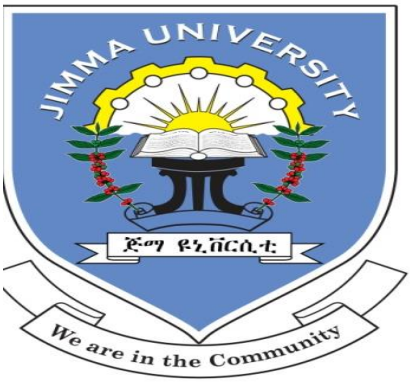
EVALUATION OF SAME-DAY MICROSCOPIC DIAGNOSIS APPROACHES FOR PULMONARY TUBERCULOSIS: WITH COMBINATION OF VARIOUS STRATEGIES IN EXISTING METHODS.



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Evaluation of Same-day Microscopic Diagnosis Approaches for Pulmonary Tuberculosis: With Combination of Various Strategies in Existing Methods.

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ACRONYM AND ABBREVIATIONS

AFB	Acid fast bacilli
ATT	Anti-tuberculosis treatment
EPTB	Extra-Pulmonary TB
LED	Light Emitting Diode
LED-FM	Light Emitting Diode fluorescence
LM	Light Microscopy
LJ	Löwenstein-Jensen
LPA	Line Probe Assays
MDR	Multidrug resistant TB
MOH	Ministry of Health
MTB	Mycobacterium tuberculosis
MTBC	Mycobacterium tuberculosis complex
NAATs	Nucleic acid amplification tests
PTB	Pulmonary TB
SSM	Sputum smear microscopy
TB	Tuberculosis
WHO	World Health Organization
ZN	Ziehl-Neelsen

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ABSTRACT

Background:- *Tuberculosis (TB) is a major cause of morbidity and mortality in relations to infectious disease. In 2011 WHO issued a policy statement recommending same-day diagnosis approach for sputum smear microscopy. Studies still clearly showed a limitation in detecting cases in same-day diagnosis approaches with conventional light microscopy. However, there is no adequate information whether introduction of bleach concentration in simple centrifugation method and using light-emitting diode-fluorescence microscopy (LED-FM).*

Objectives: - *This study was conducted to assess the performance of same-day microscopic diagnosis approaches for pulmonary tuberculosis: with different combination of techniques and methods, at Jimma University Mycobacteriology Research Center, Southwest Ethiopia*.

Methods: - *A comparative study was conducted among 126 tuberculosis presumptive patients from February 2016 to November 2016. From each participant, two spot sputum on the day of visit at one-hour interval and one morning and one spot on the next day were collected. Two direct smears were prepared from each specimen and two extra smears were prepared from pooled Spot-1 and spot-2 after concentrated with 5% bleach. The smears were stained by Ziehl-Neelsen (ZN) and fluorescence (LED-FM) staining. Finally the remaining pools of sputum specimens were processed for culture on LJ medium. The sensitivities, specificities, likelihood ratio negative (LR-) and likelihood ratio positive (LR+) with their respective 95% confidence interval (CI) were determined using culture as the gold standard.*

Result: - *From 126 selected participants only 120 were eligible for analysis. Of the 120, 34/120 (28.3 %) were culture-positive for M.tuberculosis complex. The same-day direct ZN strategy detected 25/120 (20.8 %) of TB suspected cases. It had significantly lower sensitivity 70.59 % compared to the conventional direct ZN strategy* 88.23 %; *(95CI: 77.41% - 99.07%). Same-day concentrated ZN strategy detected 32 (26.7 %) and increased the yield of same-day direct ZN strategy by 5.84 %. Same-day direct fluorescence strategy detected 30 (25 %). Same-day direct fluorescence strategy had comparable sensitivity and specificity compared to conventional direct fluorescence strategy and same-day concentrated fluorescence strategy. Same-day concentrated fluorescent strategy increased yield of Same-day direct fluorescence strategy by 1.67 % (2/120).*

Conclusions and recommendation: - *Same-day direct ZN strategy had significant lower performance compared to the conventional direct ZN strategy. However, Same-day concentrated ZN strategies (Bleach treated Same-day) compensate the limitation of same-day direct ZN*

strategy. Same-day direct fluorescence strategy has comparable performance compared to conventional direct fluorescence strategy and same-day concentrated fluorescent strategy. Same-day diagnosis approaches can be applied with same-day concentrated ZN, same-day direct fluorescence strategy and same-day concentrated fluorescent strategy. Same-day direct fluorescence strategy better than the other strategies.

CHAPTER ONE: INTRODUCTION

1.1. Background

Mycobacterium Tuberculosis Complex (MTBC) are nonmotile, nonsporulating, weakly gram positive, that appear microscopically as straight or slightly curved rods. They express unique lipid-rich mycolic acids cell wall envelope that play a critical role in the structure and function of the cell wall and it confers many of the unique characteristics of this genus: acid-fastness, extreme hydrophobicity, resistance to drying, acidity/alkalinity, and many antibiotics (1,2). *Mycobacterium tuberculosis* complex consists closely related bacteria which includes *M. tuberculosis*, *M. canettii*, *M. africanum*, *M. microti*, *M. bovis*, *M. caprae*, *M. pinnipedii*, *M. mungi*, and *M. orygis* (3). MTBC are the causative agent of infectious diseases is called tuberculosis (TB) in humans and animals (4)

Mycobacterium tuberculosis (MTB) is one of the member of MTBC and the main agent of TB in humans (3). It is an intracellular pathogen and infects the resident alveolar macrophages primarily, and the infected macrophages can then either remain in the lung or are disseminated to other organs in the body finally develop TB (2,5).

Mycobacterium tuberculosis most commonly affect lugs and it causes pulmonary TB (PTB); nevertheless, in a widely variable proportion of cases, MTB can also affect other body sites and causes Extra-Pulmonary TB (EPTB) (6). The commonest presenting symptoms of pulmonary TB are cough with or without sputum production, chest pain, hemoptysis, unintentional weight loss, fatigue, malaise, fever, night sweats and loss of appetite and breathlessness (7,8). TB is primarily a disease of the respiratory system and transmitted through inhalation of MTB containing droplets (<5 microns) come from a TB patient through coughing, sneezing and spitting (9,10).

Tuberculosis can be diagnosed by medical history, physical examination, chest x-ray, and laboratory tests (11). One of the major goal of the global control of TB in humans is the laboratory diagnosis of MTB (12). Laboratory tests for the diagnosis of TB require detection of Acid Fast Bacilli (AFB) by smear microscopy, mycobacterial culture and molecular MTB-specific Nucleic Acid Amplification tests (NAATs). Mycobacterial culture is the gold standard of diagnostic testing for TB but Culture and Nucleic acid amplification tests (NAATs) are expensive and not available in many settings (13–17).

Smear microscopy is rapid, inexpensive, identifies the most infectious TB patients and it is highly specific in areas with a very high prevalence of tuberculosis, although it remains the only same day diagnostic test within the budget of all laboratories (18,19). Acid fast bacilli sputum smear microscopy remains the most frequent diagnosis of PTB in the most setting particularly in developing countries and is widely applicable in various populations with different socio-economic levels. Hence, it has been an integral part of the global strategy for TB control. AFB smear microscopy relies on the detection of AFB in sputum smears using an either conventional light microscope or fluorescence microscopy(18,20–24). The recently invented Light Emitting Diode (LED) FM increases the sensitivity of AFB sputum microscopy in 10%. Moreover the examination time is reduced about 10-fold in selected settings, it had qualitative, operational and cost advantages (24–27).

On top of introducing of LED-FM most laboratories prepare a direct smear which is cheap, rapid, and highly specific. AFB sputum specimen concentration by chemical and/or physical methods can further increase the detection rate of AFB (27,28). One of the chemical used to concentrate sputum specimen is bleach (sodium hypochlorite). It is one of bactericidal chemical and adding bleach to sputum may sterilize it, there by potentially protecting staff from tuberculosis infection during processing and it is an ideal chemical processing agent for use in low-income countries. It also used to increase the sensitivity of smear microscopy through digestion of the mucus and debris in sputum, resulting in a clearer microscopy field (29–31).

In 2011 WHO recommended new diagnostic approaches for high burden countries: these are examination of two specimens collected on the same-day instead of two days especially in settings where patients are likely to default from the diagnostic pathway (32–34). As a result most countries in the world including Ethiopia endorsing the same-day strategy by rejecting the conventional strategy which was used three sputum specimens collected within two days (spot-morning-spot) (35,36). But studies clearly showed that, there is a limitation in detecting cases in same-day approaches. However there is no adequate information whether the introduction of bleach concentration in simple centrifugation method and LED-FM can compensate the limitation of same-day. Therefore, early evaluation of same-day diagnosis approach using combination of various existing methods is crucially important.

1.2. Statement of the problem

Tuberculosis is the first cause of death and a major cause of morbidity *in* relations to infectious disease. According to WHO 2015 report, 9.6 million new cases of TB were registered around the world. Additionally in 2014, there were an estimated 1.5 million deaths from TB (37).

The global burden of death and disease caused by TB is concentrated in low-income countries particularly in sub-Saharan Africa. Ethiopia ranks tenth among the 22 TB high burden countries in the world and fourth in Africa (37). Tuberculosis is the leading cause of morbidity, the second cause of death and the third cause of hospital admissions in Ethiopia (38).

The most essential component of tuberculosis control is the early detection of infectious cases. This is most commonly sputum smear positive pulmonary TB. The primary role of detecting infectious cases is to reduce the transmission of infection in the community. It is estimated that a single infectious person who remains undetected and therefore untreated can infect between 10 and 15 people every year (39).

Sputum smear microscopy (SSM) has been the primary method for the diagnosis of pulmonary TB (8). In Ethiopia SSM is the most used way of identifying sources of tuberculosis infection, and the primary tool for diagnosing TB. It can also use for monitoring and defining cure. Light emitting diode (LED) microscopy is a newly introduced diagnostic tool to complement the conventional light microscopy. It is recommended for centers with high case load as it saves time and improves sensitivity (8,40).

The conventional SSM procedure requires at least two days visits (8). Up to 5.8 % of patients fail to return on the second day to provide a second specimen or receive results and are called as "diagnostic defaulters" who continue to move in the community and contribute to disease spread (34). The major obstacle in conventional approach is patient dropouts and patients are spending significant amounts of money on travel and food besides the loss of daily wage in order to reach the TB microscopy Centre (41,42).

If the total process could be completed on the "same-day" it does not require the patient to visit the health facility multiple times for diagnosis of TB. Thereby, it will reduce the economic burden of an additional visit and thus will lead to lesser drop-out rates during diagnosis and the

report of the sputum examination is made available on the same-day, it ensures no delay in the initiation of treatment. In addition, this change could reduce the workload of laboratory technicians (LTs) and technicians could spend more time to examine each slide, thereby could reduce the false-negative rate. Furthermore it save sample containers, slides, and reagents (33,41,43,44). As a result In 2011 WHO issued a policy statement recommending that "countries that have implemented two-day-specimen case-finding consider switching to same-day diagnosis (34).

However, few studies still showed that same-day strategies have no effective as the conventional strategy in detecting sputum smear positive TB (45–50) and most previously conducted studies indicated the same-day diagnosis approaches using conventional light microscopy have no statistical significant difference with conventional approach (34,51–56). Nevertheless, the studies clearly shown that, there is a limitation in detecting cases in same-day approaches in different methods. However, there is no adequate information whether the introduction of bleach concentration in simple centrifugation method and the use of LED-FM can compensate the limitation of conventional light microscopy for implementation of same-day diagnosis approaches. As to our knowledge no study has been conducted on this topic in the study area.

Therefore, the aim of this study was to assess the performance of same-day microscopic diagnosis approaches for pulmonary tuberculosis: with various combination techniques in existing methods, at Jimma University Mycobacteriology Research Center, Jimma, South west Ethiopia.

1.3. Significance of the study

Most countries in the world including Ethiopia endorsed the same-day strategy but studies clearly showed that, there is a limitation in detecting cases in same-day approaches However, there is no adequate information whether the introduction of bleach concentration in simple centrifugation technique and the use of LED-FM on same-day strategy. Therefore, the study will provide information on performance of same-day diagnosis approaches in different combination of existing strategies. The findings can also be used for policy makers, national TB control program and other stakeholders. Besides, the study will help to look for more sensitive and applicable microscopic diagnostic strategies for the diagnosis of PTB. Moreover, the result will be used by others researchers as a base line data for further investigation.

CHAPTER TWO: LITERATURE REVIEW

2.1. Magnitude of tuberculosis

Globally in 2014, an estimated of 9.6 million incident cases of TB (37). Ethiopia is one of the 22 high-burden countries (HBCs). According to the Global TB report 2015, about 200,000 (160–240 per 100,000 populations) incident cases of TB in Ethiopia in 2014. According to the same report the prevalence of TB estimated to be 190,000 (160–240 per 100,000 populations) and 32,000 deaths (22–43 per 100,000) due to TB (37,57).

2.2. Same-day diagnosis approaches vs. Conventional diagnosis approaches

Sputum smear microscopy is the most widely available diagnostic test for pulmonary tuberculosis in countries with a high burden of the disease and is available in most primary health-care laboratories at health-center level (34).

In Ethiopia Sputum microscopy is the primary tool for diagnosing tuberculosis infection and it is also used for monitoring and defining cure cases. According to the conventional approach, three sputum specimens must be collected (spot- morning-spot) and it is examined in two consecutive days (8,40). Smear microscopy may, however, be costly and inconvenient for patients, who have to make multiple visits to health facilities. Several previous studies try to address the diagnostic accuracy of conventional strategies in comparison with two consecutive sputum specimens ('spot-spot') are examined on the same-day.

2.2.1. Same-day approach vs. Conventional approach with ZN staining

Overall, in a meta-analysis published by WHO in 2011 report, same-day diagnosis on average 2.8% less sensitive than the conventional approach, indicating that this strategy would be no more than 5% worse than the conventional approach. The specificity of the two approaches (with culture as the reference standard) was identical 98% (95% CI, 97–99%) (34).

A study with cross sectional design conducted in India in 2014 among 1605 patients 9.43% showed sputum smear positivity (SSP) in SM (spot-morning) scheme with ZN staining technique and 9.37% sputum smear positivity in SS 2 approach. This result indicated that no statistical significant difference between the two schemes ($P > 0.05$) (58). A similar study conducted in

India in 2012 among 658 participants showed 64 Acid Fast Bacilli (AFB) was detected from the first two smears of sputum in conventional approach, whereas in same day scheme 62 AFBs was detected. In this study there is no statistical difference between the two schemes ($p > 0.05$) was reported (51). Another study conducted in India using ZN, MZN (modified ZN) and LED FM method in 2015 among 3186 patient's showed conventional direct ZN sputum smear positivity was 297 (9.3%) and for same-day direct strategy sputum smear positivity was 294 (9.2%). In this study the difference was statistically not significant ($p > 0.05$) (55).

A study conducted at a tertiary care hospital in Bareilly among 300 patients with ZN methods showed 70 AFB cases were detected in conventional approach whereas 67 cases detected in same-day approach. In this study only 3 TB cases missed by same-day approach but no statistical difference between the two approaches was reported ($p > 0.05$) (52).

The evaluation of same-day approaches using ZN stained microscopy with cross sectional study from Uganda in 2015 showed spot- spot and spot-morning schemes have similar yields. According to the findings of this study the same-day diagnosis using the spot-spot algorithm is equivalent to conventional case-finding strategies by microscopy was reported (59). Similar study conducted in Uganda to compare same-day and conventional strategy with ZN and LED in 2011 reveled that there was no difference in sensitivity between the single specimen and two-specimen LM (Light Microscopy) strategies (55 vs. 56%; difference, 21%; 95% CI, 25to 12%). There was also no significant difference in specificity between the two strategies (98 vs. 97%; difference, 1%; 95% CI, 21to 12%) (42). Another study from Abuja, Nigeria in 2007 using ZN revealed that 45 and 40 AFBs cases of the 78 culture-positive cases in conventional and the same-day approaches respectively In this study no statistical differences between the two approaches was reported ($P > 0.5$) (53). A related study from Nigeria showed that the smear microscopy using the conventional scheme (S–M) yielded 181 [98.9%] patients, whereas the spot-spot scheme (S–Xs) yielded 176 (96.2%) patients. In this study no significant difference between the two schemes was reported ($p > 0.5$) (54). Evidence from Libya to evaluate frontloaded approaches in 2013 with cross sectional study design showed that Spot-Xspot and spot-morning smear microscopy had, respectively, 65% and 66% sensitivity and 97% and 96% specificity (p.0.5). Same-day smear microscopy is similar to that of the conventional smear

microscopy scheme and There were no statistically significant differences in sensitivity and specificity between the schemes (60).

In Ethiopia to asses one-day verses two-day approaches with cross sectional study conducted using ZN stained microscopy at 2006 showed that 51 patients were detected using the conventional method and 49 by the same-day method three cases missed by the same-day method were detected by the second day smears, while the same-day approach detected one case missed by the conventional approach. If the diagnosis of smear positive TB based on at least two positive sputum smears. Using this case definition, the sensitivity for the same-day and conventional methods was 87% (95% confidence interval [CI] 74–94) and 89% (74–95), respective ($P > 0.5$). The result suggesting that PTB can be effectively diagnosed in a single day (43) Another study done in Ethiopia, Using the case definition smear- positive TB be based on at least two positive sputum smears, the sensitivity for the same-day and conventional methods was 87% (95% confidence interval [CI] 74–94) and 89% (74–95), respectively (P > 0.5) (56).

On the other hand studies conducted in the world showed that discrepancies between the two approaches. A study conducted in India with cross sectional design using direct ZN in 2013 among 250 patients showed conventional two-day strategy detected 101 positive cases whereas same-day method detected 95 patients out of total 101 diseased when matched to two-day strategy. The one-day test method failed to detect 6 cases. In this study Statistical analysis showed that same-day approach slightly inferior to two day in detecting positive cases among diseased ($p < 0.01$) was reported (45). Similar study conducted in New Delhi, India in 2011 out of 330 TB suspects, showed that the conventional approach detected 61/330 (18.48%) and , whereas in same-day approach was detected 43/330 (13.03%). Sensitivity of the conventional and same-day approach was 58.25% and 40.07% respectively. In this study significant difference between the two approach was reported ($p < 0.05$) (46). Another study conducted in India in 2015 among in 2013 with cross sectional study in India using ZN method among 2551 presumptive TB participants showed the conventional sputum smear positivity was 431 (17%) whereas the same-day microscopy was 360 (14%). Considering the results of 433 smear positive TB cases, same-day microscopy missed 73 (17%) and 2 (1%) by the conventional method. In this study there was statistically difference between the two approaches was reported ($p<0.001$) (47). A related study conducted in India to evaluate same-day over the conventional approaches

showed that among the 2168 patients a total of 429 smear-positive results was obtained, smear microscopy using the same-day approach yielded 403 (18.6%) whereas the conventional approach yielded 427 (19.7%) however 26 (6.0%) cases were missed by the same-day approach as compared to only 2 (0.5%) missed by the conventional approach was reported ($P < 0.001$) (48).

2.2.2. Same-day approach vs. Conventional approach with LED-FM staining

A study conducted in India using ZN, MZN (modified ZN) and LED FM with cross sectional design in 2015 on 3186 patient's results was found that conventional direct fluorescent strategy sputum smear positivity was 343 (10.8%) and for same-day direct fluorescent strategy sputum smear positivity was 338 (10.6%). In this study the difference was statistically not significant ($p > 0.05$) (55).

A study conducted in Uganda using LED stained microscopy at 2012 showed that the sensitivity of both same-day and the conventional approaches was 91%. The specificity of the same-day approaches was 86.2% and the conventional approach was 91.7%. The positive and negative predictive value by the same-day approaches was 87.2% and 90.4% respectively. The PPV for the conventional approach was 91.9% while the NPV was 90.9%. The level of agreement (Kappa Statistic) between the two approaches was 0.87. Findings from this study indicate that there were no statistically significant differences between the two approach (61). A related study from Uganda to compare same-day and conventional strategy with LED in 2011 showed that there was no difference in sensitivity between the single- specimen and two-specimen LED FM strategies (61 vs. 64%; difference, 23%; 95% CI, 27to 11%). There was also no significant difference in specificity between the two strategies (96 vs. 97%; difference, 21%; 95% CI, 22to 11%) (42).

On the other hand few studies showed disagreements between the two approaches. A study with cross sectional design using LED-FM conducted in India in 2014 among 1716 presumptive TB patients showed 218 (13%) were smear-positive from this 200 (11.7%) using same-day microscopy and 217 (12.7%) using the conventional approaches. The same-day method missed 8% of smear-positive cases; This result was reported that the same-day method was inferior to the conventional method (49). A similar study conducted in Cambodia in 2013 reveled that out

of the 3306 (71.6%) participant, 188 (6.4%) were smear positive. The same-day (spot- spot) and the conventional (spot-morning) missed 28/188 (14.9%, 95%CI 10.1–20.8) and (5.9%, 95%CI 3.0–10.2) cases respectively and the difference in the proportion of missed TB cases was 9.0% (95%CI 2.7–15.4). In this study there was also significant statically difference between the two strategies was reported (P=0.006) (50).

2.2.3. Concentrated same-day vs Direct same-day and Conventional Approaches

A study conducted in India to evaluate same-day diagnosis approaches after applying concentration technique showed that among 3186 participants, smear positivity was respectively 9.6%, 9.8% and 10.8% for ZN, MZN and FS smears using spot and morning smears. Using same-day smears, smear positivity was respectively 9.5%, 9.8% and 10.6% for ZN, MZN and FS smears. In this study Smear positivity increased to 16% was reported with the concentration method (62).

2.3. Diagnostic defaulter rate in conventional approaches

Conventional case-finding approaches usually involve microscopic examination of 'spot-morning' sputum specimens The majority of sputum results are therefore available only on the second or third day after the patient presents to a health service. A number of TB control programs have found high rates of initial patient default as a result, with high mortality recorded in several resource-limited settings. Patients assigned to same-day diagnosis were more likely to submit both specimens (drop-out, 2%) than patients screened conventionally (drop-out, 5.8%) (34).

A study conducted in India to compare ZN and FN staining methods reported that out of 3,328 participants all submitted Spot-1 and Spot-2 but 143 (4.3%) did not submit Morning sputum (44). Another study in India, shows those during the study period a total of 1605 patients were included and 68 (4.2%) patients dropped out (58). Similarly a study conducted in India showed that of 1845 presumptive TB patients enrolled, 1716 (93%) provided all three samples hence 7% of the patients failed to provide all three specimens (49).Another study conducted in India in 2012 showed Of the 2283 adult presumptive TB patients 51 (2.2%) were excluded because they provided only one sputum sample, and 23 (1.0%) provided two same-day samples only Among the 23 defaulters, three had smear-positive results(48). Another study in India in India in 2015

showed conventional approach dropped out rate was 4.3% (55). Similarly 4.2% dropped out rate was reported from India in 2014 (58) . A study conducted in Cambodia showed that of the 4616 presumptive TB patients enrolled 3306 (71.6%) provided all sample; 28.4% defaulter rate was reported (50).

Evidence from Libya in 2013 showed out of 412 participants 39 patients did not return the second day (60). A study conducted in Nigeria showed that out of 1487 patients 163 (11%) dropped out was reported in conventional scheme (S–M) (54) . A cross sectional study conducted in Uganda at 2015 from 316 TB suspects. Out of the 316 patients recruited in the study only 253 submitted the morning sample, and out of 63(20.0%) defaulters 6 (15%) patients with a positive spot 1 was reported (59). Another study conducted in 2012 in Uganda showed out of 229 patients 20 (9%) patients did not return the next day to provide the second sample Of these, six were smear positive on the first smear while four were culture positive (61).

A study reported from Ethiopia among 392 patients revealed 30 (7.65%) participants failed to summit second day sputum sample was reported (56).

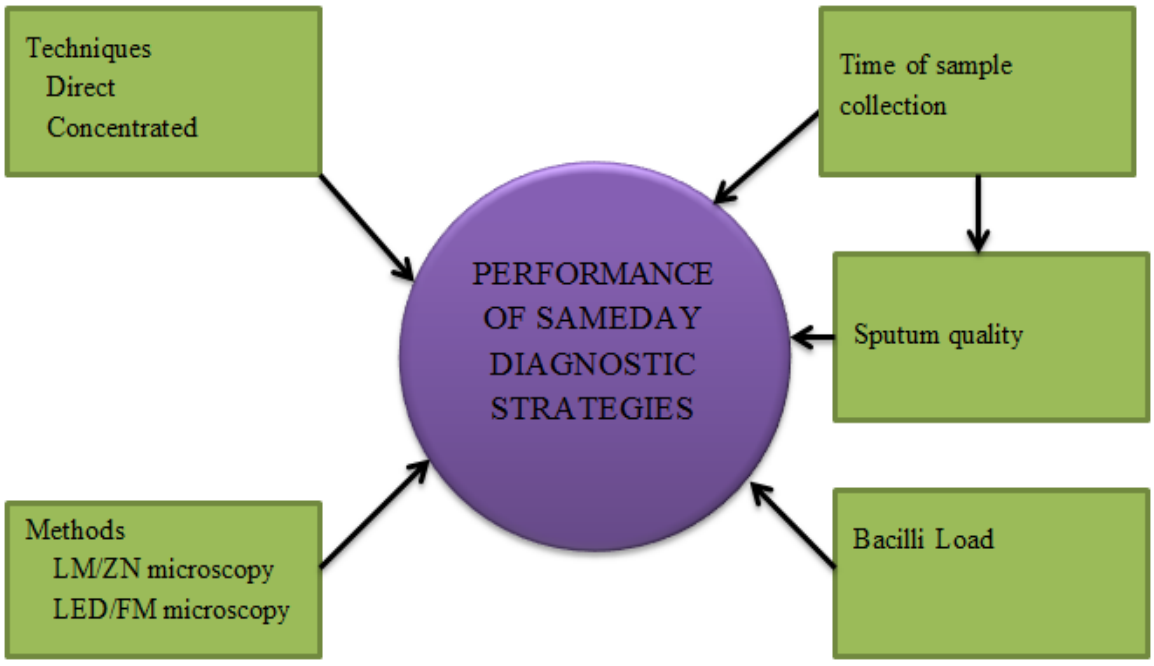

Figure 1 Conceptual framework**.** (Source: Prepared by principal investigator)

CHAPTER THREE: OBJECTIVES

3.1. General Objective:

To assess the performance of same-day microscopic diagnosis approaches for pulmonary tuberculosis: with different combination of techniques and methods at Jimma University Mycobacteriology Research Center, South west Ethiopia.

3.2. Specific objectives:

- To determine performance of same-day diagnostic approaches for pulmonary tuberculosis using ZN stained from direct sputum sample and from bleach concentrated sample.
- To determine performance of same-day diagnostic approaches for pulmonary tuberculosis using LED-FM stained from direct sputum sample and from bleach concentrated sample.

CHAPTER FOUR: MATERIALS AND METHODS

4.1. Study area and period

The study was conducted at Jimma University Mycobacteriology Research Center (JUMRC) from February, 2016 to November 2016.

Mycobacteriology research center of Jimma University was established as part of IUC-JU collaborative research project between Jimma University and consortium of Flemish Universities from Belgium. The establishment began in 2009 and became operational in November 2010.

Description: The center activities are mainly focused on basic research, training and service in the field of Mycobacteriology. The research component of the laboratory is mainly on basic and applied research which ranges from optimization of detection methods to molecular level research. The laboratory is also open for training on Mycobacteriology techniques up on request by academic departments. It is involved in the provision of service to the patients as a part of national Mycobacteriology laboratory network.

Available technologies: AFB conventional microscopy, LED-Fluorescent Microscopy, Culture to detect MTB using solid media (Lowenstein-Jensen and Middlebrooke media), Culture to detect MTB using BACTEC MGIT 960 TB detection system, Antibiogram on solid media (Lowenstein-Jensen) and liquid media using BACTEC MGIT 960 TB detection system and GeneXpert MTB/RIF (molecular automated MTB detection and resistance profiling against rifampicin) and Line Probe Assay, or GenoTypeMTBDRplus assay (63).

4.2. Study Design

A comparative cross-sectional study was employed.

4.3. Population

4.3.1. Source population

All presumptive TB patients who were attending at Jimma University Mycobacteriology Research Center during study period.

4.3.2. Study population

Presumptive PTB patients who were requested for sputum smear microscopy during the study period and fulfill the inclusion criteria.

4.4. Eligibility criteria

4.4.1. Inclusion criteria

- Patients whose age $\geq$ 15
- New tuberculosis presumptive Patients who can be able to produce enough sputum sample (4 ml for each sample collection)

4.4.2. Exclusion criteria

- Patients that unable to give enough amount of sputum sample or can't produce sputum sample
- Patients who were on anti-TB treatment at the time of data collection.

4.5. Sample size determination and Sampling technique

4.5.1. Sample size determination

Available information indicated that for methods/approaches comparisons 40-100 sample size is recommended. For new method validation, 100 to 200 are recommended to assess whether the new method's specificity is similar to that of the comparative method (29,64–67). Therefore, the sample size was 126.

4.5.2. Sampling technique

Consecutively identified presumptive pulmonary tuberculosis (PTB) patients who fulfilled the inclusion criteria were enrolled until the required sample size was met during the study period.

4.6. Study variables

4.6.1. Dependent variables

Performance of Same-day diagnosis approaches for sputum smears microscopy.

4.6.2. Independent variables

- Direct sputum smear microscopy
  - LM/ZN microscopy
  - LED/FM microscopy
- Concentrated smear microscopy
  - LM/ZN microscopy
  - LED/FM microscopy
- Time of sample collection, Sputum quality and Load of the Bacilli.

4.7. Data collection instruments and procedures

4.7.1. Demographic data

Data collection tools (questionnaire / variables; biological measurements / lab investigations): After obtaining written consent as specified **(Annex II)** data on demographic characteristics and clinical information data were collected using a structured questionnaire as specified (**Annex III**). This questionnaire is adapted from other similar studies and prepared in English and back translated into Afan Oromo and translated back into English to check its consistency.

Before the actual data collection, the Afan Oromo and Amharic questionnaire version were pretested in other settings to ensure that respondents are able to understand the questions and to check the wording, logic and skip order of the questions. Improvements were made after pre-testing.

4.7.2. Laboratory data collection

4.7.2.1. Sputum sample collection

From each study participants four sputum specimens (each of measure 4 ml) were collected after explaining the purpose, benefit and risk of the study by PI (Annex **III)**. After the first routine spot-1 specimen, patients were requested to produce a second specimen one hr. after the first spot sample collected and one container were given to the patients for collecting third an early morning sample at home on the next day. The second routine on-the spot (spot-3) specimen were obtained on following day, when the early-morning specimen submitted. Finally participants were provide four sputum specimens in two days, on the first day spot-1 and spot – 2 and on the

second day early morning sample and spot-3. The specimens were registered and macroscopically examined for their physical appearances and two smears was prepared from each samples on labeled slides and fixed.

Sample storage

If any delay was anticipated, the sample was stored at 2-8^{0}c and processed within three days.

4.7.2.2. Direct smear stained with ZN and LED-FM

After preparation of two direct smear from each sample one Ziehl-Neelsen (ZN) staining was performed as Standard Ziehl-Neelsen staining procedures were applied (carbolfuschin) as a primary stain, the acid alcohol for decolourisation and the counter stain methylene blue). Stained slides were examined for acid-fast bacilli under a 100x oil immersion objective. AFB results were reported for the presence or absence of acid-fast bacilli (AFB) using the WHO/IUATLD scale, with a positive result corresponding to ≥ 1 AFB per 100 high-power fields.

LED fluorescent staining was performed with fluorescence staining that utilizes fluorescent dye (auramine-O) as a primary stain, the acid alcohol for decolourisation and the counter stain (Potassium permanganate). Stained slides were examined for acid-fast bacilli by light emitting diode (LED) fluorescent microscope under a 20x or 40 x objectives. AFB results were reported for the presence or absence of acid-fast bacilli (AFB) using the WHO/IUATLD scale, with a positive result corresponding to ≥ 1 AFB per 20x for screening and 40 x for confirmation (68–71).

4.7.2.3. Bleach concentrated smear stained with ZN and LED-FM

Additionally after mixing spot-1 and spot-2, it was treated with 5% NaOCl and a simple concentration technique was performed. Two smears were performed from the sediment and stained with ZN and LED florescence. AFB results were reported for the presence or absence of acid-fast bacilli (AFB) using the WHO/IUATLD scale

4.7.2.4. Culture on LJ

Finally from early morning sputum sample and the left over specimen of pools were processed by NALC-NAOH method by specimens were digested and decontaminated by the sodium hydroxide and N-acetyl-L-cysteine (NaOH/NALC) method, with final concentrations of 1% for

NaOH. Equal volume of NaOH-NALC-sodium citrate solution and sputum specimens were added together in sterile 50 ml falcon tubes (BD, Becton Drive, Franklin Lakes, NJ, and USA). Tubes were vortexed lightly for about 15-30 seconds and allowed to stand for 15 minutes at room temperature. After complete digestion, phosphate buffer (*p*H 6.8) added up to 45 ml mark of falcon tube. The tubes containing the mixture were centrifuged at the speed of 3000 revolution per minute (rpm) for 15 minutes. After decanting the supernatant, the pellet was re-suspended by 1 ml of phosphate buffer (*p*H 6.8) and 3 drops (0.2 ml) of the suspended was used for inoculation on L-J media. The inoculated L-J slants were incubated at 37℃ and examined weekly for 3-8 weeks. Growth of the mycobacteria was confirmed by visual detection of colonial morphology and by microscopic examination of the colonies for acid-fast bacilli (AFB). For confirmed growth, identification was done by the para-nitrobenzoic acid inhibition test.

4.7.2.5. Para-nitrobenzoic acid (PNB) susceptibility test

Finally Differentiation of MTBC from NTM was performed by para-nitrobenzoic at 500 micro gram /ml susceptibility test for AFB positive colonies. The inoculum from LJ media was transferred to tube containing 1ml of sterile distilled water. Then, turbidity was adjusted to McFarland standard #1. Finally, two LJ media (one with PNB and the other drug-free control) were inoculated with 100 µl of the suspension and incubated at 37^0c. Reading of the LJ tubes was done on the 14^{th} and 28^{th} day. Strain with observable growth at 28^{th} day of incubation was considered resistant.

Interpretation of results for the approaches

The participants were taken as ‘smear positive’ if one of (spot-1 and spot-2) specimens was positive for same-day approaches and a participants was taken as ‘smear positive’ if any of the two from (spot-1, early morning and spot-3) specimens was positive for conventional approaches. Participants with positive cultures were considered to have confirmed TB to calculate sensitivity, specificity, positive predictive value and negative predictive value of the two approaches.

All smears were graded using the WHO/International Union against Tuberculosis and Lung Disease (IUATLD) scale or Smears were graded negative, scanty and 1+ up to 3+ To assess grading, for sameday approaches (the higher grade of one of the spot-1 and spot-2 and for

conventional approaches (one the higher grade of one of the spot-1, early morning and spot-3) was taken for all strategies.

Each participants provided four specimens (SPOT-1, SPOT-2, Early morning and SPOT-3) specimen. From these specimens one of the best from SPOT-1 or SPOT-2 was taken for physical examination of same-day diagnosis approaches. One of the best specimens from early morning or SPOT-3 was taken for physical examination of conventional approaches.

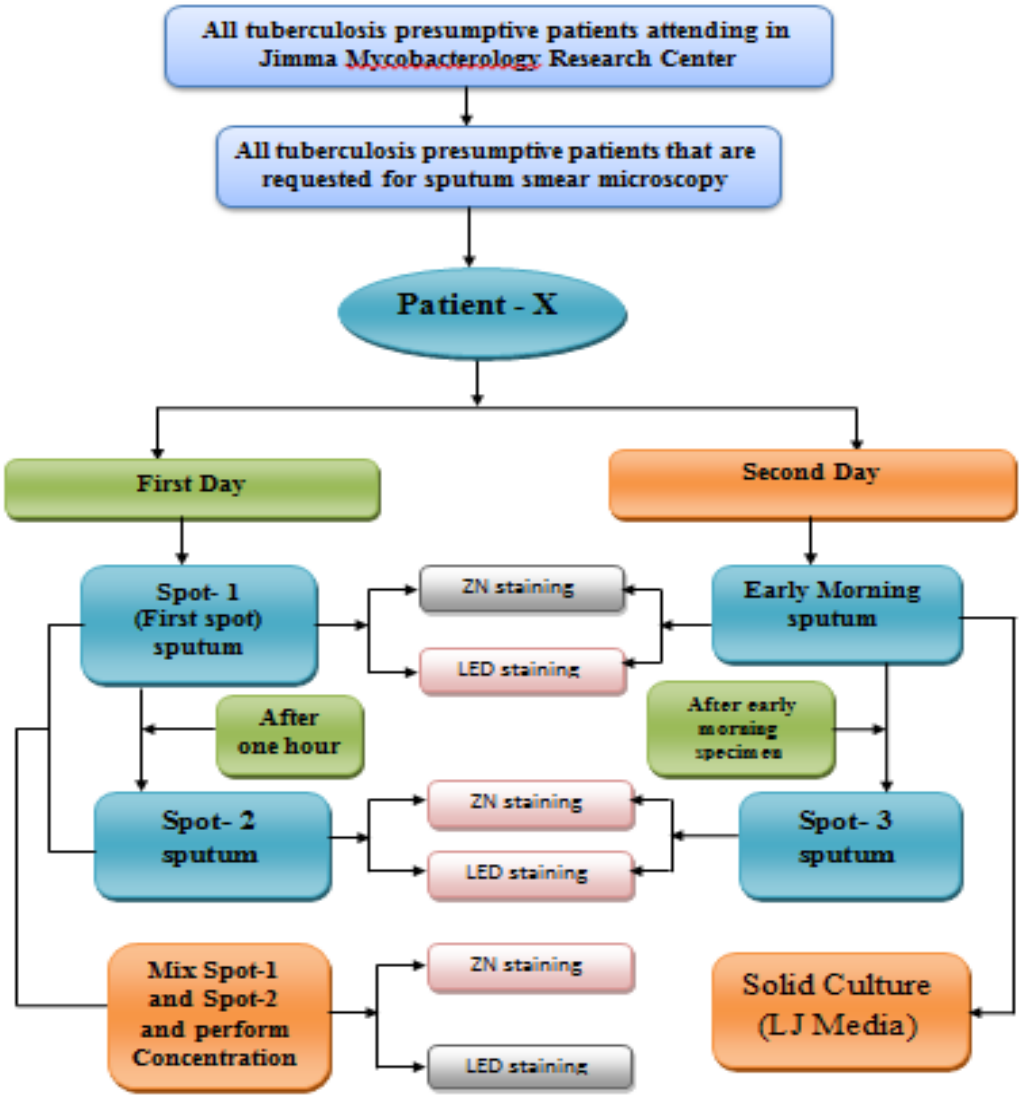

Figure 2 Flow chart of showing study participant recruitment and laboratory work flow at JUMRC, Jimma, southwest Ethiopia. (Source: Prepared by principal investigator)

4.8. Data management and statistical analysis

The questionnaire and laboratory sheet were checked for consistency and completeness. The data was entered using Epidata 3.1 and exported to SPSS version 20 computer software. The data were analyzed using SPSS version 20 computer software. Descriptive statistics was used to describe the study variables. The sensitivities, specificities, likelihood ratio negative (LR-) and likelihood ratio positive (LR+) of sputum smear microscopy for both approaches was determined using culture as the reference standard and 95% CI was calculated for statistical significance. For other variables, kappa statistic was used to determine the level of agreement between the two approaches. Kappa result was interpreted as follows: values $0 \leq$ as indicating no agreement and 0.01–0.20 as none to slight, 0.21–0.40 as fair, 0.41– 0.60 as moderate, 0.61–0.80 as substantial, and 0.81–1.00 as almost perfect agreement (72).

4.9. Data quality assurance

To ensure the quality of data, different quality control activities were involved, including standardization of procedures. All laboratory tests were done by strictly following the standard operation procedures (SOPs) and manufacturer instructions. Reagent was checked for their expiry date before any analysis is started, known positive +1 grade sputum sample and negative sputum sample was used to check the quality of ZN staining reagents and fluorescence staining reagent at each batch of reagent preparation and H37Rv strain was used to check the performance of LJ tube for supporting the growth of *M. tuberculosis.*

4.10. Ethical consideration

Ethical clearance obtained from Institutional Review Board Committee (IRB) of Jimma University, Institute of Health. Formal letters obtained from IRB and summited to Jimma University Mycobacteriology Research Centre to get permission and cooperation in order to conduct the study. Written consent and ascent (for < 18 age years) obtained from each study participants after clear discussion on the purposes and procedures of the study. All information of the participants kept confidential and the test result used only for the research and the laboratory results were reported back to the physicians/Nurse for decision making as early as available.

4.11. Dissemination and utilization of results

The results of the study will be presented to the Institute of Health, School Of Medical Laboratory Sciences as part of M.Sc. thesis. It will be presented in different national and international conferences. The final draft will be sent to different scientific journals for publication.

4.12. Operational and Standard definition

Conventional approach: is a way collecting three sputum specimens within two days or (spot-morning-spot) and the results are reported on the second day.

Same-day approach: is a way of collecting two spot specimens in one hour interval on one/single day and the results are reported on first day.

Same-day Direct ZN strategy: is a same-day approach with direct smear and stained with ZN and examined with Light microscopy.

Conventional Direct ZN strategy: is a conventional approach with direct smear and stained with ZN and examined with Light microscopy.

Same-day Concentrated ZN strategy: is a same-day approach with 5 % bleach concentrated smear and stained with ZN and examined with Light microscopy.

Same-day Direct LED-FM strategy: is a same-day approach with direct smear and stained with fluorescence stains and examined with LED-FM microscopy.

Conventional Direct LED-FM strategy: is a conventional approach with direct smear and stained with fluorescence stains and examined with LED-FM microscopy.

Same-day Concentrated LED-FM strategy: is a same-day approach with 5 % bleach concentrated smear and stained with fluorescence stains and examined with LED-FM microscopy.

Turnaround time: is duration of time from sputum sample collection up to result dispatch

Tuberculosis presumptive Patients: A person who presents with symptoms and/or signs suggestive of tuberculosis, in particular cough for two weeks or more.

New case of TB - A patient who has never been treated for TB or who has taken anti-TB drugs for less than one (<1) month.

Pulmonary tuberculosis (PTB) - Tuberculosis involving only the lung parenchyma.

Smear-negative PTB - At least two sputum specimens negative for AFB by direct microscopy.

Smear-positive PTB - One sputum smear examination positive for Acid Fast Bacilli (AFB) by direct microscopy.

Relapse (R): A patient declared cured or treatment completed of any form of TB in the past, but who reports back to the health service and is now found to be AFB smear-positive or culture positive.

Diagnostic defaulters: are presumptive TB patients failing to return on the second day to provide a second specimen or receive results.

Sensitivity: The ability of the approaches to identify correctly those **who have** the pulmonary tuberculosis disease.

Specificity: The ability of the approaches to identify correctly those **who do not have** the pulmonary tuberculosis disease.

Likelihood ratio negative (LR-) - is the ability of the approaches to changes the probability of the absence of disease from test results.

Likelihood ratio positive (LR+) - is the ability of the approaches to changes the probability of the presence of disease from test results.

Bacteriologically confirmed case of TB - A patient from whom a biological specimen is positive by smear microscopy, culture or WHO-approved rapid diagnostic tests.

CHAPTER FIVE: RESULT

5.1 Socio-demographic characteristics and medical history of participants

A total of 126 tuberculosis presumptive patients were enrolled for this study and out of the total 3 participants were defaulters and 3 participants samples were rejected from final analysis because of repeated contamination during culture. A total of 120 participants included in the analysis. Of the 120 participants included in the analysis, 34 (28.3 %) were culture-positive for *M. tuberculosis* complex (MTBC) on LJ culture.

The mean age of the participants was 39.17 years with (SD of ±16.45 years), ranging from15-73 years old. From the total participants that were enrolled in the study 73 (60.8 %) were male and 86 (71.7%) were from urban settings. The baseline characteristics of the study participants were summarized in table 1 below.

Table 1: Socio-demographic characteristics of the participants

Characteristics	Frequency (%)
Age	
15-19	12 (10)
20-24	15 (12.5)
25-29	12 (10)
30-34	12 (10)
35-39	11 (9.2)
40-44	16 (13.3)
45-49	11 (9.2)
Above 50	31 (25.8)
Mean ± SD = 39.17 ±16.446 years	
Min-max = 15 – 73 years	
Sex	
Female	47 (39.2)
Male	73 (60.8)
Residence place	
Rural	34 (28.3)
Urban	86 (71.7)

Symptoms presented by the participants also assessed 51 (42.5%) had only (cough and chest pain) , 40 (33.3 %) had (Cough, chest pain, fatigue, fever and night sweats), 21 (17.5 %) had a (Cough, chest pain, fatigue, fever, night sweats, loss of appetite and weight loss), 8 (6.7 %) had

(Cough, chest pain, fatigue, fever, night sweats, loss of appetite, weight loss, bloody sputum and shortness of breath.

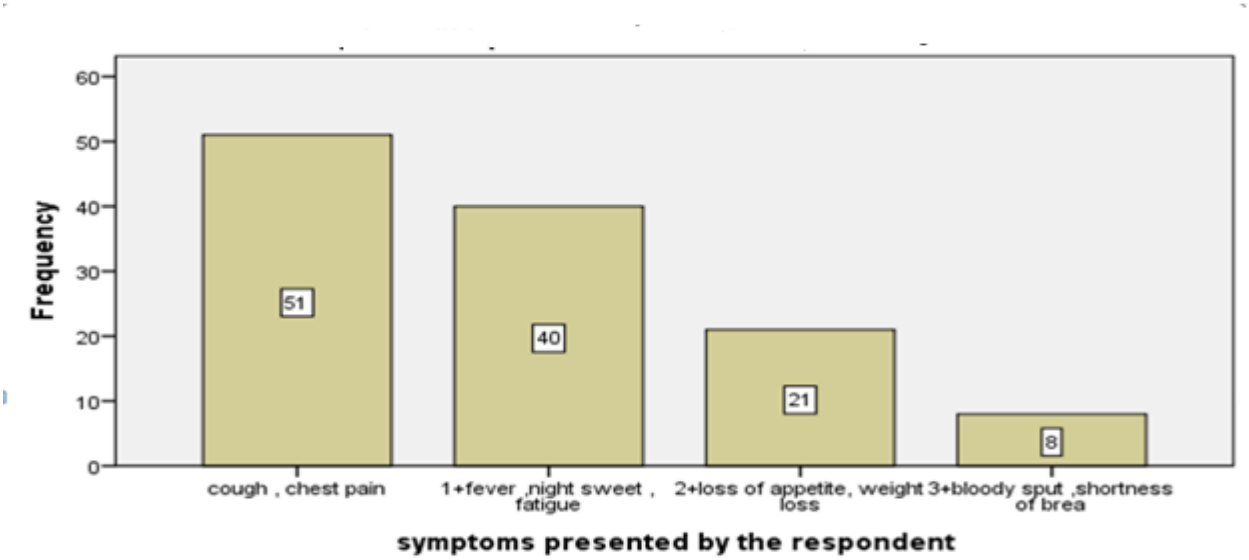

Figure 3 Showing frequency of sign and symptoms presented by respondents at JUMRC, Jimma, southwest Ethiopia from February, 2016 to November 2016 (n=120)

5.2. Examination of physical appearance sputum specimen between approaches

From the 120 eligible participants, 87 (72.5%) of the collected sputum were mucoid for same-day diagnosis approaches as shown in (**Table 2**). In conventional diagnosis approaches the majority 88 (73.3%) sputum sample were mucoid as shown in (**Table 2**). The quality of sputum was 81.2 % similar (kappa value 0.812).

Table 2: Comparison the appearance of sputum sample between same-day and Conventional Strategy

Quality of sample	Same-day strategy		Conventional strategy	
	Frequency	Percentage	Frequency	Percentage
Muco-salivary	14	11.7%	8	6.7%
Mucoid	87	72.5 %	88	73.3%
Purulent	11	9.2%	15	12.5%
Bloody	8	6.7%	9	7.5%
Total	120	100 %	120	100 %

5.3. Detection rate

Sputum was collected from 120 tuberculosis presumptive patients for this study. Of 120 participants samples, AFB was detected in 25/120 (20.8 %) of cases by same-day direct ZN strategy, 31 (25.8 %) of the cases on conventional direct ZN strategy and 32/120 (26.7 %) of the cases by same-day concentrated ZN strategy. Acid fast bacilli were also detected in 30/120 (25 %) of the cases on same-day direct fluorescence strategy, 31/120 (25.8 %) of the cases on conventional direct fluorescence strategy and 34/120 (28.3 %) of the cases by same-day concentrated fluorescence strategy (**Figure 4**).

Based on result of LJ (gold standard method), 35 cases were culture positive cases. In this study species identification was done using para-nitrobenzoic acid inhibition test. Accordingly out of 35 culture positive cases, 34 (97.15%) were *Mycobacterium tuberculosis complex* (MTBC) and the rest 1 (2.85%) were *Non-tuberculosis mycobacteria* (NTM).

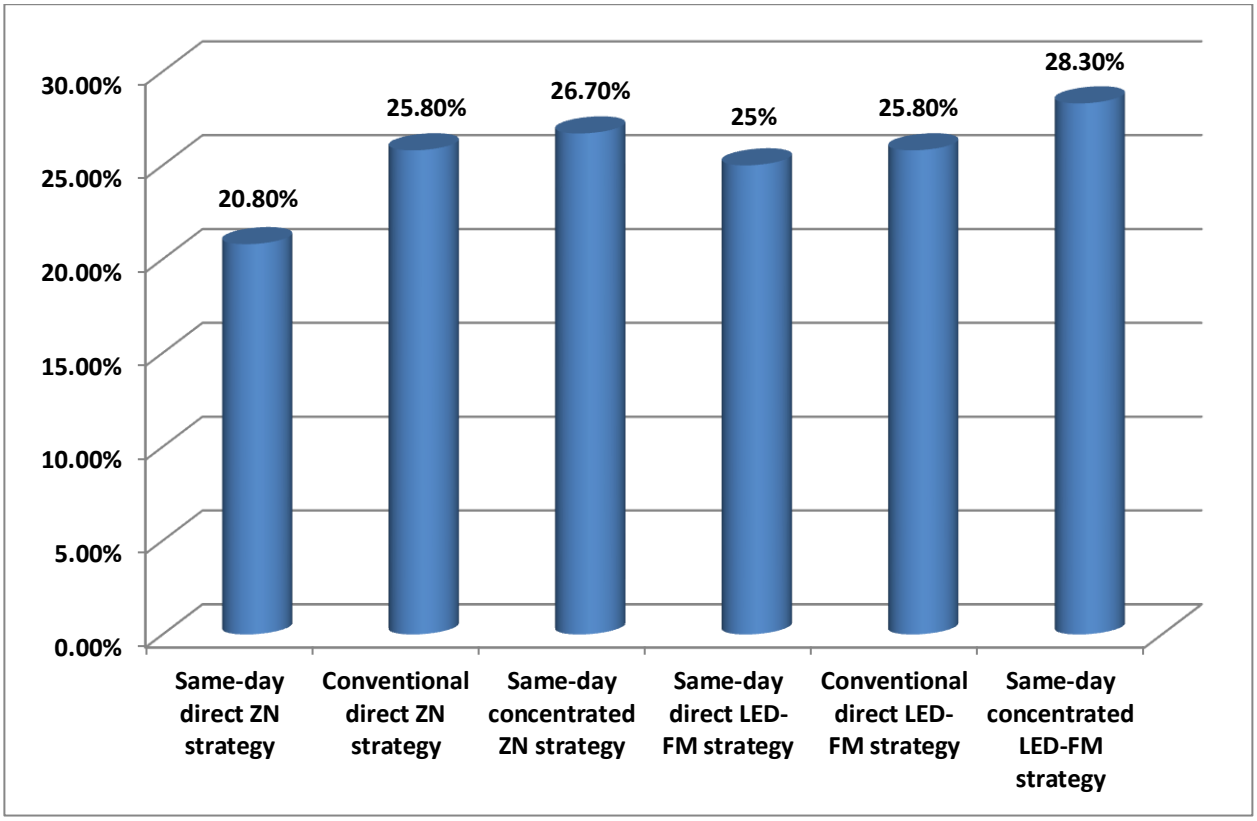

Figure 4. Detection rate of sputum smear microscopy in different strategy at JUMRC, southwest Ethiopia, from February, 2016 to November, 2016

5.4. Same-day diagnosis approaches vs. conventional diagnosis approaches

5.4.1. Same-day approach vs. Conventional approach with ZN staining

Out of 120 respondents eligible for analysis the same-day direct ZN strategy detected 25/120 (20.8 %) AFB cases with its sensitivity (SEN) 70.59 %, specificity (SPC) 98.84 %, likelihood ratio positive (LR+) 60.71% and likelihood ratio negative (LR-) of 29.76% against the gold standard culture. The conventional direct ZN strategy detected 31 (25.8 %) AFB cases with its SEN (88.23 %; 95CI: 77.41% - 99.07%), SPC 98.84, LR+ 75.89% and LR- of 11.90 % (**Table 3**). The same-day direct ZN strategy missed six cases when compared to conventional direct ZN strategy and the same-day direct ZN strategy had significantly lower sensitivity than conventional direct ZN strategy.

Same-day concentrated ZN strategy detected 32/120 (26.7 %) AFB cases with its SEN, SPC, LR+ and LR- of 91.18 %, 98.84 %, 78.42% and 8.93 % respectively (**Table 3**). It has comparable performance compared to conventional direct ZN strategy. The same-day concentrated ZN strategy detected seven cases that were missed by the same-day direct ZN strategy. The same-day concentrated ZN strategy increase an incremental yield of same-day direct ZN strategy by 5.84 % (7/120).

5.4.2. Same-day approach vs. Conventional approach with fluorescence staining

Out of 120 respondents the same-day direct fluorescence strategy detected 30/120 (25 %) AFB cases with its SEN, SPC, LR+ and LR- of 85.30 %, 98.84 %, 73.36 % and 14.88 % respectively. The conventional direct fluorescence strategy detected 31/120 (25.8 %) AFB cases with its SEN, SPC, LR+ and LR- of 88.23 %, 98.84 %, 75.89% and 11.90 % respectively (**Table 3**). The Same-day fluorescence strategy missed only one patient that was detected by conventional direct fluorescence strategy. So, no difference was found between the two strategies.

Same-day concentrated fluorescence strategy detected 34 (28.3 %) AFB cases (i.e. 3 of 34 were culture negative) with its SEN, SPC, LR+ and LR- of 91.18 %, 96.6 %, 26.14 % and 9.15 % respectively. The same-day concentrated fluorescence strategy had comparable performance compared to same-day direct fluorescence strategy and conventional direct fluorescence strategy.

The same-day concentrated fluorescence strategy detected two patients that were missed by the same-day direct fluorescence strategy. The same-day concentrated fluorescence strategy increases an incremental yield of same-day direct fluorescence strategies by 1.66 % (2/120) (**Table 3**).

5.5. Turnaround time (TAT) of the different strategies

Turnaround time were also assessed and in our study the average time to dispatch the result using same-day direct ZN strategy was 109 min/patient whereas the same-day concentrated ZN strategy was 139 min/patient. The average time to prepare sputum smear was 2.1 min/ slide for same-day direct ZN strategy and 0.83 min/slides for same-day concentrated ZN strategy. The average time to read sputum smear was 2.81 min/ slides for same-day direct ZN strategy and 1.72 min/ slides for same-day concentrated ZN strategy (**Table 4**).

The average time to dispatch result for same-day direct fluorescent strategy was 122.08 min/ patient whereas the same-day concentrated fluorescent strategy was 153.6 min/patient. The average time to prepare sputum smear was 2.1 min/ slide for same-day direct fluorescent strategy and 0.83 min/slides for same-day concentrated fluorescent strategy. The average time to read sputum smear was 1.8 min/ slides for same-day direct fluorescent strategy and 1.72 min/ slides for same-day concentrated fluorescent strategy (**Table 4**).

Table 3: Accuracy of Strategies in Different Existing Methods and Techniques against culture

Validity of Results		Strategies in Different Existing Methods and Techniques											
		SMDZN		**CODZN**		**SMCZN**		**SMDFN**		**CODFM**		**SMCFM**	
		Pos	**Neg**	**Pos**	**Neg**	**Pos**	**Neg**	**Pos**	**Neg**	**Pos**	**Neg**	**Pos**	**Neg**
Culture	**Pos (n=34)**	24	10	30	4	31	3	29	5	30	4	31	3
	Neg (n=86)	1	85	1	85	1	85	1	85	1	85	3	83
Sensitivity **[**95%CI**]**		70.59 % [55.28- 85.91]		88.23 % [77.41 - 99.07]		91.18 % [81.65 – 100]		85.30 % [73.39 - 97.20]		88.23 % [77.41 - 99.07]		91.18 % [81.65 – 100]	
Specificity **[**95%CI**]**		98.84 % [96.58 – 100]		98.84% [96.58 – 100]		98.84 % [96.58 – 100]		98.84 % [96.58 – 100]		98.84% [96.60 – 100]		96.60 % [92.64 – 100]	
Likelihood ratio positive **[**95%CI**]**		60.71 % [8.54 – 100]		75.89 % [10.77 – 100]		78.42 % [11.14 – 100]		73.36 % [10.39 – 100]		75.89 % [10.77 – 100]		26.14 % [8.55 – 79.83]	
Likelihood ratio negative **[**95%CI**]**		29.76% [17.67 - 50.11]		11.90 % [4.74 - 29.89]		8.93 % [3.02 – 26.30]		14.88 % [6.62 – 33.44]		11.90 % [4.74 - 29.89]		9.15 % [3.10 – 26.95]	

CODFM = Conventional Direct fluorescence strate
CI = Confidence Interval
SMCZN = Sameday Concentrated ZN strategy
SMCFM = Sameday Concentrated fluorescence strategy
% = in percent
n = in number
Pos = Positive
Neg = Negative
CODZN = Conventional Direct ZN strategy
SMDFM = Sameday Direct fluorescence strategy
SMDZN = Sameday Direct ZN strategy

Table 4: Turnaround time of strategies in Different Existing Methods and Techniques

Diagnostic strategies	Sample collection average time	Sputum centrifugation average time	Sputum smear average time	Smear drying average time	Staining and drying time	Result reading average time	Average turnaround time
Same-day Direct ZN strategy	1hr + 10min (70 min)/ patient	_	4.2 min/ (2 smears)	9.38 min/ slide	11.3 + 8.5 (19.8 min)/ slides	5.62 min/ (2 slide)	109 min/patient (1.82 hr/ patient)
Same-day Concentration ZN strategy	1hr + 10min (70 min)/ patient	32 min/ (1 patient)	0.83 min/ (1 smear)	14.65 min/ slide	11.3 + 8.5 (19.8 min)/ slides	1.72 min/ slide	139 min/patient (2.32 hr/ patient)
Same-day Direct LED-FM strategy	1hr + 10min (70 min)/ patient	_	4.2 min/ (2 smears)	9.38 min/ slide	26.4+8.5 (34.9 min)/ slides	3.6 min/ (2 slide)	122.08 min/ patient (2.04 hr/ patient)
Same-day Concentration LED-FM strategy	1hr + 10min (70 min)/ patient	32 min/ (1 patient)	0.83 min/ (1 smear)	14.65 min/ slide	26.4+8.5 (34.9 min)/ slides	1.22 min/ slide	153.6 min/ patient (2.56 hr/ patient)

hr = hour
min = minutes

5.6. Smear Grade of both approaches

Same-day direct ZN strategy, a total of 25 cases was AFB positive and half of them 44% (11/25) were 2+ as shown in (**Table 5**). Whereas from 31 AFB positive cases on conventional direct ZN strategy 41.9% (13/31) graded 3+ as shown in (**Table 5**). Concordance between the two approaches was moderate (Kappa value = 0.49).

The same-day direct ZN method strategy, a total of 25 cases were AFB positive and 44% (11/25) were 2+as shown in (**Table 5**). Whereas, among 32 AFB positive cases on same-day concentration ZN method strategy, 50 % (16/32) of the cases graded 3+ as shown in (**Table 5**). Among 7 smears which were negative by the same-day direct ZN method strategy, 6 were changed to 1+ and 1 were changed to 2+ by the same-day concentrated ZN method strategy. Of one smear scanty by the same-day direct ZN method strategy increased to 1+ by the same-day concentrated ZN method strategy. Of 7 smears graded as 1+ by the sameday direct ZN method strategy, 5 were changed to 2+and 2 were increased to 3+ by the same-day concentrated ZN method strategy. Of 11 smears graded as 2+ by the same-day direct ZN method strategy, 3 remained 2+, and 8 were increased to 3+ by the same-day concentrated ZN method strategy. So same-day concentration ZN method strategy had significant higher grade of smear result (Kappa value = 0.144).

On same-day direct fluorescence strategy, a total of 30 cases were AFB positive and the majority of them 60% (18/30) were 3+ as shown in (**Table 5**). Whereas from 31 AFB positive cases on conventional direct fluorescence strategy, 77.4% (24/31) graded 3+ as shown in (**Table 5**). Agreement between the two approaches was good (Kappa value = 0.65)

On same-day direct fluorescent strategy, a total of 30 cases were AFB positive and the majority of them 60% (18/30) were 3+as shown in (**Table 5**). Whereas among 34 AFB positive cases on same-day concentration fluorescence strategy, majority of the cases 85.3 % (29/34) were graded 3+ as shown in (**Table 5**). Three of the smears (i.e. - two of the three were culture negative) which were negative by the same-day direct fluorescent method strategy 2 of them were changed to 1+ and 1 were changed to 2+ by the same-day concentrated fluorescent strategy. Of 2 smears graded as 1+ by the same-day direct fluorescent strategy, 1 were increased to 2+ and 1 changed to 3+ by the same-day concentrated fluorescent strategy. Of 10 smears graded as 2+ by the

sameday direct fluorescent strategy, all or 10 of the 10 changed to 3+ by the same-day concentrated fluorescent strategy. Hence, same-day concentration fluorescence strategy had higher grade of smear than same-day direct fluorescence strategy (Kappa value = 0.03).

Table 5: AFB grading of smears on Different Strategies in Existing Methods and Techniques

Grades of Results	Strategies in Different Existing Methods and Techniques					
	SMDZN n (%)	CODZN n (%)	SMCZN n (%)	SMDFN n (%)	CODFN n (%)	SMCFN n (%)
Scanty	1 (4%)	2 (6.5%)	-	-	-	-
1+	7 (28%)	5 (16.1%)	8(25%)	2 (6.7%)	1 (3.2%)	2(5.9 %)
2+	11 (44%)	11(35.5%	8(25%)	10(33.3%)	6(19.4%)	3(8.8 %)
3+	6 (24%)	13(41.9%)	16(50%)	18(60%)	24(77.4%)	29(85.3%)
Total	25 (100%)	31(100%)	32(100%)	30(100%)	31(100%)	34(100%)

5.7. Diagnostic defaulter rate

During the study period 126 participants were selected. Participants who present on first day only and absent on the second day were 3/126 (2.4 %). Two of the three had smear negative for spot-1 and spot-2 and one of the three had smear positive for spot-1 and spot-2.

CHAPTER SIX: DISCUSSION

Sputum smear microscopy remains the most frequent diagnosis of PTB particularly in developing countries (18). However, greater than 5000 bacilli per ml of sputum are required for reliable detection of TB bacilli by smear microscopy (19). Many studies have reported that the overall yield of smear microscopy for acid-fast bacilli (AFB) was reported to be superior with multiple specimens than with a single specimen. Processing of samples by centrifugation generally leads to higher yield by concentrating the bacilli. Bleach centrifugation found to be inexpensive, quick, and effective. Beside this, its disinfectant properties could improve infection control in laboratories lacking adequate biosafety facilities (31,73–75). However, in a country like Ethiopia, where smear-negative pulmonary TB is significantly high, the performance of the same-day diagnostic approach and the role of other alternative approaches such as bleach concentration have not been systematically investigated. Therefore, an attempt has been made to assess the performance of same-day microscopic diagnosis strategy with various methods and techniques in our study.

In our study the same-day direct ZN strategy (spot-spot) missed six TB cases and had significant lower sensitivity when compared to conventional direct ZN strategy (spot-morning-spot). The present finding is comparable with the study done in India (46) .The lower sensitivity and missing cases may due to the same-day strategy lost morning specimens and in our study showed same-day approach had 18.8 % disagreement sputum quality than the conventional approach. Morning specimens have mostly shown greater bacillary load and high sensitivity than spot samples (76). The low sensitivity of light microscopy and the lower bacillary load of the specimens collected at other periods led to more likely to be graded negative than specimens collected at morning specimens (53). If those six patients remain untreated may result in more extensive disease, resulting in suffering, and more complications that lead to a higher increase the risk of mortality. It also leads to an increased period of infectivity in the community because most transmissions occur between the onset of cough and initiation of treatment (77). Even though, the same-day direct ZN strategy has lower sensitivity, it has the following advantages: reduced laboratory workload, decreases repeated health center visit and health center expenses. It might also increase the proportion of TB cases treated through a reduction in the number of patients who had chance to drop out during the diagnostic process.

It has been well researched that the performance of AFB smear microscopy on sputum specimen can be significantly improved if the sample is liquefied with one or more chemical reagents and then concentrated by centrifugation or sedimentation before acid fast staining. In our study, same-day concentrated ZN strategy detected seven cases that were missed by the same-day direct ZN strategy approaches and increased the yield by 5.84% (7/120). Bleach treated same-day ZN strategy compensates the limitation of same-day direct ZN strategy. The increased sensitivity of the same-day ZN strategy after bleach concentration may be attributed to higher bacillus concentrations and clearer microscopic fields. To the best of our knowledge, there is no published data that evaluated the performance of same-day concentrated ZN strategy using bleach concentrated technique. But there were one study that had comparable result with our result which was conducted in India and showed that the performance of same-day concentrated ZN strategy increases the yield of TB cases by 16 % using the *N*-acetyl-l-cysteine-sodium hydroxide concentration technique (62). In our study, we use simple centrifuge which is cheap and available in all health facilities including remote health centers. We also used a bleach technique which is easily available near market and can kill the bacilli. Its disinfectant properties could recover infection control in laboratories lacking adequate biosafety facilities. The above study used N-acetyl-l-cysteine-sodium hydroxide for concentration technique which is expensive and cannot kill the bacilli. Furthermore, the centrifuge they used was not available in in all health facilities.

In addition to that our result showed the same-day concentrated ZN strategy significantly increased the result of smear grading. The grade difference may be due to the application of bleach-concentration through digestion of mucus and debris in sputum, resulting in a clearer microscopy field and it may have an advantage for same-day microscopic diagnosis by making the AFB simply detectible within a smaller time without compromising accuracy and can greatly reduce the delayed reading time needed for examination of smears, thereby increasing the number of smears to be examined besides increasing the incremental yield.

In contrast to the same-day direct ZN method that reported to has lower sensitivity compared the conventional direct ZN strategy. Our study showed that same-day direct fluorescence strategy missed only one patient that was detected by conventional direct fluorescence strategy and no significant difference was found between the two approaches. The comparability between the

two approaches may be due to the higher sensitivity (10 % more) of LED-FM microscopy over LM-ZN microscopy and the bacilli shine as bright yellow against a dark background, which makes them easily identifiable even in lower load of bacilli in sputum smears. Our study finding also similar with the study done in Uganda (42). Our result also showed that same-day direct fluorescence strategy detected one case that was lost by conventional direct fluorescence strategy due to "diagnostic defaulters" and both approaches were equally effective. Therefore, the same-day direct fluorescence strategy, reduced laboratory workload, decreases repeated health center visit and health center expenses and also save "diagnostic defaulters" who continue to move in the community and contribute significantly in disease spread.

Our result reveled same-day direct fluorescence strategy missed two cases when compared to same-day concentrated fluorescent strategy. The same-day concentrated fluorescent strategy increases yield of same-day direct fluorescent strategy by 1.67 % (2/120). Therefore reassuring these "incremental yield improvements" is better because missing a single infectious person who remains undetected and therefore untreated can infect between 10 and 15 people every year (39). Our current incremental yield improvements result were in-line with previous study conducted in India showed that the performance of same-day concentrated fluorescence strategy increases the yield of TB cases by 16 %. (62).

In addition to that in our study the same-day concentrated fluorescence strategy significantly increased the result of smear grading and it may have an advantage for same-day microscopic diagnosis by making the low bacilli load samples to concentrate and thereby, the AFB simply detectible within a shorter time and reduce the delayed reading time besides increasing the incremental yield.

Due to additional work associated with the bleach concentration technique one would think that the bleach treated same-day strategy may be more likely to delay smear result than direct same-day strategy. In contrast our result showed that examining slides with same-day bleach concentrated strategies (for both ZN and LED-FM) took less time than by same-day direct strategies (for both ZN and LED-FM) this may be due to application of bleach which caused clearer microscopic fields and higher bacillus concentrations. Our study also showed that the smearing time of same-day bleach concentrated strategies took less time than same-day direct

strategies this difference may be due to digestion of mucus and debris in sputum with bleach and decrease the viscosity of the sediment and easily applied on the slide. In addition our result also showed that same-day bleach concentrated strategies took extra (30 minutes for same-day ZN and 34.52 minutes for sameday fluorescence strategy) when compared to same-day direct strategies but the difference was just a matter of minutes as a result it didn't change/affect the turnaround time and a patient's waiting time so the patient can get the results in one day as with same-day direct strategy. Therefore, adding bleach centrifugation to same-day strategy changes a patient's turnaround time (for two day conventional strategy), thereby reduce travelling costs, economic burden of an additional visit and drop-out rates during diagnosis and ensures no delay in the initiation of treatment.

One of the major disadvantage of conventional strategy is patients drop out from diagnosis follow-up, during the study period 126 participants were selected but 3/123(2.4%) were diagnostic defaulter. From these defaulters who present on same-day only and absent on the second day, two of the three had smear negative for spot-1 and spot-2 and one of the three had smear positive for spot-1 and spot-2. Our result was comparable with a study conducted in India, 2.2 % (48). In contrast our result was less than in half with WHO report (5 %) (34) and less than in different proportion reported from Ethiopia 7.65% (56), Libya 9.4 % (60), Uganda 20.0% (59); 9% (61), India 7% (49); 4.3% (44); 4.2% (58) 6.3% (51) and Cambodia 28.4 % (50). Reason for the lower defaulter rate in our findings, might be due to socio-demographic features. It also due to methodological variation (sample size) or it may be due to day to day drive to participants to comeback on the next day.

Same-day direct fluorescence strategy has comparable Performance when compared to same-day concentrated ZN strategy and same-day concentrated fluorescence strategy but same-day concentrated ZN strategy and same-day concentrated fluorescence strategy increase the work load of technicians and use additional reagents and materials when compared to same-day direct fluorescence strategy. As a result same-day direct fluorescence strategy has better than the other strategies.

Limitation of the study

- HIV test result was not available for all of the patients suspected of pulmonary tuberculosis.
- Due to limited resources, mycobacterial species identification was done only by para-nitrobenzoic acid (PNB) test. On the other hand, we neither could sequence-confirmed MTBC species nor perform biochemical assays, which are regarded as gold standards for MTBC identification.

CHAPTER SEVEN: CONCLUSION

Of the 120 participants included in the analysis, 34 (28.3 %) were culture-positive for MTBC. The performance of the same-day direct ZN strategy was significantly lower than the conventional direct ZN strategy. However, bleach pretreatment of sputum sample followed by concentration (bleach treated same day ZN method) significantly increases the sensitivity and compensates the limitation of same-day direct ZN approach.

The same-day diagnostic approach with direct fluorescence microscopy showed comparable performance with the conventional direct fluorescence strategy, thereby reducing the diagnostic delay and patients visit to health center.

Our findings demonstrated that treatment of the sputum sample with bleach followed by centrifugation didn't significantly increase the performance of LED-fluorescence microscopy. Even if the two strategies had no significant difference the same-day concentrated fluorescent strategy increased an incremental yield of same-day direct fluorescence microscopy.

Same-day diagnostic approaches can be applied with same-day concentrated ZN strategy, same-day direct fluorescence strategy and same-day concentrated fluorescent strategy. Same-day direct fluorescence strategy better than the other strategies.

CHAPTER EIGHT: RECOMMENDATION

Based on the findings of this study the following points are recommended;

- Though the Ethiopian Ministry of Health has changed its policy from conventional smear microscopy (spot-morning-spot) to same-day strategy (spot-spot), alternative innovative approaches such as bleach concentration method should be in place to compensate the limitation of the same-day direct ZN strategy where shortage of LED-FM.
- The stakeholders should also take into consideration the LED- based fluorescence microscopy with or without bleach treatment for efficient diagnosis of pulmonary TB.
- I would recommend same-day direct fluorescence strategy.
- The diagnostic performance of the different approaches among HIV-positive and – negative patients need to be addressed through further study.
- Finally we suggest studies with a larger sample size to clarify findings for bleach concentration in line with same-day strategy.

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ANNEXES

ANNEX I: INFORMATION SHEET

A. English Version

Title of the project: Same day diagnostic approaches for pulmonary tuberculosis: Various Combination Strategies of existing Methods, at Jimma University Mycobacterology Research Center, South west Ethiopia

Name of Principal Investigator: Shemsedin Mohammed

Organization: Jimma University (Medical laboratory science and pathology department)

Name of sponsor: Jimma University

This information sheet is prepared for tuberculosis suspected patients attending Jimma University Mycobacteriology Research Center who will be involved in project entitled above. We are going to tell you about the whole process that will happen in the study and requesting you to participate voluntarily.

Description and Purpose of the study

Tuberculosis (TB) is the first cause of death due to infectious disease in the world. The existing conventional sputum smear microscopy procedure requires a TB suspect to give a spot sputum sample and another early morning sample which requires at least two visits. WHO issued a policy statement recommending that "countries that have implemented the current WHO policy for two day finding consider switching to same-day diagnosis. Information on same-day microscopy is limited and conflicting and no study has been conducted on this topic in this area.

Therefore, the aim of this study is aimed to determine the performance of same day diagnostic approaches for pulmonary tuberculosis: with various combination strategies of existing Methods, at Jimma University Mycobacteriology Research Center, South west Ethiopia.

Procedures

If you are willing to participate in the study, you will be asked to sign a consent form and the following procedures will be done.

- You will provide us 5 minutes interview
- We will take specimen for laboratory investigation

- The collected sample will be processed in Jimma University Mycobacteriology research Centre

Risks and discomforts

There is no any possible risk or discomfort during sample collection. All samples will be collected aseptically following Standard operational procedures.

Benefits and Compensation

By participating in this study, there will not be direct financial benefit. Based on the laboratory result you will be referred to the TB and ART clinic, Jimma University for further care and treatment.

Confidentiality

All information that all be collected from the study subjects will be kept confidential. Any information about the participant that will be collected from the study will be stored in a file that will not bear a name on it, but only a number assigned to it instead.

Voluntary participation and withdrawal:

Your participation in this study is voluntary. You may decide not to participate or you may leave the study at any time. Your decision will not result in any penalty or loss of benefits to which you are entitled. Your decision will not put at risk at any present or future medical care or other benefits to which you otherwise entitled. You should ask the study investigators listed below any questions you may have about this research study. You may ask questions in the future if you do not understand something that is being done.

Use the following address for any question.

Mr Shemsedin Mohammed, Phone No +251-911-906679, Email: shemsmoha@gmail.com

Dr Gemeda Abebe , Phone No +251-911-991285, Email: gemeda.abebe@ju.edu.et

Mr Keder Abdella , Phone No +251-938-341367, Email: kediroab2@gmail.com

For the success of our study, we will be asking you to give the correct answer for the respective questions. Thank you for your assistance. Continue answering the questions.

B. Afan Oromo version

Duudha Infoormeeshinii afaan oromootiin

Mata duree pirojektiicha:- Akkataa qoranoo akkee microscopidhan guyyaa tokko keessatii yaalii adda addatii fayyadamudhaan itti godhamu.Yuniversitii Jimmaati research Centeri Mycobacteriology.

Maqaa invasigaatara jalqabaa: Shaamsadeen Mohaammed

Waldaa: yuunivasiitii Jimmatti koolejji fayyaa muummee medical laaboraatorii fi paatooloogii.

Maqaa ispoonsaraa: yuunivarsiitii Jimmaa

Duudha informeeshina kun kan qophaayee Dhukkubsatoota warra akkee microscopiadhan akka sakatasisan ajajamaniif hordoffanuuf kan qorannookanarratti hirmaatanif ta'a. kanatti aansine adeemsa qoranno kana keessatti ta'u siif ibsine fedhii hirmaannakee sigaaffana.

Ibsafi kaayyoo qoranno kana: Standardiin yeroo amma jiru qorannoo akke microscopidhan godhuuf guyya lamma barbachisa. Waldaan fayyaa adunyaa guyyaa tokko keessati akka hojjetamu murteesera..Hata'u malee garaa garteen guyyaa tokko giduu jiru siriti qulqulaa'ee hin beekamu .Kanaafu kaayoon qorannoo kanaa garaa garumaa isaanii madaalu ta'a.

Adeemsa: qoranno armaan oliitti ibsame kanarratti hirmachuuf yoofedhiiqaabaattee gaaffii qorannookanaan walqabate sigaaffachuf daqiiqaa 10 fi nuuf obsii. Hancuffa yaalii dhukkuba tiibiitif ta'u nuuf laatta isa dura mallattokeetiin nuuf ibsi.

Balaa gahuu danda'u: qorannoo kanarratti hirmaachuukeetiif balaan sirratti qaqabu hinjiru

Faayidaa: hirmaattonni qorrannoo kana yalii ni argatuu qoranno hancufasaanirrati hundaa'un akkasumas dhibee tiibii ittisuuf gara dhimmi isaa ilaalatutti ni ergamu.

Iccitii : infoormeeshina hirmaattota irra argame iccittiinsa kan eegame ta'a akka nama sadaffatti hindabarre akkasumas informeeshinni kun kaninni kusaamu koodiidhan ta'a.

Hirmaanna fedhiirratti hundaayeef yeroo fedhanitti addaan kutuu: hirmaannan qoranno kana fedhiirratti kan hundaayeedha. Yeroo feetanitti addankutuuf mirga guutuu qabdu kanaaf murtoon keessan dhiibbaatokkoyyuu isinirraan hinga'u. Waan isinii hingalle yoo jiraate ittigaafatamtoota qorannoo kana gaditti caqasaman gaaffachu ni dandeessu.

Gaaffii yoo qabaattan odeeffanno armaan gadii fayyadama

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Bakka ga'umsa qorranno kanaatiif deebii sirrii ta'e waan nu laattaniif galatoomaa

C. Amharic version

የጥናቱ ርዕስ፡ የአንድ ቀን የሳንባ (ቲቢ) በሽታ ምርመራ በተለያዩ አይነት ዘዴዎች እና መሳርያዎች በጅማ ዩኒቨርሲቲ በማይኮባክቴርዮሎጂ ምርምር ላቦራቶሪ

የዋና ተመራማሪ ስም: ሸምሰዲን መሀመድ

የድርጅቱ ስም፡-ጅማ ዩኒቨርሲቲ / የህክምና ላቦራቶሪ ሳይንስ እና ፓቶሎጅ ትምህርት ክፍል

ይህ የመረጃ ቅጽ የተዘጋጀዉ ከላይ በተጠቀሰው ጥናት ለሚሳተፉ ተሳታፊዎች ሲሆን በአጠቃላይ በጥናቱ ውስጥ ልናካሂዳቸው ስለፈለግናቸው ጉዳዮች እና ስለጥናቱ ጠቅላላ ማብራርያ ይሰጣል:: በመሆኑ በጥናቱ የሚሳተፉት በራስዎ ፍላጐት ብቻ መሆኑን በትህትና እንገልፃለን፡፡

ስለጥናቱ በጥቂቱ

የሳንባ ነቀርሳ በሽታ በአለማችን በገዳይነቱ ከታወቁ በመድኃኒት ልድኑ ከሚችሉ በሽታዎች መካከል አንደኛዉ ነዉ፡: በሽታው በአሁን ግዜ በ ሁለት ቀን የአክታ ምርመራ እየተደረገ ይገኛል ነገር ግን የአለም ጤና ድርጅት ወደ አንድ ቀን እንዲቀር ያዘዘ ሲሆን ነገር ግን የተጠና ጥናት የለም፡: ስለሆነም የዚህ ጥናት ዋና አላማ በጅማ ዩኒቨርሲቲ በማይኮባክቴርዮሎጂ ምርምር ላቦራቶሪ ውስጥ በአንድ ቀን እና በ ሁለት ቀን የአክታ ምርመራ ያለውን ልዩነት ማጥናት ይሆናል፡፡

የጥናቱ ሂደት ዝርዝር

በጥናቱ ለመሳተፍ ከተስማሙ የሚከተሉትን መረጃዎችና ናሙና እንወስዳለን፡

- ከራስዎ አንደበት የ 05 ደቂቃ ቃለ መጠየቅ ይደረግሎታል፡:
- እንዲሁም ናሙና ይወሰዳል ምርመራም ይደረጋል ::

የተሰበሰበዉ ናሙና በጅማ ዩኒቨርሲቲ በማይኮባክቴርዮሎጂ ምርምር ላቦራቶሪ ከጥናቱ ጋር የተያያዙ የኤፍቢ እና የካልቸር ምርመራዎች ይካሄዳሉ፡፡

ስጋትና ጉዳት

በአጠቃላይ ከላይ የተጠቀሰዉን ናሙና በሚወሰድበት ጊዜ ሊያጋጥም የሚችል አደጋ አይኖርም፡፡

ሊያስገኛቸው የሚችሉት ጥቅሞች

በዚህ ጥናት ውስጥ በመሳተፍዎ በጥሬ ገንዘብ የሚደረግ የካሳ ክፍያ አይኖርም፡፡ የጥናቱ ተሳታፊዎች በምርመራ ዉጤት ላይ በመመርኮዝ የሳንባ ነቀርሳ ከተገኘበት ለበለጠ ህክምና እና እንክብካቤ ወደ የሳንባ ነቀርሳ እና ኤአርቲ ክሊኒክ ይላካሉ፡፡

የጥናቱ ምስጢራዊነት

ማንኛዉም በጥናቱ የሚገኙ መረጃዎች በምስጢር ይጠበቃሉ:: የጥናቱ መረጃዎች በሙሉ የሚቀመጡት ከእሶም ጋር ሳይሆን ለጥናቱ ተብሎ በሚሰጠው ስውር ቁጥር ሲሆን ጥናቱን ከሚያስከሄዱት ባለሙያዎች በስተቀር ማንም ሊያውቅ አይችልም፡፡ የእርስዎን ማንነት በሚገልጥ መልኩ የተዘጋጀውን መረጃ በፈርማዎ የተረጋገጠ ፍቃድ ሳናገኝ ይፋ አናደርግም:፡ ይህ ጥናት ሳይንሳዊ መረጃ እንደ መሆኑ መጠን በወረቀት ታትሞ ቢወጣ ወይንም በሚድያ ቢነገር የእርስዎ ስም በምንም መልኩ አይጠቀስም፡:

ያለመቀበል ወይም ጥሎ የመውጣት መብት

በዚህ ጥናት ዉስጥ የሚኖርዎት ተሳትፎ ሙሉ በሙሉ ፈቃደኝነት ላይ የተመሰረተ ይሆናል:፡ በማንኛውም ጊዜ ይህንን ጥናት የማቋረጥ መብትዎ ሙሉ በሙሉ የተጠበቀ ነው:: በጥናቱ ባለመሳተፍዎ ወይም ከጥናት በመገለልዎ ምክንያት በአሁኑ ወይም የወደፊት የህክምና እርዳታ ላይ ተፅዕኖ አይኖረውም፡፡ ከዚህ በፊት ሲያገኙ ከነበሩት ጥቅሞችአንዳች ነገር አይጐሎቦትም፡፡ ጥናቱን የሚያከናውነው አካል ወይም ድጋፍሰጭ አካል ከእራስዎ ጥቅም ሲባል በጥናቱ እንዳይሳተፉ ሊከለከል ይችላል፡፡

ጥያቄ ካለወት

ስለ ጥናቱ ማንኛዉም ጥያቄ ወይም እርስዎ በዚህ ጥናት ውስጥ ለሚኖርዎት ድርሻ፣አሳሳቢ ጉዳት ወይም ቅሬታ ካለዋት የሚከተሉትን ስልኮች ወይም ኢሜል አድራሻ በመጠቀም የጥናቱን ባለቤት እና አማካሪዎች ማነጋገር ይችላሉ፡፡

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ANNEX II: CONSENT FORM

A. English Version

Participants name _____

I am informed fully in the language I understand about the aim of above mentioned research. I understood the purpose of the study entitled with "Same day diagnostic approaches for pulmonary tuberculosis: Various Combination Strategies of existing Methods, 3 month cross sectional study at Jimma University Mycobacteriology Research Center, South west Ethiopia" I have been informed that specimen will be taken and there will be interview. In addition I have been told all the information collected throughout the research process will be kept confidential. I understood my current and future medical services will not be affected if I refused to participate or with draw from the study.

Agree_____ Not agree_____

Therefore I give my consent freely for my participation in this study.

Name of participant: _____Signature:_____

Name of researcher: _____Signature_____

Date_____

B. Afan Oromo version

Akkam bulten /akkam olten

Yommuun qorannoo kana irratti hirmaadhu afaan naaf galuun natti himameera ykn naaf ibsameera. Faayidaa qorannoo ‘‘Akkataa qoranoo akkee microscopidhan guyyaa tokko keessatii yaalii adda addatii fayyadamudhaan itti godhamu.Yuniversitii Jimmaati research Centeri Mycobactriology.” naaf galeera. Waa’ee dhibee sombaa akkan gaafatamuu fi akke(akkita) yoking namuna qoronof kan ta’u akka kennamu naaf himameera. Odeeffannoon qorannoo kana irraa argamu hunduu iccitiin akka kaa’amus irratti walii galleerra.qorannoo kana hirmaachuu yoon hin barbaadne ykn yoon addaan kute ,ammas ta’ee fulduraaf fayyadamummaa kiyyarratti rakkoo tokkoollee akka hin uumnee naaf himameera.

Nan barbaada…… hin barbaadu……

Kanaafuu, odeeffannoo kana fedhii kiyyaan nan kenna.

Maqaa dhukkubsataa…… mallattoo …… guyyaa…….

Maqaa qo’ataa…….. mallattoo…… guyyaa……

C. Amharic version

የተሳታፊው ልዩ መለያ ቁጥር _____

የተሳታፊው ስም _____

እኔ ስሜ ከላይ የተጠቀሰው ተሳታፊ በታሰበው ምርምር ላይ በሚገባኝ ቋንቋ በቂ መረጃ አግኝቻለሁ፡፡ የህክምና መረጃና ናሙና ምንም አይነት ጉዳት በማያደርስ መልኩ እንደሚወሰድ ተረድቻለሁ፡፡ በተጨማሪም የሚወሰዱ ማናቸውም መረጃዎች በሚስጢር እንደሚያዙ ተነግሮኛል፡፡ እንድሁም የሚጠየቀውን መረጃ ያለመስጠትና በጥናቱ ያለመሳተፍ መብት እንዳለኝ እንድሁም ከጥናቱ በማናቸውም ወቅት ራሴን ማግለል እንደምችል የተገለፀልኝ ሲሆን ይህንንም በማድረጌ ወደፊትም ሆነ አሁን የማገኛቸው የህክምና ግልጋሎቶች እንደማይጓደሉብኝ ተነግሮኛል፡፡

እስማማለሁ _____ አልስማማም _____

የታካሚ/ የተሳታፊ ስም _____ፊርማ ---------- ቀን ----------

የተመራማሪ ስም ---------- ፊርማ ---------- ቀን ----------

ANNEX III: QUESTIONNAIRE

A. English version

Card no: _____

Lab-no: _____

Address: _____

General instruction

i. For all questions that have a pre- coded response,

- ✓ Circle the responses that best match with your response

SN	Variables	Category/response	Skip to
	Part I: Socio-Demographic characteristics		
01	Age in year	_____	
02	Sex	0. Female 1. Male	
03	Residence	0. Rural 1. Urban	
	II. Symptoms of pulmonary TB		
201	Do you have cough?	0. No 1. Yes	If No skip to 203
202	If yes, specify the duration of your cough from the first date of onset in week	_____ week	
203	Currently, what kind of symptoms (complaints) do you have? NB: **multiple choice is possible**	0. Night sweat 1. Weight loss 2. Bloody sputum 3. Chest pain 4. Intermittent fever 5. Loss of appetite 6. Shortness of breath 7. Fatigue 8. others(specify)___	
	III. Types of Sample		

	- Spot-1 or A _____ - Spot- 2 or A_2 _____ - Early morning or B _____ - Spot -3 or C _____	1- Saliva 2- Mucoid 3- Purulent 4- Bloody	

B. Afan Oromo version

Lakk-Cardii: _____

Lakk-Lab: _____

Tesso/Nanno: _____

Qajeelfaamoottaa buurra

Gaaffille dhiyaattaaniif fillanoowaan keenaammaan kessaa sirri kanjaadhan itti maraa yookaan mallottoo √ kaa

Lakk	Gaaffii	deebii	Itti dhabarfadhu
	I: Ammalaa naanno fi haawaassa		
01	Ummrii	_____	
02	Saalaa/dhalaa	1 Dhirra 2 Durbaa	
03	Teessoo	1 Baaddiyaa 2 maagaalaa	
	II. Mallottollee dhukubaa soommbaa		
201	Quffa qaabdaa?	1 Lakkii 2 Eyye	Yoo Lakkii dhabarfedhu lakk. 203
202	Yoo eyye, Guyaa meeqaa	_____Guyyaa/toorbee	
203	Amma, maaltuu sinitti dhagaamaa? **Mallotton yoo tokko-olli ibbssaa**	1 Hallkkan issin dheeffqississa 2 Ulffinaa hirrdhisu/qaamnaa 3 Dhigaa kan wallin makkee issin-tufssissa 4 Dhukuuba laphee 5 Afurra issin dhawwaa 6 Niyyaatta issin dhawwa 7 Guubbaa gizze adaa adda 8 Dhadhabee qabdaa 9 Kan biraa ___	
	III. Goossa akke		

	- Spot-1 or A _____ - Spot- 2 or A_2 _____ - Ganamma or B _____ - Spot -3 or C _____	1 Akkee 2 Akkee fi borrorra 3 Akkee,dhigaa walmekee 4 Akkee furrdaa (purulent)	

C. Amharic Version

ካርድ ቁጥር: _____

ኮድ: _____

አድራሻ: _____

<u>አጠቃላይ መመሪያ</u>

ii. ለሁሉም ጥያቄዎች አማራጮች ያሉት ሲሆን እርሶ ተገቢ ነዉ በሚሉት ላይ ያክብቡ ወይም √ ምልክት ያስቀምጡ

ተቁ	መጠይቆች	ምላሾች	ይዝለሉ
	ክፍል I: ማህበራዊና አከባብያዊ ኑሮ ባህሪያት		
101	ዕድሜ በአመት	_____	
102	ፆታ	0. ሴት 1. ወንድ	
103	የት ይኖሩ ነበር	0. ገጠር 1. ከተማ	
	II. የሳንባ ነቀርሳ (ቲቢ) ምልክቶችና ተያያዥ መንስኤዎች		
201	ሳል አለብዎት?	0. የለም 1. አዎን	ወደ ጥያቄ 203 ይዝለሉ
202	ለጥያቄ 201 መልስዎ አዎን ከሆነ ከጀመርዎት በሳምንት ምን ያህል ግዜ ሆነዎት	_____ ሳምንት	
203	አሁን ምን አይነት ምልክቶች ይታይብዎታል? **ከአንድ በላይ ምልክቶች ካሉ ይጥቀሱ**	0. ማታ ስተኙ ማላብ 1. ክብደት መቀነስ 2. ደም የተቀላቀቀለበት አክታ 3. የደረት ላይ ህመም 4. አልፎ አልፎ የሚከሰት ትኩሳት 5. የምግብ ፍላጎት የለመኖር 6. የትንፋሽ ማጠር 7. ድካም 8. ሌላ ካለ ይጥቀሱ ___	
	III. የናሙናው አይነት		
	- ስፖት-1 or A _____ - ስፖት-- 2 or A 2 _____ - የጠዋት or B _____ - ስፖት--3 or C _____	1. ምራቅና አክታ 2. አክታ 3. ደም የተቀላቀቀለበት አክታ 4. ወፍራም ወይም ፕሩለንት	

ANNEX IV: LABORATORY PROCEDURE

1. Specimen collection

Materials

- Gloves
- Marker
- N95 mask
- Sterile container

Procedure

Four sputum specimens should be collected from each patient each with new sputum cup clearly labelled with TB lab code, patient ID number, visit number and the date and time of specimen collection.

The following procedures should be used to collect sputum specimens

- Provide the patient with a labelled specimen container with the appropriate identifying information
- Inform the patient that saliva and upper respiratory/nasal secretions are not sputum and are not acceptable specimens.

Instruct the patient to:

- Breathe deeply a number of times and then cough from deep down within the lungs.
- Lean forward, breathe in and out slowly twice, hold breath for 2-3 seconds each time, and on third time forcefully cough to bring up the sputum.
- Collect sputum in container provided (For early morning sputum Collect the sputum after getting out of bed, before the morning meal, and prior to taking any medications) in well-ventilated area such as by an open window or outside. .
- Avoid touching inside or edge of specimen container or lid with fingers.
- Replace lid after collection and close tightly to avoid leakage.
- After collecting sputum thoroughly clean hands with soap and water.
- Bring container to the clinic as soon as possible, preferably in insulated cooler.
- Inform the patient that they must write down the time of collection on the specimen container or bring this information when he/she brings back the specimen.

Preparation of smear from direct sputum

Prepare smears from all direct specimens before concentrating for microscopy and culture.
The procedure is outlined as follows:

a. After taking the sputum sample take the sputum with applicator stick and put on a clean microscope slide.
b. Spread the smear about 1½ cm x 1 cm.
c. Allow the smear to air dry completely.
d. Perform all the above procedures in a biological safety cabinet. Handle the smear carefully since mycobacteria may still be viable.

Smear preparation: A new unscratched slide will be selected for smear preparation. Smear will be prepared with sterile loop. A good smear is spread evenly, over a size of 2x3 cm and is neither too thick nor too thin then allowed to air dry for 15-30 min and fixed by passing it over a blue flame 3-4 times with in safety cabinet.

2. Staining methods

A. Ziehl-Neelsen staining

Principle

Sputum smear is heat –fixed, flooded with a solution of carbolfuschin (a mixture of basic fuschin and phenol) and heated until steam rises. The heating allows melting of cell wall of mycobacteria which facilitate entrance of the primary stain into the bacterium. After staining, an acid decolorizing solution is applied. This removes the red dye from the background cells, tissue fibres, and any organisms in the smear except mycobacteria which retain (hold fast to) the dye. Following decolorization, the smear is counter-stained with methylene blue or malachite green which stains the background material, providing a contrast colour against which the red AFB can be seen.

Materials and Reagents Required:

Procedure

- Flood the slide with carbolfuchsin stain.
- Heat gently until steam rises (electric stainer may be used for heating.) Do not allow the stain to boil or dry. Keep adding stain as it dries during heating.
- Stain for 5-10 minutes; allow cooling.
- Wash gently with water.
- Decolorize with acid alcohol for 3 minutes or until no more colour appears with acid alcohol.
- Wash gently with water. Drain excess water.
- Pour Methylene Blue (counter stain) on the smear and leave for 1 minutes.
- Wash gently with water. Drain excess water.
- Air-dry and observe under microscope. Do not blot dry as it may remove smear accidentally.

Results

- AFB..... Red, straight or slightly curved rods, occurring single or in a small groups
- Cells..... Blue
- Background Material …….. Blue

Reporting system

- 0 AFB/100 field ……..……Negative
- 1-9 AFB/whole smear……Scanty positive.
- > 10 AFB/100 fields……..… 1+
- 1-9 AFB/ 1 fields…… …… 2+
- >10 AFB/ 1 fields…… …… 3+

N.B: AFB means number of acid fast bacilli seen.

B. LED fluorescence

Principle

Acid-fast mycobacteria resist decolorization by acid-alcohol after primary staining owing to the high lipid (mycolic acid) content in their cell walls. Primary stain (Auramine) binds cell-wall mycolic acids. Intense decolourization (strong acids, alcohol) does not release primary stain from the cell wall and the mycobacteria retain the fluorescent bright yellow colour of auramine. Potassium permanganate is used to quench fluorescence in the background.

Materials and Reagents Required:

Reagents: Reagents may be purchased commercially or prepared in the laboratory.

A. Fluorochrome acid-fast stain

1. Auramine O
2. 0.5% Acid-alcohol
3. Counterstain (potassium permanganate or acridine orange)
4. Demineralized Water (CAS 7732-18-5) ..... 870.0 ml

B. Supplies

a. Glass slides (25 by 75 mm), frosted ends desirable

b. Coverslips (22 by 22 mm; no. 1)

c. Sterile 50-ml conical polypropylene screw-cap tubes (aerosol free and graduated)

C. Equipment: Optional materials, depending on specimen source of laboratory protocol

a. Binocular microscope with 10X, 40X, and 100X objectives (or the approximate magnifications for low power, high dry power, and oil immersion examination).

b. Centrifuge with aerosol-free sealed centrifuge cups

d. Vortex mixer

Procedure

1. Make a thin smear of the material for study and heat fix by passing the slide through the flame of a Bunsen burner or use a slide warmer.
2. Flood the smear with TB Auramine O for 15 minutes.
3. Rinse with demineralized water and drain.
4. Decolorize with TB Decolorizer for 2-3 minutes

5. Rinse with demineralized water and drain.
6. Flood smear with TB Potassium Permanganate counterstain for no longer than 2-4 minutes.
7. Rinse with demineralized water and allow to air dry.
8. Examine microscopically under low power (25 X objective) using a fluorescent microscope; confirm under oil immersion (400-630X magnification)..

Results

Positive Test - Acid-fast positive organisms fluoresce bright yellow-green against a dark background.

Negative Test - Nonacid-fast organisms will not fluoresce or may appear a pale yellow, quite distinct from the bright yellow acid-fast organisms.

Reporting system

IUATLD/WHO scale (1000x field = HPF) Result	Microscopy system used		
	Bright-field (1000x magnification: 1 length = 2 cm = 100 HPF)	Fluorescence (200–250x magnification: 1 length = 30 fields = 300 HPF)	Fluorescence (400x magnification: 1 length = 40 fields = 200 HPF)
Negative	Zero AFB / 1 length	Zero AFB / 1 length	Zero AFB / 1 length
Scanty	1–9 AFB / 1 length or 100 HPF	1–29 AFB / 1 length	1–19 AFB / 1 length
1+	10–99 AFB / 1 length or 100 HPF	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+	>10 AFB / 1 HPF on average	>100 AFB / 1 field on average	>50 AFB / 1 field on average

3. Bleach conc tech

Principle

Bleach cause digestion of the mucus and debris in sputum, resulting in a clearer microscopy field and the PBS neutralizes the bleach and dilutes.

Materials and Reagents Required:

A. Reagents: Reagents may be purchased commercially or prepared in the laboratory.

a, 5 % Bleach (readymade or prepared in the laboratory),

b. PBS solution prepared in the laboratory

B. Supplies

a. Glass slides (25 by 75 mm), frosted ends desirable

b. Pasteur pippets

c. Sterile 50-ml conical polypropylene screw-cap tubes (aerosol free and graduated)

C. Equipment: Optional materials

a. Binocular microscope with 10X, 40X, and 100X objectives (Light Microscope or LED Fluoresence Microscope).

b. Simple and routine Centrifuge with aerosol-free sealed centrifuge cups

c. Timer, Beaker and Vortex mixer if available or can be done with hand ,

Procedure

1- A sample (1 mL) of the pooled sputa (spot-1 and spot- 2) was transferred to a 15mL screw-capped tube and mixed with an equal volume of NaOCl (5%) for liquefaction to occur.

2- The tubes were incubated at room temperature for 15 min and shaken by hand at regular intervals (1,7 and15 minutes).

3- Addition of PBS up to 8 ml graduated conical tubes.

4- The tubes were centrifuged at 3000 g for 15 min (in simple routine centrifugation method).

5- The supernatant of each tube was carefully removed.

6- The sediment was mixed with remaining fluid and two smears were prepared by applying a drop of the re-suspended sediment with a sterile pipette to a slide.

7- The slides were dried in air

8- Stained by the Ziehl-Neelsen and Fluorescence technique.

4. Culture

Preparation of Lowenstein-Jensen (LJ) Medium

LJ medium for artificial culturing of *Mycobacterium tuberculosis*.

LJ medium: Lowenstein-Jensen egg-based solid medium specific for Tuberculosis bacterial culture.

Procedure

Media preparation must be done in the media preparation room. Care must be taken to ensure that the room is absolutely kept clean, free from dirt or dust and that the benches are swabbed daily with 5% Lysol/ phenol solution or 70% alcohol. During at all stages of media preparation, the door must be kept closed, to avoid contamination of the media. All equipments, sterile glassware etc., as listed, must be available before commencing media preparation.

Equipment and glassware are to be sterilized prior to the day of media preparation, labeled indicating the date of sterilization and stored in a clean place. Equipment must be re-sterilized, if more than one week elapses between date of sterilization and date of use.

Strict adherence to aseptic techniques must be observed at All times, i.e., flaming of the mouths of flasks etc., before and after removal of the stopper, holding the stopper with the little finger and palm to avoid contamination and by working as near to the flame as possible. Weighing and measuring should be accurate. All used glassware must be sent for cleaning as soon as possible.

Material required:

Note: One batch of LJ medium is 1600 ml of solution.

1. Warring Blender (mixer) with Sterile Jar marked of 1000 ml capacity, along with sterile lid.
2. Sterile funnel with double layer' of gauze fixed over mouth.
3. 1 x 1lit stainless steel jars.
4. 2 x 1 litres sterile round, flat bottomed flask.
5. 500 ml methylated spirit.
6. Sterile McCartney bottles (28 ml universal containers) (~300 per batch of medium).
7. Sterile Mineral salt solution, 600 ml with malachite green.
8. Fresh hen's eggs (24 to 28 eggs per batch of 1000 ml fluid). Quality of media depends on the freshness of the hen’s eggs.

a. Eggs should be obtained always from a reliable source. The eggs should not be more than a day or two old, at the time of purchase.
b. The eggs required for a week should be indented in one lot, soaked in water, scrubbed gently and washed in running water, followed by dematerialized water and placed in a drain board for draining.
c. Cracked and broken eggs should be rejected. Soak the eggs in Methylate spirit for 10 min. Wipe off, with sterile gauze and store in sterile container till used.

9. Inspissator.
10. Mineral salt solution

Ingredients:

Potassium dihydrogen phosphate anhydrous (KH_2PO_4) A.R. 2.4 g

Magnesium sulphate ($MgSO_4.7H_2O$), A.R. 0.24 g

Magnesium citrate 0.6 g

Asparagine 3.6 g

Glycerol (reagent grade) 12 ml

Malachite green, 2% solution* 20 ml (Malachite green dye 2.0 g + Distilled water 100 ml)

Dissolve the dye in distilled water by grinding the dye with water using a mortar and pestle. Filter and store in refrigerator

Dissolve the ingredients in order in about 300 ml distilled water by heating. Add glycerol, malachite green solution and make up 600 ml with distilled water.

Autoclave at 121^{o} C for 30 minutes to sterilize. Cool to room temperature. This solution keeps indefinitely and may be stored in the refrigerator.

11. Inspissator, thermostatically maintained at 85ºc.
12. Clean bench surfaces and a Bunsen burner

Preparation of complete medium:

1. Arrange Mineral salt solution, eggs in a sterile container, egg breaking flask, 500ml measuring cylinder, blender container, and funnel with gauze inside the bio-safety cabinet, at a corner.
2. Individually, break the eggs by means of sharp rap on the side of the sterile 1 lit steel jar.
3. Transfer egg yolk and white carefully into the jar. Take care that there are no stale eggs.

4. Break 24 eggs, at each time. Transfer approximately 500ml egg fluid from steel jar to blender container; place the Lid on the top.
5. Place the container with egg solution on the blender, and switch on the blender mains Give 1 to ½ min breaks (once or twice) which is sufficient to homogenize the egg fluid. Do not give long breaks, froth would be formed in the solution which would affect medium slopes! Do not allow fluid to touch the lid.
6. Filter the homogenized egg solution into sterile 500ml measuring cylinder though gauze covered (2 layers) glass funnel.
7. Transfer filtered homogenized egg solution 2 liters round bottom flask.
8. Repeat filtration, of another 500ml egg solution, and transfer to round bottom flask.
9. Add 600ml of mineral salt solution total 1 lit of homogenized egg solution in the round bottom flask. Mix thoroughly by gentle agitation for 5 min, till uniform pale green color is obtained.

Media pouring/dispensing:

1. Arrange the sterile Mc-Cartney bottles in the racks.
2. Open the cap, pour 5-8ml of LJ medium into each bottle
3. Reject any chipped, cracked or dirty bottles
4. Recap the bottles tightly.

Inspissation:

1. Inspissator should be switched on sufficiently early so that required temperature is reached. Leave the inspissator racks inside the inspissator.
2. The media bottles are sloped on the inspissator racks, and transferred to inspissator.
3. Slopes are left in the inspissator at 85°c for 85 min.
4. The maintenance of the temperature by the thermostat needs to be inspected at the time of loading, once in the middle, and at the end. Ensure that fan is working inside the inspissator.
5. Switch OFF the inspissator after the work.
6. Check tubes at the back side of the slope. If large number of transparent ‘holes’ in the opaque pale green back-ground, it indicates the over-inspissation (more than adequate temperature for coagulation of egg medium). If the bubble holes are there on the surface of the slope, it indicates insufficient temperature for inspissation.

Sterility Test:

1. All the slopes of medium prepared in a day are incubated for 48 hours at 37°c.
2. From a batch of medium 10 slopes are randomly selected and incubated at 37°c for 14 days.
3. If bacterial and fungal contamination is noted, the entire batch is to be rejected.

Quality control:

The quality of drug-free culture media can be tested most simply by using a rapid growing *Mycobacterium* such as *M. fortuitum*. If no growth is observed within five days after inoculation, the medium does not have the properties required.

Storing:

Media slopes are packed in a saran-warp, packed in boxes or iron meshes/racks, labeled with date of preparation, batch number and stored at the 4°c refrigerator. Although media stored at refrigerator can be used for 2 months, stock only one month's requirement, at a time.

Results

Physical characteristics of the media

Color

Media-containing tubes from the same batch showing different shades of green color may be due to poor homogenization or to the presence of material residues in the tubes. A very dark tone of green can be caused by an excess of Malachite green or to a very low (acidic) pH. Yellowish media can indicate poor quality Malachite green or a very high (alkaline) pH.

Texture

If the medium disintegrates easily, the inspissation temperature might have been too low. This can be detected by tapping on one's hand one or two tubes randomly chosen from the inspissated batch. Tubes containing media of poor texture are not suitable for culture inoculation.

Homogeneity

If bubbles in the medium appear during inspissation, it is possible that the medium might have been subjected to excessive temperature, thus losing quality. The presence of clumps in the media indicates poor homogenization.

Sensitivity

Serious problems affecting the quality of culture medium can be detected by seeding a 1/10.000 dilution of a suspension of *Mycobacterium tuberculosis* equivalent to that of a bacterial

suspension containing 1 mg/ml of tubercle bacilli. Five tubes of a previous batch of medium and 5 tubes of the new batch of medium are inoculated with 0.2 ml of the diluted suspension. If the number of colonies obtained on the recently prepared or purchased batch is significantly lower than on reference batch of medium, the sensitivity of the new medium, whether prepared or purchased, is not adequate.

Safety Conditions

Practice good aseptic technique in the media preparation room.

Documentation

The following registers are to be maintained:

1. Media register
2. Egg stock register, and
3. Inspissation register

Sputum sample processing to culture in LJ

Processing sputum specimens has two objectives: decontamination of bacteria other than mycobacteria and liquefaction of mucous and organic debris in the specimen.

Sodium hydroxide- N-Acetyl-L-Cystein (NaOH-NALC) procedure is the standard recommended procedure to be used with LJ solid media.

Materials and Methods

Materials Required:

1. Incubator, 37°C.
2. Refrigerated centrifuge with covered bucket inserts for 50 ml conical tube with a minimum 3000-3500x g force
3. Vortex mixer, shaker
4. Bio-safety cabinet.
5. Waste receptacle, splash-proof for liquids.
6. Two 5 mm wire loops (Nichrome wire of 22 SWG).
7. One Enamel bin with lid for disposable waste.
8. Wire baskets or Clean racks to hold 16 tubes for 50 ml centrifuge tubes.
9. Wire racks for holding 100 universal containers.
10. Stock of clean, sterile Disposable 50 ml plastic tubes (Falcon tubes)

11. Stock of LJ slopes.
12. Sterile NaOH-NALC-sodium citrate solution:
13. Sterile Phosphate buffer pH 6.8 (0.067M).
14. Two Sterile 250ml conical flasks.
15. Grease marker pencils.
16. Timer.
17. Pipettes/transfer pipettes

If specimen is not collected in a 50 ml centrifuge tube, transfer it to a 50 ml centrifuge tube with a screw cap.

Preparation of processed sputum for solid culture

For LJ culture, 2 ml of sputum is added to 2 ml of sterile 4% NaOH solution in a 20 ml plastic Universal bottle (sputum with a volume of less than 2 ml is made up to 2 ml with sterile distilled water). The specimens are then vortexed briefly before incubation at room temperature for 10 minutes. They are then vortexed briefly for a second time and incubated at room temperature for a further 10 minutes. Next, 16 ml of sterile distilled water is added to the specimens, and they are then centrifuged at 2000 rpm for 20 minutes. The supernatant is then discarded, the deposit is mixed gently, and two drops of the deposit are dispensed directly onto solid Löwenstein-Jensen (LJ) media containing pyruvate. The cultures are incubated horizontally at 37^{o}C overnight to allow the inoculum to disperse evenly over the surface of the medium, after which they are incubated vertically at 37^{o}C for a further 10 weeks (during which time they are reviewed every 2 weeks). Cultures are examined macroscopically, and any with growth of one or more colonies.

Specimen Digestion, Decontamination and Concentration

Principle

N-acetyl-L-cysteine (NALC), a mucolytic agent, is used for rapid digestion, which enables the decontaminating agent, NaOH, to be used at lower final concentration (in sputum). NALC loses activity rapidly in solution, so it is made fresh daily. Sodium citrate exerts a stabilizing effect on the NALC by chelating heavy metal ions present in the specimen. The phosphate buffer neutralizes the NaOH and dilutes the homogenate to lessen the viscosity and specific gravity

prior to centrifugation. Mycobacteria have a low specific gravity and may remain buoyant during centrifugation.

In this procedure, the initial concentration of NaOH is 4%. This 4% NaOH solution is mixed with an equal quantity of sodium citrate solution (2.9%) to make a working solution (NaOH concentration in this solution is 2%). When an equal quantity of NaOH-NALC-citrate and sputum are mixed, the final concentration of NaOH in the specimen is 1%.

Procedure

- Add NaOH-NALC-sodium citrate solution in a volume equal to the quantity of specimen. Tighten the cap.
- Vortex lightly or hand mix for about 15-30 seconds. Invert the tube so the whole tube is exposed to the NaOH-NALC solution.
- Wait 15-20 minutes (up to 25 minutes maximum) after adding the NaOH-NALC solution. Vortex lightly or hand mix/invert every 5-10 minutes or put the tubes on a shaker and shake lightly during the whole time.
- Make sure the specimen is completely liquefied. If still mucoid, add a small quantity of NALC powder (30-35 grams) directly to the specimen tube. Mix well.
- At the end of 15-20 minutes, add phosphate buffer (pH 6.8) up to the top ring on the centrifuge tube (plastic tube has a ring for 50 ml mark). Mix well (lightly vortex or invert several times). Addition of sterile water is not a suitable alternative for the phosphate buffer.
- Centrifuge the specimen at a speed of 3000 g or more for 15-20 minutes. Use of refrigerated centrifugation at a higher speed is known to increase recovery of mycobacteria.
- After centrifugation, allow tubes to sit for 5 minutes to allow aerosols to settle. Then carefully decant the supernatant into a suitable container containing a mycobactericidal disinfectant. Make sure the sediment is not lost during decanting of the supernatant fluid. Add a small quantity (1-2 ml) phosphate buffer (pH 6.8) and resuspend the sediment with the help of a pipette or vortex mixer.
- Use the resuspended pellet for making smears and for culture and microscopy.

Preparation of smear from processed sputum

Prepare smears from all prcessed specimens before inoculation into culture medium.

The procedure is outlined as follows:

a. After digestion/decontamination, concentration and resuspension of the pellet mix the specimen well with a pipette and place about one drop or 2-3 loopfulls on a clean microscope slide.
b. Spread the smear about 1½ cm x 1 cm.
c. Allow the smear to air dry completely.
d. Perform all the above procedures in a biological safety cabinet. Handle the smear carefully since mycobacteria may still be viable.
e. Stain with ZN stain and Oramin O stain.

Results:

For preliminary identification of tubercle bacilli the following characteristics apply:

- Tubercle bacilli do not grow in primary culture in less than one week and usually take three to four weeks to give visible growth
- The colonies are buff coloured (never yellow) and rough, having sometimes appearance of bread crumbs or cauliflower. However, morphology of the colonies vary if specimen are very old, or specimens obtained from patients while on treatment,
- They do not emulsify in the saline used for making smears but give a granular suspension
- Microscopically they are frequently arranged in serpentine cords of varying length or show district linear clumping. Individual cells are between 3μm and 4μm in length

Examination schedule

All cultures should be examined 72 hours after inoculation to check that liquid has completely evaporated, to tighten caps in order to prevent drying out of media and to detect contaminants. Thereafter, cultures are to be examined weekly, or if this is not operationally feasible, on at least three occasions, *viz*

- After one week to detect rapidly growing mycobacteria which may be mistaken for *M. tuberculosis*
- After three to four weeks to detect positive cultures of *M. tuberculosis* as well as other slow growing mycobacteria which may be either harmless saprophytes or potential pathogens
- After eight weeks to detect very slow-growing mycobacteria, including *M. tuberculosis*, before judging the culture to be negative

Certain contaminating organisms produce acid from constituents of the medium and the lowering of pH unbinds some of the malachite green from the egg (indicated by the medium changing to dark green). Tubercle bacilli will not grow under these conditions and cultures should be discarded.

Reading of cultures

The label should show the Lab Numbers of first and last specimen in a rack, date of inoculation and date of 4th and 8th week reading.

Typical colonies of ***MTBC*** are rough, crumbly, waxy, non-pigmented (cream coloured) and slow- growers,

The acid-fastness should be confirmed by Ziehl-Neelsen (ZN) staining. A very small amount of growth is removed from the culture using a loop and gently rubbed into one drop of sterile saline on a slide.

5. Identification of *MTBC*

The process describes the differentiation of tubercle bacilli (*MTBC*) from other mycobacteria (*NTM*) and the phenotypic identification of ***MTBC***.

Principle of procedure

Inability to grow in the presence of PNB is one of the key elements in the phenotypic differentiation of tubercle bacilli from other mycobacterial species and is part of the identification process for *M. tuberculosis.*

M. tuberculosis and other tubercle bacilli will not grow on culture medium containing PNB, 500 µg/ml; other mycobacterial species, with the exception of *M. gastri* and some strains of *M. kansasii* and *M. marinum*, will grow in the presence of PNB.

The identification process comprises the phenotypic identification of cultures of acid-fast bacilli grown on solid medium based on the combination of observation of colony morphology, inability to grow on a culture medium containing p-nitrobenzoate.

Samples

Cultures grown on solid media. These could be primary cultures if growth is abundant. Pure cultures of acid-fast bacilli grown on solid media, age 21–28 days, with more than 50 colonies. The test must be carried out on pure cultures otherwise it will yield false results.

Detailed procedure

The entire procedure must be carried out in a biological safety cabinet.

The inoculum was prepared by scraping freshly grown colonies from the surface of the LJ medium. A few colonies are emulsified in one flask containing glass beads and 2 ml of sterile distilled water to obtain turbidity greater than McFarland 1 standard. The content of the flask is homogenised, allowed to stand for 5min to allow larger clumps to settle, and then 1 ml of the supernatant suspension was transferred to another tube, where the turbidity is adjusted to McFarland 1 standard adding sterile distilled water. This bacterial suspension is used as the work suspension. LJ medium is inoculated with 100 µl of the work suspension.

- Inoculate a slope of LJ medium containing PNB and a slope of LJ medium with no PNB (control) with bacterial suspension adjusted to McFarland turbidity standard No. 1.

PREPARATION OF McFarland STANDARD No.1 (Paik, G. 1980)

1. Prepare 1% aqueous barium chloride and 1% sulphuric acid (AR) solutions. (100 mg of Barium chloride (anhydrous) in 10 ml of SDW and 0.1 ml of sulphuric acid (AR) in 10 ml of SDW).
2. Add 0.1 ml of 1% Barium Chloride to 9.9 ml of 1% Sulphuric acid to obtain the McFarland standard No.1, which matches with 1 mg/ ml of *M. tuberculosis*.
3. Seal the tube with parafilm and label as McFarland standard No.1 with date of preparation.

- Incubate at 37 °C and compare growth on both slopes after 28 days of incubation.

4.6 Result and interpretation

- Abundant growth on both slopes – mycobacterial strain other than tubercle bacilli.
- Abundant growth on the control tube and little or no growth on PNB medium – *M. tuberculosis* complex strain.
- No growth on either slope – non-interpretable result, test to be repeated.

Interpretation and identification

If all the above criteria are met, the culture is identified as *MTBC*.

ANNEX V: CULTURE MONITORING LOG

S.No	TB Lab Code	Date Specimen inoculated	Date on which growth observed								Visual observation	AFB Smear from culture	Culture result	Remark
			W-1	W-2	W-3	W-4	W-5	W-6	W-7	W-8				

Sameday Microscopy Diagnosis Reporting format

S/N	Lab-no	Res-code	AFB Microscopy result with grade										Culture result	Controller	Remark
			Spot-1or A		Spot-2 or A^2		Early – mor or B		Spot-3 or C		Bleach conc ($A + A^2$)				
			ZN	FR	ZN	FR	ZN	FR	ZN	FR	ZN	FR			

ANNEX VI: REQUEST FORM OF TB LABORATORY

Request and reporting form for AFB microscopy, and TB culture

Patient identification (ID):

Surname and first name of patient:_____ Age (yrs):_____ Sex:____

Card No: _____ Address: _____

*HIV-status: Pos / Neg / Unknown _____

TB Disease type and treatment history

Site: □pulmonary History: □ new
□Extrapulmonary (specify):_____ □ relapse
□ failure
□ return after default

Person requesting examination: Name: _____ Position: _____

Date specimen was collected: ____/____/20____

Specimen : □ sputum
Specimen Appearance: mucous / saliva / muco-purulent / bloody / other_____
Specimen volume_____

Microscopic examination result

Direct smear: 1st ____ 2nd ____ 3rd ____
Reported on date-------/-------/20------

From concentrate

ID #	Neg	1-9	1+	2+	3+

Reported on date-------/-------/20------

Culture result: reported on date _____/_____/20___

ID #	Contaminated	Neg	MTB complex	NTM (species)

Date: _____/_____/20_____ Signature: _____

ANNEX VII: ASSURANCE FORM

ASSURANCE OF PRINCIPAL INVESTIGATOR

I, the undersigned, MSc Medical Microbiology student declare that this thesis is my original work in partial fulfillment of the requirement for the degree of master science in Medical Microbiology. Where others work has been used, it has been carefully acknowledged and referenced in accordance with the requirements.

Name of the Investigator: _____

Signature _____ Date._____

APPROVAL OF THE FIRST ADVISOR

Name of the first advisor: _____

Signature _____ Date._____

APPROVAL OF THE SECOND ADVISOR

Name of the second advisor: _____

Signature _____ Date._____

APPROVAL OF THE INTERNAL EXAMINER

Name of the internal examiner: _____

Signature _____ Date._____