direct FM smear results showed that, direct LED FM sensitivity was significantly higher than concentrated ZN microscopy (63.63% vs 71.59%,P<0.001) but FM specificity was slightly lower(98.3% vs 100%).*

Conclusion and Recommendation:- *our results strongly suggest that the optimum detection of AFB is achieved by the auramine-O fluorescent staining using Light Emitting Diode Fluorescent Microscopy the concentrated Ziehl-Neelsen And .Federal Ministry of Health and Tigray region health bureau in collaboration with other stakeholders should encourage further implementation LED based fluorescent microscopy. They should strengthen and support pulmonary tuberculosis prevention and control programs.*

Keywords/Phrases**:** *Pulmonary tuberculosis* (*PTB) , Light Emitting Diode Fluorescent Microscopy (LED-FM) , Ziehl-Neelsen (ZN) , Auramine O/Phenol , Sodium Hypochlorite (Household bleach) ,Mycobacterium tuberculosis, Lowenstein -Jensen(LJ)*

ACKNOWLEDGEMENT

First of all I would like to acknowledge Department of Epidemiology, Jimma University for organizing this opportunity to conducting the survey and for their contribution in providing the necessary information. I would like thank and appreciate the Aksum St. merry Hospital and Tigray regional laboratory for letting the use of its facilities. I gratefully acknowledge to the Regional Health Bureau for funding the study and for providing me such an ample information and supported with full cooperation and willingness. I am indebted to my supervisors for their close supervision at each and every step of the study, critical evaluation of the thesis proposal draft and immediate feedback with valuable comments that it given me a remarkable experience and exercising how to concern health problems. At last but not the least, I am indebted and gratefully acknowledged to all staff and patients participated in this thesis.

TABLE OF CONTENT

List of tables **pages**

List of figures **pages**

List of abbreviations

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
CPC	Cetylpyridinium chloride
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
NA	Not applicable
NALC	N-acetyl L-cysteine
NaOCl	Sodium Hypochlorite
NaOH	Sodium Hydroxide
NPV	Negative predictive value
NTM	Non Tuberculosis Mycobacteria

OPD	Out Patient Departmen
PBS	Phosphate Buffer Solution
PPV	Positive predictive value
PTB	Pulmonary Tuberculosis
QC	Quality Control
SOPs	Standard Operating Procedures
SDG	Sustainable Development Goals
TB	Tuberculosis
UV	Ultra Violet
WHO	World Health Organization
XDR	Extensively Drug Resistant
ZN	Ziehl-Neelsen

CHAPTER ONE: INTRODUCTION

1.1 Background

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)(1).Transmission occurs through airborne spread of infectious droplets. The source of infection is a person with TB of the lungs who is coughing(2).The diagnosis of TB infection is vital both clinically and epidemiologically. The target of WHO a 70% case detection rate and 85% treatment success (WHO, 2006(3) are, but not likely to be achieved with the existing methods of smear microscopy(6).

The greatest difficulty in diagnosing tuberculosis and other mycobacterial infections by sputum microscopy is lack of sensitivity (4) so that one of the Efforts to maximize the yield and sensitivity of smear microscopy have led to changes in specimen collection, processing, and microscopy techniques((5); (6); (7)).

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 newer alternative technique to Z-N smear microscopy, FM is known to increase the sensitivity (10% higher) when compared with Z-N microscopy methods while speeding up the whole process to consume much lesser time(8). The Recent development of simple FM systems based on light-emitting diodes (LED-FM) which have long life spans , do not produce UV light, and have minimal power requirements could facilitate the implementation of FM in high burden and limited resources countries(9). And also Fluorescent AFB can be seen at lower magnification than Z-N stained AFB and FM smears can be examined in a fraction(about 25%) of the time needed for Z-N smears as well. There is thus a definite need for alternative culture methods that would rapidly detect and identify mycobacteria, including their drug-susceptibility patterns , from clinical specimens (10).Culture on Lowenstein Jensen (LJ) medium remains the gold standard for the diagnosis of TB however the facility is not available on its full extent in developing countries (11). Hence required special procedures and need skilled workers. Culture on LJ medium is time consuming but cheaper than radiometric and molecular based techniques and a handy approach in the

diagnosis of TB in developing countries(12).Traditional or conventional methods for mycobacterial culture utilize media containing egg or potato base (Middlebrook 7H10 or 7H11) or albumin (Lowenstein - Jensen medium, LJ). Although these media support the growth of mycobacteria, several weeks (2-8 weeks) of incubation maybe necessary before the growth can be detected. This duration may further be prolonged in the case of paucibacillary specimens. Laboratory culture can be done which is considered as the gold standard test, but the recovery period of *M. tuberculosis* is long (6-8 weeks)(13).

1.2. Statement of the Problem

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(43). The Federal Ministry of Health (FMOH) 2010 report data shows that the TB case detection rates in Addis Ababa 63% and the lowest detection rates with 19% in Somali, 23% in Amhara and 25% in Tigray regions and the treatment success rates, Tigray (79%), Addis Ababa (72%), and Harar (64%) were average so the detection rate remains low, especially Tigray as compared with WHO target (31). 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 sustainable Development Goals (SDG).but According to those few previous studies are not adequate to show which one the best method finding on laboratory to improved TB case detection rate to goes to the high rate and also there is no study occurred in our county to compared about sensitive, specific and feasible technique between Direct LED fluorescence microscopy with concentrated Ziehl-Neelsen microscopy techniques to know which one the best. Because of this our county, especially Tigray Still there is a gap in the trend of case detection rates low on TB in health facilities.

1.3. Significant of the study

This study were improved service quality at all health facilities identifying major gaps on TB case detection rate on laboratory and scale up best performances from the evidences to be found from facility participated by proceed both method of Direct LED fluorescence microscopy in the diagnosis of pulmonary tuberculosis in comparison to Concentrated ziehl-neelsen microscopy from patients suspected of pulmonary tuberculosis by know which one is the best method to achieved the objective of detection rates of TB detection case .And the outcome of this study were hopefully used to design effective messages which one is the best method to used on health facilities and appropriate strategies design to use according highly sensitive, specific and feasible technique used on laboratory of health facilities for combating TB in our country and also as baseline information for the other detailed studies . So the Federal Ministry of Health and regional health Bureau were used the findings to further put in place service quality improvement actions on TB case detection and also this finding were help in the health facilities performance in improving on TB combating program by introduced the best method finding on laboratory to improved TB case detection.

CHAPTER TWO : REVIEW OF THE LITERATURE

2.1. Epidemiology of Tuberculosis

2.1.1. Global Epidemiology

In 2011, Tuberculosis(TB) incident cases were estimated 8.7 million new cases, among them approximately 1.1 million HIV positive new TB cases and 1.4 million deaths associated TB infection in 2011,so that tuberculosis (TB) remains a serious worldwide health problem and one of the leading causes of death from infectious diseases, especially in developing countries (30). In 2012, Tuberculosis(TB) incident cases an estimated 8.6 million people developed TB from those 0.5 million infected children and 2.9 million infected women and 1.3 million died from the disease .(27). And in 2013, Tuberculosis(TB) incident cases an estimated 9.0 million people developed TB and 1.5 million died from the disease and the prevalent rate was estimated 11 million prevalent cases(range, 10 million–13 million) of TB in 2013 ,equivalent to 159 cases per 100 000 population(14). TB in 2013, more than half (56%) of the developed TB were in the South-East Asia and Western Pacific Regions. A further one quarter were in the African Region, which also had the highest rates of cases and deaths relative to population. (14).But globally, the incidence rate was relatively stable from 1990 up until around 2000, and then started to fall and to achieving the MDG target ahead of the 2015 ,deadline each year between 2000 and 2013 and it is estimated that 37 million lives were saved between 2000 - 2013 and an average rate of decline per year was 1.5%(14).

2.1.2. National perspectives

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)(31).According to the national 2009 Tuberculosis and Leprosy annual report, the prevalence of all forms of TB is estimated at 261 per 100,000 population, leading to an annual mortality rate of 64 per100 000 so Ethiopia has the seventh highest burden of TB globally and ranks third in Africa(31).According to the 2011 Ministry of Health (MOH) report, TB is the eighth leading cause of hospital admissions and the third leading cause of hospital deaths in Ethiopia (32).According to the WHO global TB report 2011, there were an estimated 261 per 100,000 incident cases of TB and prevalence of TB was estimated to be 394 per 100,000 in Ethiopia in 2010(29). When we see to the FMOH 2011 report the estimated overall

bacteriologically confirmed prevalence of PTB in the year 2010/11 the national TB Prevalence estimate of 277 per 100,000 (32). And also the overall weighted prevalence (culture and/or smear) of PTB in the Tigray region of Ethiopia in 2010 was found to be 216 per 100,000 (33). Currently in 2012,Ethiopia has been stilll one of the highest TB burden countries; ranking among the top five in Africa with respective incidence and prevalence rates of 247 and 470 cases per 100,000 as WHO 2013 report (27). And when we come to the recent estimated epidemiological burden of TB in 2013,Ethiopia was found 224 and 435 cases per 100,000 in the respective of incidence and prevalence rates as WHO2014 report (14).

2.1. Clinically methods of Tuberculosis

The diagnosis of tuberculosis infection is vital both clinically and epidemiologically. A presumptive diagnosis is crucial to guide treatment, to limit the person to person spread of the disease and to assess the degree of activity of the disease. Acid-fast bacilli (AFB) microscopy, which is a means of detecting/screening of pulmonary tuberculosis, has been used worldwide as a mainstay of case finding(27).Prompt detection, isolation, identification, and susceptibility testing of *Mycobacterium tuberculosis* from clinical samples are essential for appropriate management of patients with TB(36).The current TB control strategy relies mainly on detection and treatment of active TB. In this framework, the use of a performing diagnostic method is crucial. Sputum smear microscopy, the most widely used test, is simple, rapid and inexpensive but it has a low sensitivity under field conditions (36).The sensitivity of microscopy is influenced by the quality of specimen collection, the number of mycobacterium present in the specimen, the method of processing, the staining technique, and the quality of the examination (37).To achieve maximum sensitivity of this diagnostic test, it is essential to have a good quality sputum sample, i.e. the sample should contain mucous or mucopurulent material and the volume should be at least 3 mL (38). Direct microscopy for AFB is widely used method for diagnosis and confirmation of pulmonary tuberculosis and when positive, defines the more infectious cases (39). This method is highly specific, faster and cheaper for detection of AFB in sputum. Acid-fast bacilli (AFB) microscopy, which is a means of detecting/screening of pulmonary tuberculosis, has been used worldwide as a mainstay of case finding (27) Conventional microscopy (CM) is inexpensive, rapid, and highly specific but has poor sensitivity, particularly in patients co-infected with HIV(7). For this limitation there are several drawbacks of this method ,First of all, ZN stain has low sensitivity relative to fluorescent stain

and culture(6). Secondly, it takes more time to scan-at least 300 field so that it have a low and variable sensitivity. In the scenarios with high burden of tuberculosis, the work load is high and therefore the amount of time spent examining smears is low, therefore the corresponding sensitivity is low. Thirdly, it needs experienced pathologist. Finally, it often miss the paucibacilary tuberculosis and when the patient is co-infected with HIV and also Technical error is also more common in case of ZN stain, in which heated carbol fuchsin is very much important(40). Also, it cannot differentiate live bacilli from inactive or dead bacilli (41).

Acid-fast bacilli (AFB) microscopy, which is a means of detecting/screening of pulmonary tuberculosis, has been used worldwide as a mainstay of case finding(41). Zheil Neelsen staining is a cheap and specific test which takes about 1 to 2 hours for reporting; however it is less sensitive and requires a large number of bacilli (up to 10,000 bacilli/ml) in the specimen. Moreover, it cannot distinguish Mycobacterium tuberculosis from Mycobacterium other than tuberculosis and is therefore, used for screening only(41).

Since Light-Emitting Diode Fluorescence Microscopy (LED-FM) for sputum smear examination and numerous reports have confirmed the superior diagnostic performance of fluorescence microscopy, compared with Ziehl-Neelsen (ZN) staining and light microscopy ((42),(43),(44)). Light-Emitting Diode Fluorescence Microscopy (LED-FM) for sputum smear examination is recommended by the World Health Organization (WHO) for detection of acid-fast bacilli in high tuberculosis (TB) burden countries(45). LED-FM has higher sensitivity (8–10%) ((46),(6)) and similar specificity(47)as compared with Ziehl-Neelsen staining based bright field sputum smear microscopy(ZN ,SSM) in detecting sputum smear positive TB cases and is more efficient ((47),(48)). Previous studies, which were specifically-designed in research settings, showed an increase of 8–10% in the proportion sputum positive patients with LED fluorescence microscopy. These studies had shown that the increase in yield in positivity was predominantly among those with scanty grade ((47),(49),(50),(51)). and also fluorescent microscopy has been shown 84% sensitivity (95% confidence interval [CI], 76 to 89) and 98% specificity (95% CI, 85 to 97) against culture as the reference standard, and has been shown to improve by 6% (95% CI, 0.1 to 13%) the sensitivity compared to the traditional Ziehl-Neelsen microscopy(78% sensitivity)(52).The increased sensitivity of FM could attributed to several reasons which include:- i) stronger absorbability of mycolic acid for carbol-auramine than carbol-fuchsin ii) larger field area examined using high power fields using FM as compared to

oil immersion fields by ZN and iii) sharper contrast between bacilli and the background enabling easier identification(53). But study conducted in India said Positivity by ZN method was 33% and 34% with FL staining. The sensitivity of ZN microscopy was 82.9% and specificity was 93.8%, similar to that observed with FL microscopy and 94.3% correlation was found between ZN and LJ positive cases. So ZN staining alone is sufficient for the detection of Mycobacterium tuberculosis , with no added advantage of FL staining (21).

And when we see the direct and concentrate sputum sample comparison, the concentrated sputum sample increase in sensitivity. In study conducted in Jimma said , the detection rate of direct smear microscopy by ZN stain was 25.1% and that of the concentration method 49.7%. The sensitivity of direct smear microscopy was 34.6%, for concentrated smear microscopy 66.1%(55).And study conducted in Bangladesh said , the sensitivity of direct and concentrated smear microscopy was different when using positive culture as the gold standard (71% vs. 83%), it showed that concentrated increases the sensitivity of microscopy by 12%. (56).

Culture on Lowenstein Jensen (LJ) medium remains the gold standard for the diagnosis of TB, however the facility is not available on its full extent in developing countries((11),(12))hence required special procedures and need skilled workers. Culture on LJ medium is time consuming but cheaper than radiometric and molecular based techniques and a handy approach in the diagnosis of TB in developing countries(12).These media support the growth of mycobacteria, several weeks (2-8 weeks) of incubation maybe necessary due to the case of paucibacillary specimens. The procedure of Culture on Lowenstein Jensen (LJ) medium can be done which is considered as the gold standard test, but the recovery period of *M. tuberculosis* is long (6-8 weeks)(13).

CHAPTER THREE: OBJECTIVES

3.1.General objective

To compared efficacy of Direct LED fluorescence microscopy with concentrated Ziehl-Neelsen microscopy techniques against Mycobacterial culture of sputum sample from patients, who were clinical and radiological data suspected of pulmonary tuberculosis for the diagnosis of tuberculosis.

3.2.Specific objectives

1. To assess the performance of direct LED Fluorescent microscopy against Culture on Lowenstein Jensen (LJ) medium techniques in the diagnosis of pulmonary tuberculosis.
2. To assess the performance of concentrated Ziehl-Neelsen(ZN) microscopy against Culture on Lowenstein Jensen (LJ) medium techniques in the diagnosis of pulmonary tuberculosis
3. To estimate the efficacy of fluorochrome stain in detecting paucibacillary pulmonary TB cases in comparison to concentrated ZN stain against Lowenstein Jensen (LJ) medium.

CHAPTER FOUR: MATERIALS AND METHODS

4.1. Study area and population

The study were conducted in St. Merry Aksum General Hospital at Aksum town . which is 320Km far from Mekele to wards west and 1080 km far from Addis Abeba towards north. The hospital serves gives regular health services for inpatient and ambulatory patients and also around 387 patients per day on OPD department give regular health services. The hospital has Facilities for tuberculosis diagnosis, treatment and monitoring.The Aksum town has 60,766 total population estimated according 2014 report of Surveillance and outbreak investigation at Aksum town Health Office.

4.2. Study design and period: A cross sectional, laboratory-based study were conducted from September - December 2015.

4.3. Study Subjects

The sputum sample from those who are clinically and/or radiologically suspected of having pulmonary tuberculosis and attended St.Merry Aksum General Hospital during the study period and who were agreed to participate in the study Consecutively.

4.4. Inclusion criteria:-Patients who were attended the Out Patient Department Room and had cough last for two or more weeks with sputum production,loss of appetite or loss of weight or chest pain or haemoptysis or fever or night sweats and radiological evidence of tuberculosis were included.

4.5. 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.

4.6. Sample size

convenient sampling technique was used for this study and the sample size calculation was based on the weighted prevalence (culture and/or smear) of PTB in Tigray region of Ethiopia in 2010 was found 21.6% for convenience(51) and based on standard sample size calculation for diagnostic test evaluation(59), assuming rough estimate a sensitivity of 70% of LED fluorescence microscopy versus the composite reference standard described above and a precision of 10 %, specimens from at least 81 TB cases was needed. Based on an expected TB prevalence of Tigray 21.6 % among TB suspects then there was 21.6 infected subjects per 100 patients seen at the clinic. So, to have 81 infected subjects, we was included 375 TB suspects patients (100/21.6 × 81). And we were added 5% contingency to the 375 sample size because

of the culture contamination rate of Tigray Regional laboratory shows that about 5% . Therefore , the over all sample size was 394.

n = $\frac{(Z\_/2)\ 2 * (1-p) * (p)}{(d)2}$ = **80.6** Where N = minimum sample size

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

P = sensitivity of a LED florescence microscopy test

N= (100/21.6 ×n) = **375.00** d= margin of error 0.1 at 95% CI

And 5%contingency of 375 was**19**,so **375.00 + 19=394.00** TB suspects patients were included.

4.7. Sampling procedure:-All Patient attending the St Merry Aksum general Hospital Out Patient Department Room were suspected TB using clinical diagnosis and radiological evidence investigation and then Patient were selected to this study to give sputum sample (spot-morning-spot) to those patients having clinical diagnosis and radiological evidence to Tuberculosis as the current guidelines of tuberculosis control program.Consecutive sampling were used continually until satisfied the sample size by using inclusion and exclusion criteria.

4.7.1. Schematic form of sampling procedure

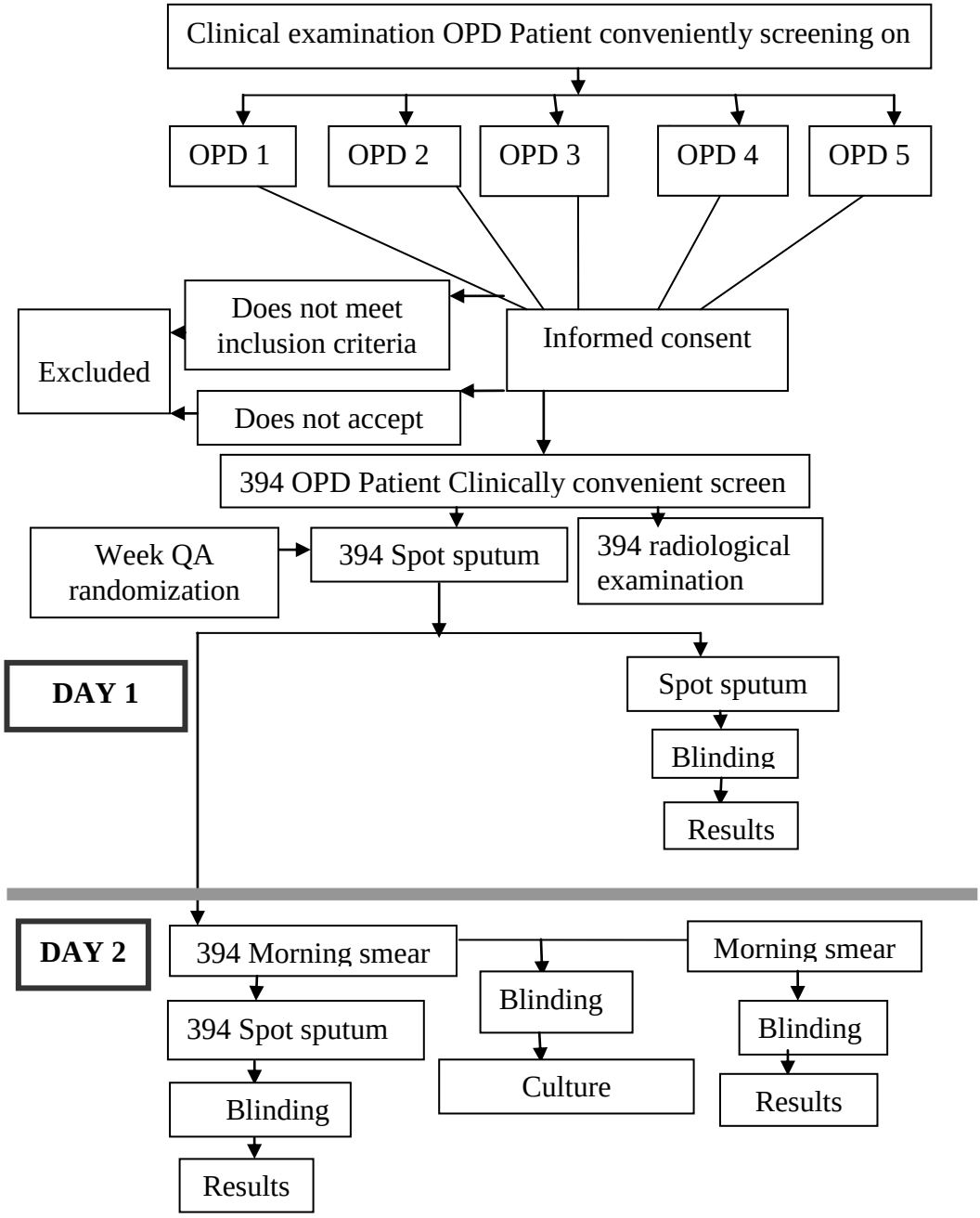


Figure 2.Schematic form of sampling procedure For Sputum Sample Collection follow

4.8.Study variables

Study variables were included the efficacy of methods on concentrated Ziehl-Neelsen and direct LED FM of sputum sample using golden standard for diagnosis of pulmonary tuberculosis by using Measurement for Efficacy for each methods were used Sensitivity, Specificity, Positive predict value, Negative predict value and age, sex , living area results of each sputum samples were collected from PTB suspected patient.

4.9.Oprational definition

Chest Pain:- is the symptom complained by patients to have either dull or sharp pain experienced at chest and chest discomfort or pain that comes on or gets worse with exercise, stress, or eating a large meal .

Cough (2 week) ;- is complain of patient as having Coughing is the body's way of removing foreign material or mucus from the lungs and upper airway passages or of reacting to an irritated airway and cough which stayed for at least two weeks

Fever ;- complain by patient as having as having a temperature above the normal range due to an increase in the body's temperature set-point. There is not a single agreed upon upper limit for normal temperature with sources using values between 37.5 and 38.3 °C .

Haemoptysis ;- is a sputum Spitting up blood or blood-tinged sputum from the respiratory tract. which has blood and the act of coughing up blood or blood-stained mucus from the bronchi, larynx, trachea, or lungs.

Night Sweat ;- symptom complained by clients as having refer to excess sweating during the night. But if your bedroom is unusually hot or you are wearing too many bedclothes, you may **sweat** during sleep, and this is normal

Mucopurulent Sputum ;- the sputum which has pus and viscous liquid (mucus). A yellow-greenish (muco purulent) color suggests that treatment with antibiotics can reduce symptoms

Production Cough ;- a cough which a patient produces sputum and produces phlegm or mucus (sputum). The mucus may have drained down the back or may have come up from the lungs.

Purulent sputum ;- the sputum which has both contains pus, composed of white blood cells, cellular debris, dead tissue, and viscous liquid (mucus) and typically yellow or green.

Saliva Sputum ;- sputum which seems saliva.

Weight loss ;- loss of weight complained by clients as having reduction in weight occurred.

4.10.Instrument and Tools

Interviewer were collected data from PTB suspected patients by administered structured questionnaire for the Clinical and Socio-demographic data by the attending physicians. And in the laboratory and on radiological methods of results also administered from recorded form were used at the study time.

4.11. 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 and were sent to the radiological examination for chest X-rays were taken.

4.12. 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. Merry Aksum general hospital. The X-ray were reported as normal, Upper lobe infiltrations (bi-lateral or uni -lateral right), Cavitations, Patchy, nodular shadows around the cavity.

4.13.Data quality assurance

Pre-test of the questioner at OPD room patients which were not included in the study were carried out for those 5% (19 TB susceptive patients) the sample size . The laboratory staff were participated in a 6-day training carried out by an expert from the Tigray health research and regional laboratory ,TB- Lab department , which covered Ziehl–Nielsen staining, LED Fluorescent microscope and reading of sputum smears, decontamination and concentrate sputum specimen , media preparation, inoculation and reading of LJ Medium. The Trainings were part of a wider project including other laboratory techniques. The staff of the clinics were participated in an initial workshop to motivate and to familiarize with the study procedures. A coordinator were conducted supervision to check adherence to the procedures every weakly and subsequently for months, mean until the end of the study the supervisor were attained randomly 13 times or in weak the supervisor were attained randomly. Pre-testing of all procedures were performed 1 week before the study roll-out. Sample collection at chest clinic and transportation were Followed national guidelines , eligible TB suspects produced three sputum samples (spot-morning-spot), after receiving thorough instructions. The first sample of

this set were used for the study. In case of failure to produce the first spot sample, the morning sample were used . Samples were collected directly in a sterile tube. In case of need to keep samples overnight, they were kept at 4°C.

4.14. 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 were prepared for each code. All data were double entered into an EPI data, cleaned, verified and Descriptive statistics were used for analysis of socio-demographic and then transferred for statistical calculation in SPSS 22.0 version. The sensitivity, specificity, positive and negative predictive values for each diagnostic technique and expressed as percentages including their 95% confidence intervals (CI) were calculated by using the sputum culture results as the "gold standard". By Chi-square test were used to assess whether difference between values obtained are significant. Inter-test agreement between Direct LED FM and LJ culture systems and also Concentrated ZN and LJ culture systems were determined by using the kappa-test. All statistical tests were considered significant if the two sided P-value (p) was <0.05.

4.15. Ethical considerations

The protocol were approved by the Department of Epidemiology, in Public Health and Medical Science College, Jimma University . Support letters were obtained from the Tigray regional of Health Bureau. The purpose of the study were clearly explained for each study participant. Written and oral consent were obtained from the subject prior to enrolment. The results of the study were communicated to the responsible physicians and all results were kept confidentially.

4.16.Plan for finding dissemination- the finding were presented to the university community, the city government health sector officials and health facility heads. And Written report were submitted to the health sector with recommendations for taking all possible solutions.

CHAPTER FIVE: RESULTS

5.1. Socio-demographic characteristics of the study population

In this study, a total of 394 pulmonary tuberculosis suspected patients were enrolled from September,2015 to December,2015 in Aksum St. Merry's General hospital. Socio-demographic characteristics such as age, sex and living area were obtained for all study subjects. Information regarding demographic characteristics of the 394 subjects were analyzed and summarized in Table 1. The patients were mainly young adults and the median age was found to be 42 years. The highest prevalence of tuberculosis suspected patients were observed in the age group of 35–44 years old. The minimum and maximum age was 18 years and 90 years respectively (range 72). There were 246 (62.4%) males, and 148 (37.6%) females with a ratio of 1.7:1. Relatively males were more often affected by tuberculosis than females (24 % versus 20%). Among the 394 patients,302(76.6%) were living in rural.

Table 1: Distribution of pulmonary tuberculosis suspected patient cases by age and sex enrolled from Sep,2015 - Dec,2015 in Aksum St. Merry's General hospital (n=394)

Age group (yrs)	Female n (%)	Male n (%)	Total n (%)
≥24	23(5.8)	36(9.1)	59(15)
25-34	26(6.6)	48(12.2)	74(18.8)
35-44	30(7.6)	47(11.9)	77(19.5)
45-54	28(7.1)	47(11.9)	75(19)
55-64	23(5.8)	31(7.9)	54(13.7)
≥65	18(4.6)	37(9.4)	55(14)
Total	148(37.6)	246(62.4)	394(100)

5.2. Test characteristics result of direct LED- fluorescent stained and concentrated *Ziehl-Neelsen* microscopy with culture

A total of 394 pulmonary tuberculosis suspected patients (1182 specimens) were enrolled. The direct LED- fluorescent stained and concentrated *Ziehl-Neelsen* smears results were compared using the result of golden standard , which were 88(22.3%) culture positive and 306(77.7%) culture negative participants (Table 2). From total of 394 pulmonary tuberculosis suspected patients , the direct LED- fluorescent microscopy were 69(17.5%) specimens positive , of these, 63(16%) were positive for both culture and Fluorescent stained microscopy but Six (1.5%) specimens were fluorescent microscopy only positive, but not culture .Over all the sensitivity, specificity, PPV and NPV achieved by direct fluorescent microscopy using culture as a gold standard were 71.59%, 98.03%, 91.3% and 92.3% respectively (Table 3).The correlation between direct LED FM and culture as gold standard method showed statistical significance (x^2= 229.38,p<0.001). Inter-test agreement between Direct LED FM and LJ culture systems determined by using the kappa-test were ((κ) = 0.754, p = 0.042), and the observed agreement was found to be also statistically significant.

When we see to concentrated *Ziehl-Neelsen* microscopy, of the 394 pulmonary tuberculosis suspected patients, the 56(14.2%)were positive by concentrated *Ziehl-Neelsen* microscopy. Out of these, 88 (22.3%) were positive by culture ,both culture and concentrated *Ziehl-Neelsen (ZN)*microscopy also 56(14.2%) were positive ,this means Zero(0%) or there was not any situation occurred like concentrated *Ziehl-Neelsen* positive, but culture negative .The concentrated *Ziehl-Neelsen* stained of the sensitivity, specificity, PPV and NPV were 63.63%,100% ,100% and 90.53 %, respectively as used culture as gold standard (Table 3).The correlation between concentrated *Ziehl-Neelsen(ZN)*microscopy and culture as gold standard method showed statistical significance(x^2= 226.99, p <0.001). Inter-test agreement between Concentrated ZN and LJ culture systems determined by using the kappa-test were ((κ) = 0.731, p = 0.044), and the observed agreement was found to be also statistically significant .

Table 2: ***Test characteristics of the direct fluorescent and concentrated Microscopy against culture results of pulmonary tuberculosis suspected patient cases enrolled from Sep,2015 - Dec,2015 in Aksum St. Merry's General hospital***

	Culture results		
	Positive (%)	**Negative (%)**	**Total (%)**
Concentrated *Ziehl-Neelsen*			
Positive	**56(14.2)**	**0(0)**	**56(14.2)**
Negative	**32(8.1)**	**306(77.7)**	**338(85.8)**
Total	**88(22.3)**	**306(77.7)**	**394(100)**
Direct LED-Fluorescent Microscopy			
Positive	**63(16)**	**6(1.5)**	**69(17.5)**
Negative	**25(6.3)**	**300(76.1)**	**325(82.5)**
Total	**88(22.3)**	**306(77.7)**	**394(100)**

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

A comparison of the Sputum processing concentrated ZN and direct FM smear results using culture as a golden standard showed that ,direct LED FM on sensitivity and negative predictive value was significantly higher than concentrated ZN microscopy (71.59 % vs 63.63 % , difference 7.96 %, And 92.3% vs90.53% ,difference 1.77%, respectively , X^2=229.38, p<0.001) but direct LED FM on specificity and positive predictive value was slightly lower than concentrated ZN microscopy (98.03% vs 100%, difference 1.97%, And 91.3% vs 100%, difference 8.7%, respectively, X^2=226.99,p<0.001). (Table 3)

Table 3: ***Sensitivity, Specificity, PPV, NPV and Test efficiency of direct FM and concentrated ZN microscopy against culture results. of pulmonary TB suspected patient cases enrolled from Sep,2015 - Dec,2015 in St. Merry's General hospital***

	DLED-FM vs Culture (%)	CZM vs Culture (%)
Sensitivity	**71.59**	**63.63**
Specificity	**98.03**	**100**
positive predictive value	**91.3**	**100**
Negative predictive value	**92.3**	**90.53**

DLED-FM=Direct **LED** Fluorescent Microscopy , **CZM**=Concentrated *Ziehl-Neelsen*

5.4. Quantifications of AFB smears reported by the different techniques

A total of 1,182 sputum specimens included in the study, 13.2% were salivary (contained no mucoid elements), 64.7% muco-purulent, 5.3% mucous and blood stained were filled for sputum consistency. (Table 4) Compares quantifications of AFB smears reported for the different techniques. Strongly positive (2+ and 3+) smears made up only 59.6% of CZN, but 50% of DFM smears. Smears with 1+ made up 35.7% for CZN and 31.8% for DFM . Smears with scanty AFB were rare with CZN(5.35%). However, smear with scanty AFB by DFM showed (15.9%) .

Table 4: Distribution of quantified smear results by different technique from pulmonary tuberculosis suspected patient cases enrolled from Sep,2015 - Dec,2015 in Aksum St. Merry's General hospital

	Percentage by technique	
Quantification grade	**C ZN(56)**	**DLED-FM(69*)***
Scanty	**3(5.35%)**	**11(15.9%)**
+1	**20(35.7%)**	**22(31.8%)**
+2	**14 (25.0%)**	**17(24.6)**
+3	**19(33.9%)**	**19(27.5%)**

D*LED*-FM= Direct LED-fluorescence microscopy CZN= Concentrated Ziehl—Neelsen

6. CHAPTER SIX: DISCUSSION

Current recommendations for the control of Tuberculosis emphasize early case detection so as to treatment of patients and limit the transmission of bacilli. ZN stain can detect bacilli when they are in the order of 105/ml of the sputum, where as a more sensitive AO stain can detect in the order of 104/ml of sputum **.** Cultivation of the tubercle bacilli is the most sensitive method for the detection o f *M. tuberculosis* and can detect 100 bacilli per ml of sputum **.** So this study we compared the performance of direct light-emitting diode (LED) based fluorescence microscopy technique and bleach concentrated sputum sample Ziehl-Neelsen stain method in patients with symptoms of pulmonary TB by using LJ culture system as the gold standard. In this study, the total sputum specimen studied were 394x3, from each clinically suspected pulmonary tuberculosis patients. These specimens were examined by direct Auramine fluorochrome stain and concentrated sputum using ZN stain and Culture .

The results of our study from concentrated Ziehl-Neelsen stain method showed statistical significant the sensitivity was **63.63** %(X^2 =226.99 ;P<0.001) and also showed a high specificity of 100 % regardless concentration Ziehl-Neelsen stain method . Similar findings have been reported were found the sensitivity of concentrated Ziehl-Neelsen smear microscopy was different when using positive culture as the gold standard (71% vs. 83%) ,SPECIFICITY both 100 (56). Other study about be conducted to find out the benefit of concentrated sample for ZN were achieved by Kumari AR and Prasanthi K , ZN was 50% and significantly (1,42). In a other similar study, the concentrated smear of ZN staining sensitivity was 51% and significantly(65) . In our study , the results from both direct Fluorescent methods and concentrated Ziehl-Neelsen stain method showed statistical significant and the sensitivity, specificity, PPV and NPV of concentrated ZN versus direct fluorochrome staining technique was significant 63.63%,100%,100%, 90.53% for concentrated ZN and for direct Auramine fluorochrome stain was 71.59%, 98.03% , 92.03%, 91.03% respectively(Table 3) . So that the sensitivity of direct fluorescent microscopy was a 7.96 % incremental yield (X^2= 229.38 , P< 0.001) but specificity slightly decreases by 1.97 % , when compared with concentrated Ziehl-Neelsen stain method . Our results strongly suggest that the optimum detection of acid fast bacilli is achieved by the application of auramine/phenol fluorescent staining a samples. And other similar study achieved by Saroj Hooja said that , AO was found the sensitivity of direct staining was 71.85% for AO. Direct fluorescent microscopy detected

9.63% paucibacillary sputum samples that were missed on ZN staining On concentration, the sensitivity was 62.22% for concentrated ZN and the specificity was 99.19% for both ZN and AO (66). Similar findings with our study have been reported, the sensitivity, specificity achieved with direct fluorescent microscopy compared to culture showed statistical significance were 72.8%, 97.1%, respectively achieved by Nebiyu Gizaw study (67).

Our study founded that FM greatly improves the diagnostic value especially in patients with a low density of bacilli that are likely to be missed on ZN stained smears, on the other hand our study also showed that, a relation between ZN missed positive smears and density of bacilli. This is in agreement with Ulukanligil and his colleague (4) . Our results also were in agreement with those of Ba (50)and Rieder(63), who reported that FM appears to be more likely to detect TB in smears which contain low-density bacilli. But LED-FM was found that decreased specificity was most common with "scanty" FM results. Sputum smears that were reported as "scanty" had less reproducible readings and were less likely to be associated with positive cultures due to many false positive results in scanty grade smears.

Our study found also direct LED-FM was more sensitive than concentrated ZN microscopy (71.59% vs. 63.63%), but less specific (98.03 % vs. 100%) means , we found that 6 (1.5 %) specimens were direct fluorescent microscopy positive, 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(62). In addition, certain non tuberculosis organism may also be detected by fluorescent microscopy. But the False-negative results of the culture may be also due to the presence of inhibitors in the specimen and in procedure.

And in this study there was a significant increase in the average number of AFB seen per microscope field in the smears prepared after concentration , Smears which were graded l+ by the direct FM method increased to 2+ after concentration with bleach ZN method (Table 4) and this result is comparable with study from Kenya (4) .

LIMITATIONS OF THE STUDY

The study area coverage is restricted to St. Merry Aksum General Hospital and sample size a little bet small because of limited resources and the study subject were selected by Convenient sampling technique , so it does not represent the whole Tigray regional state health institution.

7. CHAPTER SEVEN: CONCLUSIONS

LED FM sensitivity in AFB detection was 71.59 % when compared with gold standard culture technique. A concetrated Ziehl-Neelsen staining with auramine/phenol staining improves detection of AFB by about 7.96 %.So our results strongly suggest that the optimum detection of AFB is achieved by auramine-phenol fluorescent staining. And in the direct LED FM were occurred positive, but culture negative were also reported and these may be due to the inorganic material that absorbs fluorochrome stains. In addition, certain non tuberculosis organism may also be detected by fluorescent microscopy. And also in 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.So ,our results strongly suggest that the optimum detection of AFB is achieved by the auramine-O fluorescent staining using LED Fluorescent Microscope.

RECOMMENDATIONS

- Aksum St. Merry General Hospital and Aksum town health office should plan and implement LED based fluorescent microscopy on prevention and control of pulmonary tuberculosis. It should also promote LED based fluorescent microscopy is sensitive and specific 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

- Federal Ministry of Health (FMOH) and Tigray region health bureau in collaboration with other stakeholders should encourage further implementation LED based fluorescent microscopy. They should strengthen and support pulmonary tuberculosis prevention and control programs.

- All tuberculosis endemic countries, like Ethiopia might be reasonably expected to improve tuberculosis case-finding by increasing sensitivity and expected decrease in time spent on microscopic examination.So this should encourage to plan and implement LED fluorescent microscopy for prevention and control of pulmonary tuberculosis
- Additional study should be done for Extra Pulmonary tuberculosis, in HIV/AIDS patients, Pediatrics.

REFERANCE

1.Kumar ,Vinay;Aasbb, Abul K.; Fausto, Nelson; Mitchell, Richard N. Robbins Basic 43 Pathology. Saunders Elsevier. (8th ed.): pp. 516-22

2.Horna-Campos ,OJ, Sánchez-Pérez , HJ., Sánchez, I., Bedoya, A. and Martín, M. Public transportation and pulmonary tuberculosis, Lima, Peru.Emerg .Infect. (2007):Dis 13:1491–3.

3.World Health Organization. Stop TB Strategy: building on and enhancing DOTS to meet the TBrelatedMillenniumDevelopmentGoals..Geneva,Switzerland:WHO, 2006: pp 6–16 .

4.Matu S, Githui, L.N., Muthami, J., Makumi, J., Ngeranwa, N. Tunge, Juma, E. . Combination of bleach and Fluorescent microscopy: a milestone in the diagnosis of smear negative tuberculosis. East. African. Medica .Journal . (2007).84 (10).

5.Mase , SR, Ramsay, A., Henry, M.. Hopewell, PC., Cunningham, J. and Urbanczik,R. . Yield of serial sputum specimen examinations in the diagnosis of pulmonary tuberculosis: a systematic review. Int. J .Tuberc. Lung. . (2007).Dis.11 (5):485-95.

6.Steingart ,KR, Henry, M., Hopewell, PC., Ramsay, A., Cunningham, J. and Urbanczik, R.(2006).Sputum processing methods to improve the sensitivity of smear microscopy for tuberculosis: a systematic review. Lancet. Infect .Dis. 6 (10):664-74.

7.Steingart ,KR,Ramsay,A.and Pai , M. Optimizing sputum smear microscopy for the diagnosis of pulmonary tuberculosis .Expert review of anti-infective therapy.(2007);5(3):327- 31.

8.Armstrong D: A. LeD-based fluoroscopy and the ParaLens system:Tuberculosis.2010;7:17–8.

9.Stop TB Partnership and World Health Organization:New laboratory diagnostic tools for tuberculosiscontrolRetoolingtaskforce.Geneva,Switzerland:WHO;http://www.finddiagnostics.org/export/sites/default/resourcecentre/reports_brochures/docs/New_Lab_Diagnostic_Tools2008

10.Lakshmi V, Patil MA, Subhadha K, Himabindu V. . Isolation of Mycobacteria by bactec 460 system from clinical specimens. Indian J Med Microbiol. 2006.

11.Iqbal S,Iqbal R, Khan MM, Hussain I, Akhtar A, Shabbir I.Comparison of two conventional techniques used for the diagnosis of tuberculosis cases.Int J AgriBioi 2003;5(4):545-7.

12.Munir MK ,Shabbir I, Iqbal R, Khan SU. Comparison of detection of acid fast bacilli inclinical samples by AFB smear microscopy and culture in the diagnosis of tuberculosis in a tertiary care setting. Pak J Chet Med. 2009;15(4):14-8.

13.Prasad R LS, Mukerji PK, Agarwal SK, Srivastava R. . Clinical utility Of Polymerase Chain Reaction in patients of Pulmonary Tuberculosis. Indian J Tub. 2001;48:135–8.

14. WHO World Health Organization Website.Global TB Report 2014. Available: http://www.who.int/tb/country/data/download/en/index.html

15.Corbett EL, Bandason T, Cheung YB, Munyati S, Godfrey-Faussett P, et al.. Epidemiology of tuberculosis in a high HIV prevalence population provided with enhanced diagnosis of symptomatic disease. .PLoS Med(2007): 4:e22.

16.Matee M ,Mtei L,Lounasvaara T,Wieland-Alter W,et al.Sputum microscopy for the diagnosis of HIV-associated PTB in Tanzania.BMC Public Health.(2008):8: pp 68.

17.Perkins MD ,Cunningham J .Facing the crisis. improving the diagnosis of tuberculosis in the HIV era. J Infect. (2007) Dis 196 Suppl 1:S15–27.

18.Arati Bhadade , PM, Swapna Kanade, Department of Microbiology SGKH, Parel, Mumbai 400012, India. Utility of light-emitting diode microscopy for the diagnosis of pulmonary tuberculosis in HIV infected patients. inernational journy Mycobacteriology. 2015;4:31-5.

19.Lidya Chaidir1* IP, Jessi Annisa1, Soni Muhsinin1, Intan Meilana1, Bachti Alisjahbana1,, Crevel3 Rv. Implementation of LED Fluorescence Microscopy for Diagnosis of Pulmonary and HIV-Associated Tuberculosis in a Hospital Setting in Indonesia. 2013;8(4).

20.Semaria Solomon1* SG, Berhanu yitayew. Comparative Analysis of Performance of PREVI Fluo TB Method by Using LED-Microscopy. Solomon, et al, J Med Microb Diagn. 2015; 4:2

21.Niladri Sekhar Das DT. Assessment of sputum staining techniques for the diagnosis of pulmonary tuberculosis.INDIAN JOURNAL OF MEDICAL SPECIALITIES.2013;4(2):243-7.

22.A.V.Deun AHS, K.J.M,Aung.M.A.Hossain N.Chambugonj, M.A Hye.et.al. performance of variation of carbol-fuchsin staing of sputum smear for AFB under field condition Interanatuonal JOURNAL OF TubercLung,Dis. 2005;9(10).

23.R.Singhal JA, M.Bhalla,P.lal,S.reza,D.Behera,et.al,.presumptive Identification of Mycobacterium Tuberculosis Complex based on cord Formationin BACTEC MGIT 960 Medium. INDIAN JOURNAL OF MEDICAL SPECIALITIES.30(2012):218-21.

24.S G-S. Evaluation of the concentration sputum smear technique for the laboratory diagnosis of pulmonary tuberculosis. Trop Doct. 2003;33(3):160–2.

25.Yassin M, Cuevas, L., Gebrexabher, H. and Squire, S. . Efficacy and safety of short term bleach digestion of sputum in case finding for pulmonary tuberculosis in Ethiopia. Int. J. Tuberc. Lung Dis. 2003;7:678-83.

26.World Health Organization Global tuberculosis control: WHOGt. surveillance, planning, financing. WHO Report (2010) WHO/HTM/TB, Geneva, Switzerland Available:http://www.who.int/tb/country/data/download/en/index.html. Accessed 2013.

27. WHO World Health Organization Website . Global TB Report 2013. Available: http://www.who.int/tb/country/data/download/en/index.html.

28.United Nations. The Millennium Development Goals Report 2010. New York, NY, USA: UN, 2010. http://www.un.org/millenniumgoals/aids.shtml Accessed March 2014

29.world health organization Control: Global Tuberculosis. WHO declares TB a global emergency.WHOReport.2011.http://www.who.int/tb/publications/global_report/2011/gtbr11_full.pdf.2.Robert K:WHO declares TB a global emergency. Health Horiz 1993,19:251-2.

30.World Health Organization Global tuberculosis control: surveillance p, financing. WHO Report (2012) WHO/HTM/TB, Geneva, (2012)

31.Ethiopia Federal Ministry of Health office. Health Sector Development Programme IV. Annual performance report. Addis Ababa, Ethiopia. (2010)

32.Federal Democratic Republic of Ethiopia Ministry of Health office. Health and health-related indicators. Addis Ababa, Ethiopia: Federal Ministry of Health. ,2011.

33.Gebretsadik Berhe FE, Elena Hailu, et.at.Population-based prevalence survey of tuberculosis in the Tigray region of Ethiopia,Berhe et al.BMC Infectious Diseases 2013; 13:448

34.Federal Democratic Republic of Ethiopia MoH. Health and health-related indicators. Addis Ababa, Ethiopia: Federal Ministry of Health, 2012.

35.Devi SB ,Naorem S, Singh TJ,. HIV and TB Ind Acad Clin Med 2005; 6(3): 2005:220-3.

36.Hasegawa N, Miura T, Ishii K, Yamaguchi K, Lindner TH, Merritt S, et al. . New simple and rapid test for culture confirmation of mycobacterium tuberculosis complex: A multicenter study. J Clin Microbiol. 2002;40:908–12. [PubMed]

37.Somoskovi A ,Hotaling JE, Fitzgerald M, O'Donnell D, Parsons LM, Salfinger M. . Lessons from a proficiency testing event for acid-fast microscopy. Chest 2001;120:250-7.

38.TB INDIA 2009.RNTCP Status Report. Central TB Division. TIRSRCT. Directorate General of Health Services.http://tbcindia.nic.in/pdfs/TB%20India%20,2009. 2009.

39.Ziaee M, Namaei M, Khazaei M. .Comparison of the value of two different sputum staining for diagnosis of acid-fast bacilli.Iranian J Clinical Infectious Diseases.2008;3(2):99-102.

40.Dinnes J ,Deeks J, Kunst H, Gibson A, Gummins E,Waugh N.. A systematic review of rapid diagnostic tests for the detection of TB infection. Health Technol Assess. 2007;11(3):1-196

41.MafijUddin MK ,Chowdhury MR, Ahmed S, Rahman MT, et al. Comparison of direct versus concentrated smear microscopy in detection of PTB;BMC Research Notes 2013; 6:291

42.Prasanthi K ,Kumari AR. Efficacy of fluorochrome stain in the diagnosis of pulmonary tuberculosis co-infected with HIV . Indian J Med Microbiol; 2005;23:179–85.

43.Kivihya-Ndugga LE vCM, GithuiWA, et al. A comprehensive comparison of Ziehl-Neelsen and fluorescence microscopy for the diagnosis of tuberculosis in a resource poor urban setting.;Int Tuberc Lung Dis.(2003);7:1163–71.

44.Ha¨nscheid T ,Ribeiro CM, Shapiro HM, Perlmutter NG . Fluorescence microscopy for tuberculosis diagnosis. .Lancet Infect Dis.(2007);7:236–7.

45.World Health Organization. Fluorescent light emitting diode (LED) microscopy for diagnosis of tuberculosis :policystatement .Available :http://www. who.int/ tb/ publications/ 2011/led microscopy diagnosis_9789241501613/en/index. html.

46.Cuevas LE , Al-Sonboli N, Lawson L, Yassin MA, Arbide I, et al. (2011) LED fluorescence microscopy for the diagnosis of pulmonary tuberculosis: a multicountry cross-sectional evaluation. Accessed 29 April 2013. [cited Accessed 29 April 2013.].

47.Bonnet M ,Gagnidze L,Guerin PJ, Bonte L,et al. .Evaluation of combined LED-fluorescence microscopy and bleach sedimentation for diagnosis of tuberculosis at peripheral health service level. PloS one 6: e20175. abstract (2011) [cited abstract Accessed 25 April 2013.].

48.Marais BJ ,Brittle W, Painczyk K, Hesseling AC, Beyers N, et al. . Use of light-emitting diode fluorescence microscopy to detect acid-fast bacilli in sputum. America Clinical infectious diseases: an official publication of the Infectious Diseases Society of America (2008) [cited Accessed 10 April 2013.]. 47: 203–207. Available: http://www.ncbi.nlm.nih.gov/ pubmed/18532893

49.Xia H, Song Y-Y, Zhao B, Kam K-M, O'Brien RJ, et al. . Multicentre evaluation of Ziehl-Neelsen and light-emitting diode fluorescence microscopy in China. . the official journal of the International Union against Tuberculosis and Lung Disease 17:107–112. (2013).

50.Ba F, Rieder HL. A comparison of fluorescence microscopy with the Ziehl-Neelsen technique in the examination of sputum for acid-fast bacilli. the official journal of the International Union against Tuberculosis and Lung Disease 3:1101–5.

51.Chaidir L, Parwati I, Annisa J, Muhsinin S, Meilana I, et al. . Implementation of LED Fluorescence Microscopy for Diagnosis of Pulmonary and HIV-Associated Tuberculosis in a HospitalSettinginIndonesia.:PloSone8:e61727.Available:http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3631225&tool = abstract; (2013) [updated cited Accessed 23 May 2013.].

52.WHO. Fluorescent Light-Emitting Diode (LED) Microscopy for Diagnosis of Tuberculosis. World Health Organization; 2011. [PubMed]

53.Richards OW . THE STAINING OF ACID-FAST TUBERCLE BACTERIA..Available http://www.ncbi.nlm.nih.gov/pubmed/17787445; [cited Accessed 30 May 2013.].

54.Gordon C, Van Deun a, Lumb R.Evaluating the performance of basic fuchsin for the Ziehl-Neelsen stain. The international journal of tuberculosis and lung disease: the official journal of the International Union against Tuberculosis and Lung Disease .(2009) 13: 130–135.

55.Mulualem Tadesse1 GA, Ketema Abdissa1, Alemayehu Bekele1, Mesele Bezabih1,Ludwig Apers2 RC, Leen Rigouts. Concentration of Lymph Node Aspirate Improves the Sensitivity of Acid Fast Smear Microscopy for the Diagnosis of Tuberculous Lymphadenitis, 2014.

56.Mohammad Khaja Mafij Uddin MRC, Shahriar Ahmed, Md Toufiq Rahman,, Razia Khatun FvLaSB. Comparison of direct versus concentrated smear microscopy in detection of pulmonary tuberculosis. 2013;6:291.

57. J Infect Dis Antimicrob Agents. 2011;29:67-74.

58.Dunlap NE BJ, Fujiwara P. Diagnostic standards and classification of tuberculosis inAdults and children. . Am J Respir Crit Care Med 2000;161:1376-95.

59.Banoo S BD, Bossuyt P et al. Evaluation of diagnostic tests for infectious diseases: general principles. Nat Rev Microbiol 2006;4:S16-S28.

60.Smith wick RW.Laboratory Manual for Acid Fast Microscopy(2nd edition) Centre forDisease Control, Atlanta.(1975).

61.Wright PW, R.J. Wallace., N.W. Wright, B.A. Brown and D.E. Griffith. Sensitivity of Fluorochrome Microscopy for Detection of Mycobacterium tuberculosis versus Non tuberculous Mycobacteria. J Clin Mirobiol. (1998); 36:1046-9.

62.WHO(1998). Laboratory services in tuberculosis control.:Part II. Geneva: 23–30

63.Rieder HL, Chonde, T.M., Myking, H., Urbanczik, R., Laszlo, A., Kim, S.J., Van Deun, A.,Tr´ebucq A, . The Public Health Service National Tuberculosis Reference Laboratory and

the National Laboratory Network. Minimum Requirements, International Union Against Tuberculosis and Lung Disease (1998).

64.Kent T, Kubica, GP. (1985). Public Health mycobacteriology. A guide for level III laboratory.Atlanta, Centre for Disease Control. Heifets L. (1997). Mycobacteriology laboratory.Clin. Chest. (1985): Med. 18: 35–53.

65.Peterson EM, Nakasone A, Platon-DeLeon JM, Jang Y, de La Maza LM, Desmond E: Comparison of direct and concentrated acid-fast smears to identify specimens culture positive for Mycobacterium spp. J Clin Microbiol 1999, 37(11):3564–3568.

66. Saroj Hooja1, Nita Pal, Bharti Malhotra, Sumit Goyal, Vipin Kumar and Leela Vyas. (2011). comparison of ziehl neelsen & auramine o staining methods on direct and concentrated smears in clinical specimens. Indian Journal of Tuberculosis.).

67. Nebiyu Gizaw, Solomon G/Selassie, Kassu Desta,: COMPARISON OF COMBINATION OF BLEACH AND LIGHT EMITTING DIODE FLUORESCENT MICROSCOPY AGAINST MYCOBACTERIAL CULTURE FOR THE DIAGNOSIS TB,2011.

68.H. Albert, L. Nakiyingi, J. Sempa, O. Mbabazi, S. Mukkada, et al, Operational implementation of LED fluorescence microscopy in screening tuberculosis suspects in an urban hivclinicinUganda,PLoSOne8(9)(2013)e72556,http://dx.doi.org/10.1371/journal.pone.0072556.

69. Khagi ,AR.,Singh, S.,Subba, S., Bajracharya, A., Tuladhar, R., Lekhak, B.,Shrestha, KB.(2009).Comparison of Different Diagnostic Method for Mycobacteria Tuberculosis in Suspected Patients .*J. Nepal. Health. Res. Counc* .7(15):84-8.

Appendix

Appendix I-Questionnaire

This questionnaire form will be intended to determine the efficacy of Direct LED fluorescence microscopy with concentrated ziehl-neelsen microscopy techniques against Mycobacterial culture of sputum sample for the diagnosis of tuberculosis from patients suspected of pulmonary tuberculosis. 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) Negative _____positive 1+_____2+_____3+____

FM (Concentrated) Negative _____positive 1+_____2+_____3+_____

18. **Culture Result**

Lowenstein-Jensen Medium positive ☐ ☐ negative

19.HIV test

Positive ☐ Negative ☐

Date and signature of laboratory technician _____

Comment:_____

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

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

Principal Investigator: Rigat solomon

Jimma University

Purpose: The purpose of this study is to compare a fluorescent microscopy against mycobacterial culture for the diagnosis of tuberculosis in St Merry Aksum general 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. Merry Aksum general 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 Department of Epidemiology, and Institutional Review Board (IRB) Ethical Review Committees , in College Public Health, Jimma University.

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

Signature: _____ Date: _____

Appendix III:- Information sheet for study subjects (Ameharic version) ሰለ ጥናቱ መረጃ ሰጭ ፅሑፍ(በኣማርኛ)

በዚህ ጥናት የርሶን ናሙና ለጥናቱ ለግብአት ጠቃሚ መሆኑን እና ለመጠቀም መፈለጋጠን የሚገልፅ ፅሑፍ ነው:

የአጥኒው ስም:- ርግኣት ሰለሞሞን

በጅማ ዩኒቨርስቲ

የጥናቱ አላማ:-የዚህ ጥናት ጠቀሜታ ለቲቢ በሽታ ምርመራ ከሚያገለግሉት ውስጥ የFM (fluorescent microscopy) የምርመራ ዜዴ ከCulture የምርመራ ዜዴ ላይ ወደ አክሱም ሆስፒታል ከመጡት በሽተኞች የአክታ ናሙናን በመውሰድ የቲቢ በሽታ የማወቅ ብቃቱን ለማወዳደር የሚያጠና መሆኑ::

የጥናቱ ውስጥ የሚካዱበት ሂደቶች:-የአክታ ናሙና ከበሽተኞች ለመታከም ከመጡት ውስጥ ሓኪሙ ባዘዘው መሰረት እና የስምምነቱን ፈርማ የሞላ በሽተኞ ከሚገኘው የአክታ ናሙና በመውሰድ ወደ ባክትሮሎጂካል ምርመራ ለመውሰድ መጠኑ 10ml የሆነ የአክታ ናሙና እና እንዲሁም የራጅ ምርመራንም በሽተኛው በማስመርመር ጭምር የቲቢ በሽታን ለማወቅ በአክሱም ሆስፒታል የተመላላሽ በሽተኛ ውስጥ በመስራት ነው::

የጥናቱ ሊዛመዱ ከሚቹሉት ተግዳሮቶች:-የዚህ ጥናት በመሳተፍ ምንም አይነት ችግርም ይሁን የከበደ ሂደቶችን ምን የረዘመ ወይም በተደጋጋሚ የመቀመጥም ይሁን የመመለስ ሁኔታ አይኖርም::

የጥናቱ ጥቅም:-ቀጥተኛ የሆነ በገንዘብም ይሁን ከገንዘብ ዙርያ ውጪ የሚገኙ የጥቅማጥቅም ሁኔታዎች አይኖሩትም ሀሆም የጥናቱ ውጤት ግን ጥሩ የሆነ ለቲቢ በሽታን ለመግታት በሚደረገው እንቅስቃሴ የጎላ የሆነ ግብኣት ይግኝበታል::

የጥናቱ ምክንያት የምናቻችለው ነገር:-ምንም አይነት የምናጣው ወይንም የምናቻችለው ነገር አለመኖሩ::በተለይ ደሞ በዚህ በጥናቱ ምክንያትም ይሁን ያክታ ናሙናዉንም በመስጠታቹሁም ይሁን የምታጡት ነገር አለመኖሩ::

ለሰጡት መረጃ ታማኝነት(ሚስጥረኝነት)መጠበቅ:-የላብራቶሪ የምርመራ ውጤቶችን ከእርሶ እና ከጥናቱ አጥኒ እንዲሁም ከሐኪሞ በሰተቀር ለማንም አሳልፈን የማይሰጥ መሆኑና የምርመራ ውጤትዎን በአግባቡ በካርድ ላይ የሚከመጥ መሆኑን::

ተጨማሪ መረጃ ነጥናቱ ዙርያ:-ጥናቱ በጅማ ዩኒቨርስቲ በ Epidemiology ክፍል እና Institutional Review Board (IRB) የስነ ምግባር የሚያጠና ኮሚቴና በማሕበረሰባዊ ጤና ሕክምና ሳይንስ ኮሌጅ የፀደቀ መሆኑን መግለፅ እንፈልጋለን::

ከላይ በተሰጠው መረጃ መሰረት እኔ በጥናት ለመሳተፍ መወሰኔ በስሜ አና ፊርማዬ አረጋግጣለሁኝ::

የጥናቱ ተሳታፊ ፊርማ እና ስም: _____ ቀን: _____

Appendix IV. Consent form (English version)

Mr/Mrs/Miss_____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 efficacy of Direct LED fluorescence microscopy with concentrated ziehl-neelsen microscopy techniques against Mycobcaterial culture for the diagnosis of tuberculosis in Aksum, 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)የስምምነት ፎርም (በአማርኛ)

አቶ/ወ/ሮ/ወ/ሪ_____የተባልኩኝ በስማሁት/ ባነበብኩት መረጃ መሰረት ስለ ጥናቱ ጥቅም በማወቄ /በመረዳቴ ;በዚህ ጥናት ለመስተፍ እናንተን ለመጠየቅ እፈልጋለሁኝ ; በመሆኑም ፍላጎቴን በመረዳት የጥናቱ አጥኒው እኔን ለማሳተፍ ፍቃደኛ መሆኑን ለመግለፅ በስምና በፊርማዎ መስማማትዎን እድያረጋግጡልኝ እፈልጋለሁ::

የጥናቱ ተሳተፊ ፊርማ እና ስም_____ቀን_____

የ አ ጥ ኚ ው ፊ ር ማ እ ና ስ ም_____ቀ ን _____

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

Scale(1000xfield=HPF)	Microscopy System Used	
Result	Bright field (1000xmagnification 1 length =2cm=100HPF)	LED fluorescence (400xmagnification:1length =40fields=200HPF)
Negative	Zero AFB/1length	Zero AFB/1length
Scanty(actual count)	1-9AFB/1 length or 100HPF	1-19AFB/1 length
1+	10-99AFB1length or 100HPF(1-9AFB/10Field)	20-199AFB/1 length
2+	1-10AFB/1HPE on average	5-50AFB/1field on average
3+	>10AFB/1Fild on average	>50AFB/1field on average

APPENDIX VII: ZIEHL-NEELSEN AND FLUOROCHROME STAINING REAGENTS PREPARATIONS

A.ZIEHL-NEELSEN STAINING REAGENTS PREPARATIONS

Carbol Fuchsin (1%)	**Quantity per liter**
Basic fuchsin	10.0 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 (0.1%)

Methylene blue chloride	1.0 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.

B.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 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 (Na2HPO4) in 1000 ml (1 liter) distilled/deionized water, using a volumetric flask.

• Dissolve 9.07 g monopotassium phosphate (KH2PO4) 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 Lowenstein-jensen medium solid and liquid Culture media

Lowenstein - Jensen Medium is used with fresh egg and glycerol for the isolation and differentiation of *Mycobacterium* spp.

Product Summary and Explanation The Reagents and solution

- 70% Ethanol
- 2% Malachite green solution
- Salt solution
- Hens' eggs

Reagents preparation:

- *2% Malachite green solution*

Ingredients	**Amount**
Malachite green dye	2 g

Sterile distilled water	100 ml

Using aseptic techniques dissolve the dye in sterile distilled water: place the mixture in the incubator at 37 °C for 1–2 hours or heat in a water-bath at 37°C. Store in dark bottles. This solution is not stable long-term: if precipitation occurs, discard and prepare a fresh solution.

- *Salt solution*

Ingredients	**Amount**
MonopotassiumDihydrophosphate (KH2PO4),anhydrous	2.4 g
Magnesium sulfate(MgSO4 ·7H20)	0.24 g
Magnesium citrate	0.6 g
L- Asparagine	3.6 g
Distilled water up to	600ml
Glycerol (ml) or pyruvate[c] (g)	12ml or 7.2g
Egg homogenate	1000ml
Malachite green (2%)	20ml
pH about	6.8

Glycerol in the LJ medium favours the growth of *M. tuberculosis*, while LJ medium without glycerol but containing pyruvate encourages the growth of *M. bovis.*

Prepare the salt solution by dissolving the components in distilled water (as indicated in the above table). Autoclave at 121 °C for 30 minutes in a screw-capped bottle

- *Hens' eggs*

Hens' eggs should be fresh (no more than 7 days old), bought from a farm that does not use feedstuffs containing antibiotics. The eggs should be medium-sized (for balanced proportions of egg white and egg yolk); about 23 eggs will be needed per litre of egg mass.

Principles of the Procedure

A variety of solid and liquid culture media have been developed for the isolation of *M. tuberculosis* on culture media. Egg-based media are widely used since they can be prepared locally and offer several other advantages – low cost, stability of the chemicals needed, long shelf-life, and characteristic morphology and luxurious growth of tubercle bacilli. However, it may be as much as 8 weeks before cultures become positive, particularly if specimens contain few bacilli. Egg mass of whole eggs is mixed with ingredients needed for growth of tubercle

bacilli. Coagulation yields a solid medium. Malachite green in the medium helps to minimize the growth of contaminants.

Precautions

1. For Laboratory Use.

2. IRRITANT. Irritating to eyes, skin, and respiratory system.

Directions

1. Dissolve 37.3 g of the medium in 600 mL of purified water containing 12 mL of glycerol.

2. Heat with frequent agitation to completely dissolve the medium.

3. Autoclave at 121°C for 15 minutes.

4. Prepare 1000 mL of a uniform suspension of fresh eggs under aseptic conditions. Avoid whipping air into suspension during the collection and mixing.

5. Aseptically mix the 1000 mL of egg suspension with 600 mL of the sterile Lowenstein-Jensen Medium cooled to 50 - 60°C, avoiding air bubbles.

6. Dispense the finished medium into sterile screw-cap test tubes. Place the tubes in a slanted position and heat at 85°C for 45 minutes.

Quality Control Specifications :-**Dehydrated Appearance:** Powder is homogeneous, free flowing, and light to medium blue-green.

Appendix.IIX.Standard operational procedure For Transport and Storage Sputum Sample

Sample type	Amount required	Transport and Storage	Stability
Sputum	3-5ml*and above	As soon as possible If not kept in the refrigerator. No later than 5 – 7 days if kept at $+4^0$C.	Up to 7 days at +40C Or at room temperature (> 20°C if CPC(Cetylpyridinium chloride) added.

Appendix X .Laboratory methods

A. Sample collection

Sputum specimens were collected from patients suspected of having pulmonary tuberculosis (PTB) attending St. Merry Aksum general Hospital, Aksum town. 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.

B. Direct smear preparation

The 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 for the auramine O phenol method(60).

C. 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,). 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 used for ZNstaining (61).

D. Concentrated ZN smears staining

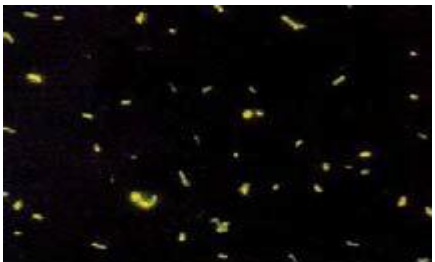
The prepared smears were placed on the staining rack and heat fixed then stained with 0.3% Carbol fuchsin, heated gently until steam rose, and left for 5 minutes, washed with gentle stream of water and flooded with 3% acid-alcohol for 1 minute, washed and flooded with 1% methylene blue for 1 minute(62).

E. Direct Flurochrome staining

The prepared slides by direct 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(62, 63).

F.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 Laboratory technologists, who were blindly and work only LED Fluorescence microscopy.Smear results were recorded before culture results were available (Appendix VI).

Figure 3.AFB Positive on Fluorescence microscopy

G. Examination and grading of ZN stained slides

ZN stained slide were 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 Laboratory technologists, who were blindly and work only ZN Conventional microscopy . Smear results were recorded before culture results were available (Appendix VI).

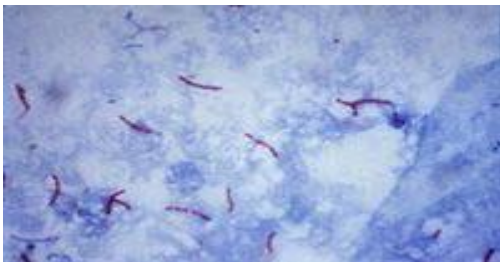


Figure 4;- AFB Positive on Conventional microscopy(Ziehl-Neelsen)

H.Laboratory procedure for sputum culture

The sputum specimens were liquefied and decontaminated by modified Petroff's methods (64). Equal amount of NaOH-NALC- Sodium Hydroxide N-acetyl-L-cysteine 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 were allowed the tubes to sit for 5 minutes to settle aerosols. After centrifugation the supernatant were decanted and add a small quantity (1-2ml) phosphate buffer (pH 6.8) and were re-suspended the sediment with the help

of a vortex mixer. Then All the specimens inoculated into L-J media were incubated at 37°C for 6 to 8 weeks in a vertical position for the better development of individual colonies. When small and buff colored colonies grew on LJ medium, the sample was considered as positive. Contaminated cultures (e.g. growth of moulds, and also those in which the medium had liquefied or turned dark green) were discarded.

The Principles of the Lowenstein-Jensen Medium is the L-Asparagine and Potato Flour are sources of nitrogen and vitamins in Lowenstein-Jensen Medium. Monopotassium Phosphate and Magnesium Sulfate enhance organism growth and act as buffers. Glycerol and the Egg Suspension provide fatty acids and protein required for the metabolism of mycobacteria. The coagulation of the egg albumin during sterilization provides a solid medium for inoculation purposes. Sodium Citrate and Malachite Green are selective agents to prevent growth of most contaminants and allow early growth of mycobacteria.

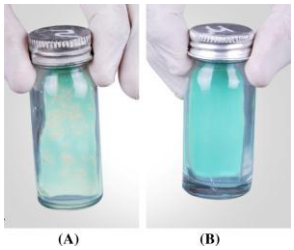


Figure 5.Comparison between positive and negative results on Lowenstein Jensen. Yellow colonies of Mycobacter tuberculosis representing positive growth are clearly seen in (A) which are absent(B)

I. Safety Precautions

Slide preparations were performed in a bio safety level II laboratories. Sputum processing, decontamination, inoculums preparation, inoculation to Culture on Lowenstein Jensen (LJ) medium, were performed in a suitable bio safety level III laboratories were dedicated for mycobacterial work with negative air pressure and with an appropriate ventilation system by sending the Sputum specimens were transported specimen transportation box (cool box) at +4 0c in the triple packaging of sample transport system, that were used to preserve the normal situation according the SOP(standard operational procedure) for sputum sample transporting system to the Tigray health research and regional laboratory, TB- Lab department. And accesses to the room were restricted when work were in progress, and proper protective gowns, gloves and respirator masks were used while handling specimens.

J. Quality control

Stained smears were read blindly by two trained laboratory technologists at different occasions and for culture examination were read blindly by one trained laboratory technologists. All smears and for LJ medium culture were read by two and one full-time laboratory technicians respectively, who have a median experience of 10 years (range 5–20 years) with ZN Staining

and LED FM Microscopy and LJ medium culture. Blinding and quality control procedures for reading smears were remain as proposed. The internal quality control procedures for AFB using known positive and negative control slides were included with each batch of slides read were continues every morning before the patient test started with each new batch of ZN and Auramin stain. Our lab also participates in EQA program for smear microscopy provided by the WHO. Finally to assure quality, all positive smears and 25% of the negative smears were cross checked by senior experienced examiner means were cross checked by quality officer. For mycobacterial culture, we monitor the contamination rate on a monthly basis, as a quality control for the decontamination process. And all positive cultures are confirmed for AFB by using ZN microscopy.

Appendix XI. Declaration

I declare that (*comparison efficacy of Direct LED fluorescence microscopy with concentrated ziehl-neelsen microscopy techniques against Mycobacterial culture of sputum sample for the diagnosis of tuberculosis from patients suspected of pulmonary tuberculosis*) is my own work, that it has not been submitted for any degree or examination in any other university, and all the sources I have used or quoted have been indicated and acknowledged by complete references.

Name of the candidate: Rigat Solomon

Signature ---------- Date -------/-------/---------- Place: Jimma

This Proposal has been submitted for ethical clearance with my approval as university advisor.

Name of the advisors:

Honok Asefa (BSc,),

Signature ---------- Date -------/-------/---------- Place: Jimma

Alemayehu Atomsa(BSc, MPH),

Signature ---------- Date -------/-------/---------- Place: Jimma

Name of the internal examiner:

Chaltu Fikru (BSc, MPH),

Signature ---------- Date -------/-------/---------- Place: Jimma