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DEPARTMENT OF MEDICAL LABORATORY SCIENCES

PERORMANCE OF SAME DAY SPUTUM SMEARS FOR DETECTION OF PULMONARY TUBERCLOSIS AND MULTI DRUG RESISTANCE TUBERCLOSIS AMONG PRESUMPTIVE TUBERCLOSIS CASES VISTING SAINT PETER SPEACIALIZED HOSPITAL ADDIS ABEBA, ETHIOPIA

A thesis to be submitted to the Department of Medical Laboratory Sciences, School of Allied Health Sciences, and College of Health Science Addis Ababa University in Partial Fulfillment of the Requirements for the Degree Of Masters in Diagnostic and Public Health Microbiology

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List of Abbreviations

AFB	Acid fast bacilli
AIDS	Acquired immune deficiency virus
BL	Bio safety level
CI	Confidence interval
DC	Denderitic cell
EMLA	Ethiopian medical laboratory association
EPHA	Ethiopian public health association
EPHI	Ethiopia public health institution
HIV	Human immunodeficiency virus
INH	Isonized
LED	Light emitting diode
LJ	Lowenstein Jensen
MDR	Multidrug resistance tuberculosis
MGIT	Mycobacterium growth indicator tube
MTB	Mycobacterium tuberculosis
MZN	Modified Ziehl Nelson
PCC	Probe cheek control
PTB	Pulmonary tuberculosis
QC	Quality control

RIF	Rifampcin
RPC	Reagent processing control
SM	Spot- morning
SMS	spot morning spot
SOP	Standard operating procedure
SSP	Sputum smears positivity
TB	Tuberculosis
WHO	World Health Organization
ZN	Ziehl Neelsen

Operational definitions

Front-loading / same day sputum smear approach: where two spot sputum samples are collected and analyzed on the same day within one hour interval for the detection of AFB.

Conventional /standard approach: uses three (spot morning spot) Sputum sample are collected and analyzed for the detection of AFB.

Presumptive TB case: persons who presents with symptoms and/or signs suggestive of tuberculosis, in particular cough of two weeks or more duration is presumed TB case.

Abstract

Background: Tuberculosis is an infectious disease caused by the bacillus Mycobacterium tuberculosis. The diagnosis of TB is highly relies sputum smear microscopy. It requires three sputum sample examinations over two days (conventional approach) and patient often abandons the diagnostic procedure owing to costs and inconvenience involved in multiple visits. The World Health Organization (WHO) recommends same day sputum microscopy (spot-spot) for the diagnosis of smear positive tuberculosis. As there is limited study on the same day sputum microscopy in our country this study aim to evaluate same day sputum smear performance for the detection of pulmonary and multi-drug resistance tuberculosis.

Objective: The aim of this study was to assess Performance of Same day Sputum Smear Approach for Detection of Pulmonary Tuberculosis and multidrug resistance among presumptive tuberculosis cases Visiting St, Peter specialized hospital Addis Ababa, Ethiopia

Method: Institution based Cross sectional study was conducted from January 2017 to May 2017 at St, peter specialized hospital, Addis Ababa. Smears was stained and examined by Ziehl- Neelsen (ZN) and florescence (FM) microscopy. Culture was done on LJ media and Multidrug resistance tuberculosis status was determined using line probe assay. In addition descriptive analysis was computed and Chi square test was used to see any association between the two methods and P value <0.05 was considered as statically significant.

Result we identified 25 patients with at least one positive smear and 9 cases were positive by conventional method and missed by the same day scheme. The same day and conventional method significant difference in case detection (P<0.001). The sensitivity of the same day method was 40.8%, positive predictive value and negative predictive value was 95.2%, 91.4% respectively.

Conclusion

The performance of same day smear microscopy for the diagnosis of pulmonary tuberculosis in the case of pulmonary tuberculosis suspect is associated with significant missed diagnosis and lower sensitivity. This has indirect impact on the number cases of MDR.

Recommendation Since same day approach missed considerable number of smear positive cases and has lower sensitivity Service providers should promote the use of spot –morning-spot sputum smear approach (conventional smear microscopy) the diagnosis of pulmonary TB

Keywords; Tuberculosis**,** MDRTB, Someday, Conventional

1. Introduction

1.1 Background

TB is an infectious disease caused by the bacillus Mycobacterium tuberculosis. It typically affects the lungs (Pulmonary TB) but can affect other sites as well (extra pulmonary TB). It causes illness among millions of people each year and is ranked as the second leading cause of death from an infectious disease worldwide. Every year, about nine million cases of active TB disease and Most of the cases of (7 million) are in Asia and Africa. (1, 2)

Multidrug-resistant (MDR) TB has become a major public health problem and presents new barriers to the control of TB. Drug-resistant TB is a man-made problem, largely being the consequence of human error as a result of poor supply management and quality of anti-TB drugs and inadequate or improper treatment, which is further exacerbated by human immunodeficiency virus (HIV). Poor infection control practice has also been identified as a major contributing factor for the spread of drug-resistant TB. Nearly half a million cases of MDR-TB emerge every year, but only 3% of them get treatment globally and 110,000 die annually. Ethiopia is one of the 27 high MDR-TB countries; it is ranked 15th with more than 5000 estimated MDR-TB patients each year .According to the WHO report, the prevalence of MDR-TB had been 2.8% in newly diagnosed patients.(3,4)

The control of TB in high-incidence countries relies upon passive case finding among individuals self-presenting to health-care facilities and diagnosis by sputum smear microscopy. Sputum smears microscopy (SSM), based on direct visualization of acid-fast bacilli, is the primary diagnostic method for identifying pulmonary TB, Conventional SMS is low cost, has limited infrastructural needs, and trained microscopists can read 20–30 slides per day. However, microscopy has highly variable sensitivity (20–80 %) and poor accuracy among persons with HIV infection. (5)

Sputum culture is another diagnostic method used for mycobacterium identification and has higher diagnostic yield in comparison with smear microscopy. Nevertheless, it is more time-consuming, the turn-around time is about 2–8 week so it is less useful to guide the clinical decision-making

process. Biomarker-Based Technologies may be ideal for diagnosing is pediatric TB patients, extra pulmonary TB patients, While existing serological, antibody tests have been shown to lack accuracy and are discouraged by the WHO. Nucleic acid amplification tests (NAATs) amplify genome specific targets, through different methods: polymerase chain reaction (PCR), transcription-mediated amplification (TMA), loop-mediated amplification (LAMP), nucleic acid sequence based amplification (NASBA), and strand displacement amplification (SDA). Are some of molecular tools under development.(5)

Despite recent advances in rapid diagnostics, the most common method for diagnosis TB worldwide is sputum smear microscopy however it has several limitations, including poor sensitivity, being labor intensive, and requiring skilled microscopists. Furthermore, the need to collect serial sputum specimens over multiple patient visits results in a protracted diagnostic process with high rates of patient dropout. Recent studies examining the yield of serial sputum specimens, usually collected as spot-morning-spot, have reported that the majority of patients with smear-positive PTB are identified by the first two sputum specimens and the World Health Organization (WHO) has recently changed its policy in this respect, reducing the minimum number of sputum specimens examined for each patient from three to two . This will result in reduced laboratory workloads in many settings, with the potential of improving the quality of sputum microscopy. The policy changes do not, however, specify the timing for the collection of the two specimens. (6)

If specimens were collected at the time of consultation (1st on-the-spot) and the morning of the following day (morning sample), the spot morning specimens would still require a minimum of two visits, which is the minimum required by the spot-morning spot scheme currently used in most diagnostic of low- and middle income countries. In addition, the spot-morning and spot-morning-spot schemes still examine a substantial proportion of samples the second day of the diagnostic process. (6)

Another technique, referred to as same day sputum smear, has been proposed where two sputum samples are collected and analyzed on the same day. Same day would offer immediate results, and hence reduce on the dropout rate and ultimately control disease spread. This technique was be able to diagnose from 76% to 97% patients with PTB in two different studies, comparable to the standard scheme which identifies from 73% to 97%, while a multi country study found the

sensitivity of the same technique at 63.6% (95% CI) which was non-inferior to spot morning 64.8%, (95% CI) recommended by the WHO. (7)

However Most of the studies conducted hitherto has been under trial conditions and there is a lack of evidence from routine program settings and limited, conflicting information from was shown. Study from South India showed that same day microscopy was as effective as the conventional strategy. Whereas another study from North India showed that same day microscopy missed a considerable number of smear positive TB patients. (8, 9)

Therefore this study provoked by this state of knowledge and aim to assess the performance of this new strategy for the detection of pulmonary TB in our setting.

1.2 statement of the problem

Tuberculosis (TB), the most prevalent disease in the world has infected one-third of the world's population. In the Sub-Saharan Africa sharp rise in the incidence of pulmonary tuberculosis (PTB) has been attributed mainly to the appearance and wide spread of Human Immunodeficiency Virus (HIV) infection on the Continent. Ethiopia is an area with high prevalence of TB infection ranked second after Nigeria in Africa and seventh among the 22 high TB burden countries worldwide. The average TB prevalence and mortality rates are 623 and 42 per 82,950 individuals respectively having experienced a major increase in the burden of TB, presents one of the most serious public health challenges. (10, 11)

Direct sputum smear microscopy is the cornerstone of the diagnosis of pulmonary tuberculosis (TB), available in most primary health care laboratories at health center level. It is simple ,economical and reliable tool used widely for identifying acid fast bacilli for diagnosis and treatment of tuberculosis (TB) however it is documented to be highly specific in areas with a high prevalence of TB but with varying sensitivity (20-80%).It is a key component of directly observed therapy short course (DOTS) and DOTS plus strategy for diagnosing tuberculosis, initiating anti tuberculosis drugs, monitoring progress of the disease and it is a valid document to declare the cure in designated health facilities situated far flung areas. (9)

Most of the national TB control programs required microscopic examination of three sputum specimens for isolation of Acid Fast Bacilli (AFB) collected over two days using Spot Morning Spot (SMS) scheme. This scheme requires at least two visits and the patient often abandons the diagnostic procedure owing to costs and inconvenience involved in multiple visits. The greatest disadvantage is that such defaulting patients continue to move in the community and finally by the time we are able to diagnose them, they have already contributed to the spread of disease in the community. Every smear positive person if left untreated has the potential to infect 10-15 persons/year. (12)

Few studies in Ethiopia have been done and published regarding Performance of Same day Sputum Smears for Detection of Pulmonary tuberculosis however as to our knowledge there was no published study which used culture as a confirmatory test identify the proportion Multi drug Resistance Tuberculosis using same day approach.

Therefore assessing the performance of the same day for detection of pulmonary TB by performing both sputum smear and confirming with culture and identification of the proportion of MDR TB by using this new strategy at country major hospital Saint Peter which aim to be center of excellence a model TB specialized hospital in east Africa could give a good insight.

1.3 Significance of the study

Implemanting the same-day-smear microscopy in place of the conventional smear would allow services to provide laboratory results with in the same day and hence early identification of TB help to initiate therapy on the same day of consultation and this will interrupt the chain of transmission, furthermore it will have cost–benefit implications for the patient and the health system. The reduced workload of the laboratory also has the potential to redirect inefficient use of time (e.g. examination of the third smear) into improving the quality of examination of two smears.

Moreover, this study may be used

- As a document for policy makers to establish the same day diagnosis of TB supporting the recommendations given by WHO
- As a base line for researchers to conduct further research

2. Literature review

Tuberculosis (TB) is one of the most common infectious disease. Direct sputum smear microscopy is the most widely used test for the diagnosis of pulmonary tuberculosis (TB), available in most primary health care laboratories. The majority of laboratories use conventional light microscopy to examine Ziehl-Neelsen stained direct smears, documented to be highly specific in areas with a high prevalence of TB but with varying sensitivity (20-80%). Besides being labor-intensive, direct sputum smear microscopy may have considerable patient costs and inconvenience associated with the need to submit multiple sputum specimens over a period of up to three days. A number of TB control programs have reported high rates of initial patient default as a result. (13)

In 2011 study conducted in India showed that out of the total 330 TB suspect, the standard and the same day could identify 61/330(18.48%), 43/330(13.03%) respectively. Sensitivity of the standard and the proposed new method 58.25%and 40.07% respectively and specificity was 99.55% in both methods. (9)

In 2009, three systematic reviews were commissioned by WHO to assess approaches to improve microscopy for TB diagnosis. Including, frontloaded or same day strategies for sputum investigation, the results from seven studies involving 7,308 patients were reviewed. Pooled summary estimates using two specimen (spot-morning) showed that the sensitivity of a front-loaded approach (64%) was similar to that of the conventional two-specimen approach (65%) Pooled specificity estimates were identical (98%) at 95%. One large randomized controlled trial (6,628 patients in four different sites) reported data on differential patient losses to follow-up for the two diagnostic approaches, indicating that patients assigned to the front-loaded scheme were more likely to submit the first two (spot-spot) specimens than patients assigned to the conventional (spot-morning) scheme (97.9% VS 94.3%; difference 3.6%.(14)

In the same study by using three specimens(spot-morning-spot) and direct ZN microscopy the sensitivity estimates for the two strategies were similar and did not differ statistically: frontloading 71% ; conventional 68%. And specificity estimates were also similar at 98% for front-loading and 99% for the conventional approach. In the same study mentioned above reporting data on differential losses to follow-up (6,628 patients, four different sites), patients assigned to the front-

loaded approach were more likely to submit the third specimen (94.2%) than those assigned to the conventional approach (92.7%), difference 1.5%. (14)

In another Systematic review and meta-analysis on eight relevant studies from five articles enrolling 7771 patients with suspected tuberculosis in low-income countries including Ethiopia, Nepal, Nigeria, and Yemen Compared with the standard approach of examination of two smears with Ziehl-Neelsen light microscopy over 2 days, examination of two smears taken on the same day had much the same sensitivity (64% (95% CI) for standard microscopy VS 63% for same-day microscopy) and specificity (98% VS 98%). They noted similar results for studies employing light-emitting diode fluorescence microscopy and for studies examining three smears, whether they were compared with two-smear strategies or with one another. Indicating Same-day sputum smear microscopy is as accurate as standard smear microscopy. (15)

A Multi-Country Non-Inferiority Cluster Randomized Trial conducted in Ethiopia, Nepal, Nigeria, and Yemen shows that to the sensitivity of spot-spot (63.6%) was non-inferior of SMS (66.4%) and the specificity spot-spot (97.6%) similar with SMS (97.5%) suggesting two spot specimens did not have sensitivity inferior to two specimens collected as spot and morning(16)

In 2011 the study conducted in the Rajahmundry, India shows that among 658 participants, the first two smears from standard approach (SM scheme) identified Acid Fast Bacilli (AFB) in 64 patients, whereas same day scheme could identify 62 cases. Therefore, same day scheme missed two patients. There was, however, no statistical difference between the two schemes ($p>0.05$). (17)

In 2015 the study conducted by the same investigator T. Jaya Chandra on Same day sputum smear microscopy for the diagnosis of pulmonary tuberculosis by using modified ZN staining versus LED FM shows from 3328 patients were 142 (4.3%) were dropped out. The remaining 3186 patient's results were given. For Spot-Morning approach sputum Smear positivity was 297 (9.3%), 311 (9.8%), 343 (10.8%) and for same day approach sputum Smear positivity was 294 (9.2%), 311 (9.8%), 338 (10.6%) respectively for ZN, MZN and FS by LEDFM .MZN and FS techniques were Not significantly associated in SM and same day approaches. (18)

Another study conducted in central India, in the state of Chhattisgarh shows of 2551 presumptive TB patients, All patients provided the first spot specimen, 2361 (93%) provided the second spot specimen, and 2435 (96%) provided an early morning specimen. 72% of specimens were muco-

purulent in conventional strategy as compared to 60% in same day strategy. The proportion of smear-positive patients diagnosed by same day microscopy was 14%, as compared to 17% by the conventional method ($p<0.001$). A total of 73 (16.9%) potential cases were missed by the same day method compared to only 2 (0.5%) by the conventional method. (8)

In a cross-sectional descriptive study conducted at three district hospitals and the National Hospital of the Federal Capital Territory of Abuja, Nigeria in this study from Two hundred and twenty-four patients with chronic cough, The same-day and internationally recommended approaches identified 44 and 45 of the 78 patients with positive cultures, respectively. Suggesting that it could be possible to diagnose TB in a single day by examining two spot specimens. (19)

A Cross- Sectional Study at a Referral Hospital in Uganda Study to evaluate the Performance of Frontloading for Smear Microscopy in the Diagnosis of Pulmonary Tuberculosis shows that sensitivity of both the frontloading or same day and standard schemes was 91.1% while their specificities were 86.2% and 91.7% respectively. There was excellent agreement between the diagnostic capacities of the two methods (kappa statistic = 0.87, P, 0.0001). The positive predictive value for the frontloading scheme was 87.2% and that for the standard approach was 91.9%, while the negative predictive values were 90.4% and 90.9%, respectively. Concluding Frontloading based on smear examination of two same-day sputum samples has a similar performance to the current standard method and would not be associated with any significant missed diagnosis. (7)

Study from Tripoli, Libya on Front-loaded smear microscopy for the diagnosis of pulmonary TB indicate that Spot-Xspot and spot-morning smear microscopy had, respectively, 65% and 66% sensitivity and 97% and 96% specificity. Concluding For the diagnosis of pulmonary TB, the sensitivity and specificity of front-loaded (same-day) smear microscopy is similar to that of the standard smear microscopy scheme. (20)

A study conducted in Hawassa showed that out of of 4099 (26%) of the suspects had at least one positive smear with 3753 (91.6%) of the first specimens being positive. A further 303 (7.4%) were negative in the first specimen but had a positive second specimen and 42 (1%) suspects had two negative specimens followed by a positive third smear. The value of the third sputum is negligible as 99% of the cases were identified from the first and second specimens. Reducing the number of specimens to two or even one would have multiple advantages in countries where laboratories are usually over-burdened and are not easily accessible to the population. Submission of two specimens on the same day could improve compliance in submitting samples and collecting results as the number of diagnostic visits would be reduced without significant loss of sensitivity (21).

Also a study conducted in Bushullo Major Health Centre (BMHC) in the Southern Region of Ethiopia out of 243 suspects were enrolled; 52 had confirmed PTB: 49 (94%) were detected by the same-day method and 51 (98%) by the standard method($P > 0.05$). The same-day approach would reduce the number of visits required for diagnosis, save resources for the health system and the patient, and ultimately improve case detection in poorer countries (22).

3. Objectives

3.1 General objective

- To assess Performance of Same day Sputum Smears for Detection of Pulmonary and Multidrug Resistance Tuberculosis among presumptive tuberculosis cases visiting Saint Peter specialized hospital Addis Ababa, Ethiopia

3.2 Specific objective

- To compare yield of pulmonary tuberculosis detection using the Same-day and conventional scheme
- To assess sensitivity and specificity of the same-day scheme
- To identify proportion of MDR cases

4. Hypothesis

There is no difference between same-day and conventional for detection of pulmonary tuberculosis

5. Material and methods

5.1 Study area

The study was carried out in St, peter specialized hospital Addis Ababa Ethiopia. St Peter, was established 1953 as part of ministry of health of federal democratic republic of Ethiopia.

Its mission is to become the center of excellence a model TB specialized hospital in east Africa. its objectives are to integrate internal medicine pediatrics services and to improve research ,training and health services .St peter offers a host of services including laboratory, dental ,maternal health, neonatal care, voluntary counseling and testing ,health facility based medical care psycho-social support ,information ,education and communication ,as well as HIV/AIDS research.

5.2 Study Design and period

The study design for this research was Institution based Cross sectional study and the data collection period was from February 2017to may 2017

5.3 population

5.3.1 Source population: All pulmonary TB suspected patient who visit the hospital during the Study time.

5.3.2 Study population: All pulmonary TB suspected cases who fulfill the inclusion criteria was involved in the study

5.4 Inclusion and exclusion criteria

5.4.1 Inclusion criteria: All patients who were clinically suspected of Pulmonary TB and able to provide sputum samples were included in the study.

5.4.2 Exclusion criteria:

- Patients on follow up and treatment

- Patients who were unable communicate due to mental problem, coma, and ambulatory.

5.5 Study Variables

Dependent variable	Independent variable
➢ Yield of same day approach ➢ Performance of same day ➢ Proportion of MDR	age, sex, quality of specimen, qualification and experience of laboratory professional, training on AFB isolation

5.6 measurement and data collection

5.6.1 Sample size determination for determination of proportion of MDR TB using the same day

Sample size of the study population were all patients who gave four consecutive sputum sample for TB microscopy included conveniently. The sample size was calculated based on single sample size estimation as shown below. the value of p taken as 50% because there was no previous study on detection of MDR TB using the same day approach in Ethiopia.

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

$$N = \frac{(1.96)^2 * (1-0.5) * (0.5)}{(0.05)^2}$$

N =384

Where N = minimum sample size

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

P = 50 % prevalence MDR using same day

d= margin of error 0.05 at 95% CI

5.6.2 Sample size determination for pulmonary TB using same day approach

The sample size was calculated based on single sample size estimation as shown below. The value of p taken as 87 %(sensitivity) as a result of previous study conducted on the performance of same day sputum smear for the diagnosis of pulmonary tuberculosis. (22)

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

$$N = \frac{(1.96)^2 * (0.87) * (1-0.87)}{(0.05)^2}$$

N =174

Where N = minimum sample size

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

P = 87% % sensitivity of same day smear

d= margin of error 0.05 at 95% CI

We use 384 as sample size for our study.

5.6.3 Sampling technique

Convenient sampling technique was used and all new pulmonary TB suspect who visits the hospital within the data collection period were included.

5.6.4 Data collection procedure

After collecting Data on demographic and clinical characteristics, using structured questionnaire the patients were explained the importance of submitting thick sputum. It was demonstrated that by taking three deep breaths, followed by a deep cough, a good quality sputum could be brought from the lungs. The Patients were explained to provide first routine on Spot sample and after one hour "extra" spot sample at the time of first visit to the hospital. And the morning sample was collected at home of the following day and second routine on the spot sample was collected again when the patient brings the morning sample.

Laboratory data was collected by examining macroscopically for the quality of the samples and additionally two trained senior medical laboratory technologists and one laboratory technician examine all sample for microscopy by Ziehl-Neelsen (ZN) and florescent microscopy (FM) and observed under oil immersion for AFB following the WHO guide line. In accordance with the standard operating procedure for AFB and reporting system of St, peter hospital, all smears with $\geq$ 1 AFB/100 high power fields (HPF) were considered positives. At least 100 microscopic fields were examined to declare a slide negative. In case of positive smear, the bacterial load was classified (1 to 9 AFB per 100 fields, 10 to 99 AFB per 100 fields; 1 to 10 AFB per field after examining at least 50 fields and more than 10 AFB per field after examining at least 20 fields) using Standard operating procedure for AFB and reporting system of St, peter hospital and WHO guidelines.

Furthermore the morning sample collected with falcon tube was transported to EPHI national TB reference laboratory to and cultured using Lowenstein-Jensen (LJ) and Culture isolates were confirmed by SD bio line TB Ag MPT64 rapid test. Multidrug resistance tuberculosis status was determined using line probe assay (Rifampicin, Isoniazid).Culture is considered as a gold standard to compare the result and evaluates the performance of the standard and the same day smear microscopy and for calculation of sensitivity, specificity, negative predictive value and positive predictive value.

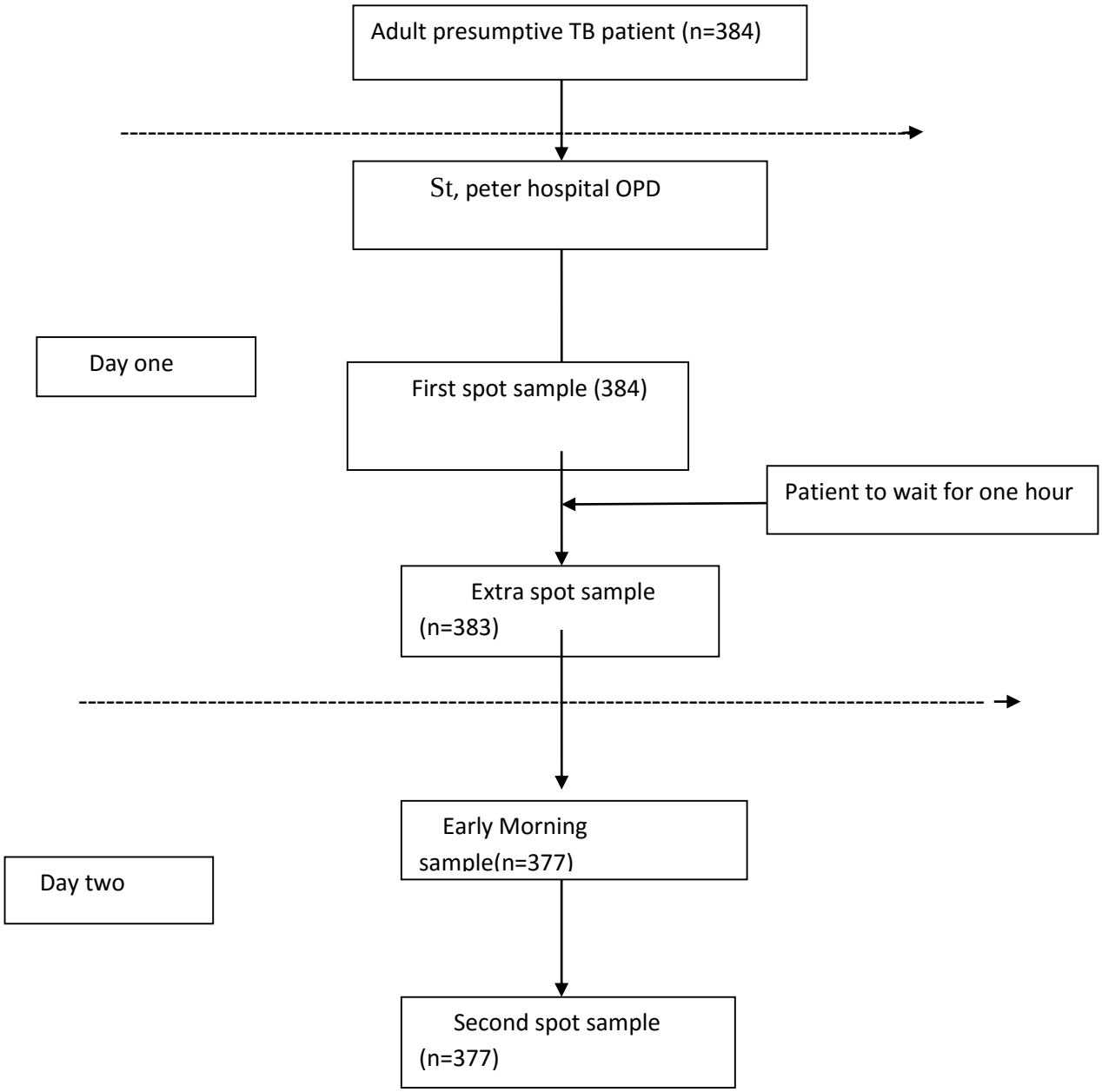

Figure 1; sample collection procedure.

5.6.4.1 Direct smear preparation

A total of four sample from each one smear of direct smears was prepared by using labeled microscopic slides and taking a small portion of the purulent or muco-purulent part of the sputum with an applicator stick, and smearing it over the area equal to 2-3cm.which was then dried ,quality control was done using positive and negative slides and documented in the air at room temperature the dried smears was stained using both the Ziehl-Neelsen (ZN) and florescent (FM) technique. (23)

5.6.4.2 Principle of Ziehl- Neelsen staining

The property of acid fastness is based on the presence of mycolic acid in the cell wall of mycobacterium. Primary stain (fuchsin) binds to cell-wall of mycolic acids. Intense decolourization (strong acid or acid alcohol) does not release the primary stain from the cell well and the mycobacterium retain the red color of fuchsine hence acid-fastness. Counterstaining (with methyl blue) will provide a contrasting background. (24)

5.6.4.3 Result Reporting and interpretation

Viewed with oil immersion AFB are red, slender rods, some time with one or more granules. The bacilli may occur singly, V-shaped forms, cords, or clumps. Fragments of bacilli often seen during treatment. (24)

observation	Reporting
No AFB found in at least 100 fields	No AFB seen
1-9 bacilli in 100 fields	Record exact number of bacilli
10-99 AFB per 100 fields	1+
1-10 AFB per fields (cheek 50 fields)	2+
More than 10 AFB per fields (cheek 20 fields)	3+

Table 2: Reporting and Interpretation of AFB

5.6.4.4 Culturing using Lowenstein-Jensen Medium

Principle

L-Asparagine and Potato Flour are sources of nitrogen and vitamins in Lowenstein-Jensen Medium. Mono potassium Phosphate and Magnesium Sulfate enhance organism growth and act as buffers. Glycerol and the Egg Suspension provide fatty acids and protein required for the metabolism of mycobacteria. The coagulation of the egg albumin during sterilization provides a solid medium for inoculation purposes. Sodium Citrate and Malachite Green are selective agents to prevent growth of most contaminants and allow early growth of mycobacteria. (25)

5.6.4.4.1 Inoculation and incubation of LJ media

Primarily condensed moisture observed at the bottom of culture medium slants were removed before inoculation. Inoculation for primary isolation, identification, and susceptibility testing of Mtb was done at BL-2 cabinet using pipettes. Each slope was inoculated 150 µl of the centrifuged sediment, distributed over the surface. Two slopes of LJ medium were inoculated per specimen. But preparation of inoculums for susceptibility testing was according to the McFarland standards of inoculums preparation procedure stated in the annex part .All cultures were incubated at 35°-37°C until growth is observed and cultures were discarded as negative after eight and six weeks for primary isolation and susceptibility testing respectively. Inoculated media was preferably be incubated in a slanted position for at least 24 hours to ensure even distribution of inoculums. Thereafter, if incubator space is needed, bottles were placed upright. Caps were tightened to minimize evaporation and drying of media. (25)

5.6.4.4.2 Culture reading

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

(colonies yielded in less than 7 days) were removed and repeated sample processing from the sediment .(25)

5.6.4.4.3 Identification of mycobacterium tuberculosis using SD Bioline TB Ag MPT64 rapid test

SD Bioline TB Ag MPT64 Rapid is used for rapid immunochromatographic identification of the M.tuberculosis complex in combination with culture systems based on liquid or solid media. The assay rapidly discriminates between the M.tuberculosis complex and MOTT bacilli.(26)

Principle

The SD Bioline TB Ag MPT64 Rapid test is an immunochromatographic method which can detect MPT64 antigens produced by M. tuberculosis complex from AFB positive MGIT or Lowenstein Jensen's media. The test cassette consists of a sample pad, a gold conjugate pad, a nitrocellulose membrane and an absorbent pad. Mouse monoclonal anti-MPT64 antibodies are immobilized on the nitrocellulose membrane as the capture material (Test Line). Another antibodies conjugated with colloidal gold particles are used for antigen capture and detection in a sandwich type assay. The test device has a letter of T and C as "Test Line" and "Control Line" on the surface of the case. Both the "Test Line" and "Control Line" in result window are not visible before applying any sample. The "Control Line" is used for procedural control. (26)

Control line should always appear if the test procedure is performed properly and the test reagents of the control line are working. As the test sample applied in the sample well flow laterally through the membrane, the antibody-colloidal gold conjugate binds to the MPT64 antigen in the sample. This complex then moves across a chromatographic carrier, and is then captured by a second fixed antibody located in the middle of the test cartridge. This reaction produces a red to purple color band which indicates a positive result for M. tuberculosis complex. In the absence of MPT64, there is no line in the test band region. (26)

5.6.5Line probe assay

This test was be used for detecting the drug susceptibility of M. tuberculosis Complex (MTBC) strains against the two first-line anti-TB drugs (INH, RMP).

5.6.5.1 DNA Extraction using Genolyse chemical method

Genolyse DNA extraction method is a chemical technique that provides a substantial decrease in time to prepare DNA from clinical samples or culture isolates. The time required to extract DNA is less than half that of the mechanical method in MTBDRplus Version 1.0. Extracted DNA is then amplified to facilitate detection of mycobacterial drug resistance.

Principle

DNA extraction is a procedure whereby DNA is fetched from bacterial cells or fragments of bacterial cells to be used for molecular biology analysis. With the Genolyse chemical method test, this implies that: the bacterial cells in the decontaminated patient sample or culture samples are chemically broken to expose the DNA by using a lyses buffer. (27, 28)

5.6.5.2 Preparation of master mix reagent for technique version 2.0

It is a premixed, ready to use solution containing Taq DNA polymerase, dNTPs, and MgCl2 and reaction buffers at optimal concentrations for efficient amplification of DNA templates by PCR

Principle

All reagents needed for amplification are included in the Amplification Mixes A and B and are optimized for the PCR step of MTBDRplus test. The AM-A contains Taq polymerase, PCR buffer and nucleotides. The nucleotides acts as DNA precursors (the four deoxynucleoside triphosphates, dATP, dCTP, dGTP and dTTP) which will be used as building blocks during the elongation of the single stranded DNA. DNA polymerase (Hot Start Taq) is required to elongate the DNA molecule by facilitating the incorporation of the free nucleotides onto the end of the primer, according to the complementary base on the single stranded target DNA. The AM-B contains biotinylated primers for the amplification of specific regions of the mycobacterial chromosome. The Mg2+ in the salts forms soluble complexes with the free nucleotides allowing for the DNA polymerase to recognize them as substrates during the amplification procedure. (28, 29)

5.6.5.3 DNA addition, amplification and detection

Genotypic DST is used for rapid confirmation of drug resistance tuberculosis in suspected patients groups. MDR-TB diagnosis can be made in as short as 48 hours as compared to conventional DST which can take as much as 1-2 months.

Principle

Genotypic DSTs were designed as alternative methods to improve the speed of diagnosis of drug resistant-TB, especially MDR/XDR-TB. Resistance to first line drugs develops through sequential accumulation of mutations in genes targeted by the respective drugs. Several genes were linked to resistance to TB drugs, the most known and used are KatG and promoter region of InhA for INH, rpoB for rifampicin resistance. Mutations in specific codons were identified and used for detecting resistance to specific drugs. Genotype MTBDRplus is based on the DNA-strip technology and consist of three steps: DNA extraction from cultures or clinical specimens, amplification of the target gene with biotinylated primers and a reverse hybridization. (29)

All reagents needed for amplification are included in the master mix and are optimized for the PCR step of MTBDRplus test. The AM-A contains Taq polymerase, PCR buffer and nucleotides. The nucleotides acts as DNA precursors (the four deoxynucleoside triphosphates, dATP, dCTP, dGTP and dTTP) which will be used as building blocks during the elongation of the single stranded DNA. DNA polymerase (Hot Start Taq) is required to elongate the DNA molecule by facilitating the incorporation of the free nucleotides onto the end of the primer, according to the complementary base on the single stranded target DNA. The AM-B contains biotinylated primers for the amplification of specific regions of the mycobacterial chromosome. The $Mg2+$ in the salts forms soluble complexes with the free nucleotides allowing for the DNA polymerase to recognize them as substrates during the amplification procedure. (29)

The membrane strips used in the hybridization or detection step are pre-coated with specific probes complementary to the amplified nucleic acids. After chemical denaturing, the single amplicons bind to the probes. Highly specific binding of complementary DNA strands is ensured by stringent conditions which result from the combination of buffer composition and a certain temperature. Thus, the probes reliably discriminates several sequence variations in the gene regions examined. The streptavidin-conjugated alkaline phosphatase binds to the amplicons' biotin via the

streptavidin moiety. Finally, the alkaline phosphatise transforms an added substrate into a dye which becomes visible on the membrane strips as a colored precipitate. (29)

5.7 Data quality assurance

After labeling, sample collection and transportation was done before starting the analysis and quality control materials was used for both microbiological and molecular analysis. For ZN reagents was controlled with scanty and +1 smear positive and negative control slides for every newly prepared staining solution to ensure the process is well credential. Physical characteristics (color, texture, homogeneity, and humidity), Sterility and Performance check of LJ media was performed. SPC and reagent processing (RPC) controls was used during processing of sputum and line probe assay while H37RV was used as a positive control for identification MTB in line probe assay. In order to minimize error standard operating procedure (SOPs) was strictly followed. The results of each and every test was properly recorded. The questionnaires and laboratory results was checked by the investigator for their consistency and completeness.

5.8 Data analysis and interpretation

Double entry of data was done using Microsoft Excel 2007 and analysis of the data is performed by SPSS version 24.The descriptive statistics such as frequency, median and percentage was calculated. Statistical significance association between dependent and independent variables will be evaluated using Chi-square test P-value < 0.05 was considered as indicator for statistical significance.

5.9 Ethical consideration

The proposal was approved by “Research and Ethical Review Committee” of the Department of Medical Laboratory Science Collage of Health Sciences Addis Ababa University Ethical Review Committee” and Permission was also obtain from the St Peter hospital research directorate and EPHI national TB lab. Written informed consent was sought from all study participants. Positive laboratory results was be communicated to Physicians.

5.10 Dissemination of the result

The results of the study was presented to the department of Medical Laboratory Sciences, School of Allied Sciences, College of Health Science, and Addis Ababa University and St, peter research and training center The principal investigator submitted the study abstract to local associations

(like EMA, EPHA and EMLA) and other international associations to present the results of the project during continuous medical education events or conferences organized by these associations. The summary of the thesis was submitted to the international or national peer reviewed journal for publication.

6. Result

Study of demographic characteristics

There were 384 participants involved in this study. The participants were mostly male 217(56.5%) and the rest 167(43.4%) were female. The age range was 8-95 years with the mean age 42 and standard deviation ± 16.8 years. About 50% of the study participant age was between 25 and 44; 95(24.7%) belongs to the age group 25-34years and 84(21.8 %) belongs to 35-44 years. out of 373 study participants 355(92.2%) were HIV negative and 18(4.7%) were HIV positive 11(2.9%) don't tested for HIV. The main clinical symptoms that the patients presented with were Cough greater than two weeks (350, 91.1%), night sweats (286, 74.5%) and chest pain (258, 67.7%). About 23.4% of the patients had past history of TB. The socio-demographic and clinical characteristics of the participants are summarized in Table 3.

(Table 3) demographic and clinical characteristics of participant's

characteristics		Number	Proportion (%)
Age (years)	0-14	02	0.5
	15-24	26	6.8
	25-34	95	24.7
	35-44	84	21.8
	45-54	57	14.8
	55-64	58	15
	>65years	62	16
sex	Male	217	56.5
	Female	167	43.4
HIV status	Negative	355	92.2
	positive	18	4.7
	Not done	11	2.9
Cough> two weeks	Yes	350	91.1
	No	34	8.9
Fatigue	Yes	253	65.9
	No	131	34.1
Fever	Yes	221	57.6

	No	163	42.4
Weight loss	Yes	199	51.8
	No	185	48.2
Hemoptysis	Yes	117	30.5
	No	267	69.5
Chest pain	Yes	258	67.7
	No	126	32.4
previous Family history of TB	Yes	90	23.4
	No	294	76.6
Night sweeting	Yes	286	74.5
	No	98	25.5

6.1 Yield of pulmonary tuberculosis using the same day method VS conventional method using FM and ZN microscopy

Out of 384 enrolled patients in the study only data from 377 participants provide all the required samples including the morning and the second spot samples. Using florescent microscopy (FM) we identified 25 patients with at least one positive smear. Of this, and 9 cases were positive by conventional method and missed by the same day scheme. The same day and conventional method using FM had a significant difference in case detection ($P<0.001$).

Moreover; using Zeil Neelson (ZN) microscopy both the same day and conventional microscopy also compared. ZN identified19 patients and 11 of them were identified only by conventional method. Same day diagnosis and conventional method using ZN also have a significant difference in case detection ($P<0.001$). The performance characteristic of the same day were compared using conventional method and culture as golden standard. The sensitivity, specificity, PPV and NPV by using smear and culture result were summarized by in Table (4).

Table (4) Sensitivity, specificity, positive predictive value and negative predictive value of the same day method compared to conventional and culture method

	conventional method		Culture result	
	FM same day method	ZN same day method	FM same day method	ZN same day method
Sensitivity	73.5	63.3	40.8	32.7
Specificity	100	100	99.7	100
PPV	100	100	95.2	90.3
NPV	97.4	96.9	91.4	100

Diagnostic value of FM over ZN

Additionally we compare the yield of the same day method by using FM and ZN. Out of 377 cases both FM and ZN identified 19 positive cases and 06 cases were positive in florescent microscopy only (P< 0.001and kappa value was 0.855).

Furthermore we compare the yield of conventional method by using FM and ZN. Both FM and ZN identified 30 cases and 04 cased missed by ZN but positive by FM (P< 0.001 and kappa value = 0.932).

6.2 Performance of smear microscopy compare to culture

A total of 377 sample which was inoculated on LJ media 49(12.8%) were positive for MTB, 308(80.2%) were negative, 19(4.9%) were contaminated and 01case was NTM. By using same day from 25 patients which were positive by FM 20 of them had positive culture. And by using conventional scheme from 34 patients which were positive by FM 29 0f them had positive culture. Number of cases detected using same day VS and conventional method by using FM and ZN microscopy compared to culture was summarized in table (5)

Table 5. Summary of same day and conventional method VS culture

Approach			Culture result				
			contaminated	Negative	NTM	Positive	total
New method	FM	Neg	16	307	0	29	352
		Pos	3	1	1	20	25
	ZN	Neg	16	308	1	33	358
		Pos	3	0	0	16	19
Conventional Method	FM	Neg	16	307	0	20	343
		Pos	3	1	1	29	34
	ZN	Neg	16	307	0	24	347
		Pos	3	1	1	25	30

Neg =Negative, Pos=Positive, NTM=Non mycobacterial tuberculosis, FM= light emitting diode florescent microscopy, ZN=Ziehl Neelsen

The proportion of MTB in sputum was high 25(6.6%) in 25-34 age group followed by age group 35-44years 9(2.3%) both age group 45-54 and 55-64years had equal proportion of MTB 5 (1.3%) The Proportion of patients with positive culture by age summarized in table (6).

	Culture result					
		contaminated	Negative	NTM	positive	total
Age(years)	0-14	0	1	0	1	02
	15-24	2	20	0	4	26
	25-34	10	58	0	25	93
	35-44	2	68	1	9	80
	45-54	3	49	0	5	57
	55-64	1	51	0	5	57
	>60year	1	61	0	0	62

(Table 6) proportion of patients with positive culture by age.

Out of 384 participants 217 were male however seven of them do not bring complete sample required for the study. Out of 210 male participants 32 (8.4%) and from167 female participants17 (4.5%) had positive culture. The Proportion of patients with positive culture by sex summarized in table (7)

Table (7) Proportion of patients with positive culture by sex

Sex	Culture result				
	Contaminated	Negative	NTM	Positive	Total
Male	12	165	1	32	210
female	7	143	0	17	167
Total	19	308	1	49	377

6.3 The proportion of MDR

From the 49 culture positive cases 44 cases had valid LPA result of which 40 were susptable for Rifampicin and Isoniazid .one of the remained four cases which was smear negative had resistance for both Rifampicin and Isoniazid and of the other 03 cases which had INH mono resistance, from these three cases 01 case was smear positive in all four sputum sample by same day and conventional method using both FM and ZN ,01 case was smear positive by the morning sample and identified by conventional method using both FM and ZN whereas 01 case was smear positive by the morning sample and identified only by conventional method using florescent microscopy.

7. Discussions

Sputum smear microscopy is the most widely used test for the diagnosis of pulmonary tuberculosis (TB), available in most primary health care laboratories. The majority of laboratories use conventional light microscopy to examine Ziehl-Neelsen stained direct smears, documented to be highly specific in areas with a high prevalence of TB but with varying sensitivity (20-80%). Besides being labor-intensive, direct sputum smear microscopy may have considerable patient costs and inconvenience associated with the need to submit multiple sputum specimens over a period of up to three days. A number of TB control programs have reported high rates of initial patient default as a result (15).

7.1 Performance of the same day scheme

In this study from 377 participants using florescent microscopy (FM) we identified 25 patients with at least one positive smear. Of this, and 9 cases were positive by conventional method and missed by the same day scheme. Using Ziehl Neelsen (ZN) microscopy both the same day and conventional method identified19 patients and 11 of them were identified only by conventional method. The same day and conventional method by using both FM and ZN had a significant difference in case detection ($P<0.001$). The Sensitivity of the same day method was 40.8% and 32.7% Specificity 99.7% and 100%, PPV 95.2% and 90.3%, NPV 91.45 and 100% by using FM and ZN respectively.

The present study indicate that the sensitivity same day method was 40.8% and 32.7% by using FM and ZN respectively. This is inconsistent with the findings of Systematic reviews conducted by WHO (2009) which reveals that the sensitivity estimates for the same day and conventional strategies were similar and did not differ statistically: the same day or frontloading 71% conventional 68%. (14)

In contrast to our finding the study conducted by Davis JL et al also indicate that examination of two smears taken on the same day had much the same sensitivity (64%) for standard microscopy VS (63%) for same-day microscopy .They noted similar results for studies employing light-emitting diode fluorescence microscopy and for studies examining three smears. Indicating Same-day sputum smear microscopy is as accurate as standard smear microscopy (15)

The smear positivity of the current study was 25(6.6%), 19(5%) and for conventional approach 34(9%), 30(7.8%) respectively for FM and ZN technique different from the finding of Chandra T.J et al which shows For Spot-Morning (standard) approach sputum Smear positivity was 297 (9.3%), 343 (10.8%) and for same day approach sputum Smear positivity was 294 (9.2%), 338 (10.6%) respectively for ZN, and FS by LEDFM .(18)

The smear positivity in our study by using both microscopic technique was inferior to the finding of Chandra T.J et al

In the present study the sensitivity and specificity was 40.8% and 99.7% respectively and by using ZN the sensitivity and specificity was 32.7% and 100% PPV was 95.5%, 90.3% respectively for FM and ZN and NPV was 91.4%, 100% respectively for FM and ZN.

This finding had similar PPV and NPV but lower sensitivity compared to study done in Uganda (Nakakawa E, et al) in this study the sensitivity of both the frontloading or same day and standard schemes was 91.1% while their specificities were 86.2% and 91.7% respectively, the positive predictive value for the frontloading scheme was 87.2% and that for the standard approach was 91.9%, while the negative predictive values were 90.4% and 90.9%, respectively .Concluding Frontloading based on smear examination of two same-day sputum samples has a similar performance to the current standard method and would not be associated with any significant missed diagnosis.(7)

Even though in most of the studies conducted worldwide and in our country showed the same day and conventional sampling method has no significant difference in smear positivity rate, sensitivity and specificity in TB diagnosis (8, 16, 20, 21 and 22) the finding from V.P.Myneedu et al contradict the above mentioned findings by showing that out of the total 330 TB suspect, the standard and the same day could identify 61/330(18.48%), 43/330(13.03%) respectively. Sensitivity of the standard and the proposed new method 58.25%and 40.07% respectively and specificity was 99.55% in both methods. Indicating that two spot sputum specimen examination had sensitivity inferior to the standard method. (9)

In the present study the same day sputum collection approach identified 25 (6.6%), 19 (5%) and conventional method identified 34(9%), 30(8%) smear positive cases and the sensitivity of the same day was 40.8%, 32.7% by using FM and ZN staining technique respectively.

Despite the microscopy technique used the same day approach missed considerable number of positive patients than conventional scheme and the sensitivity was P value < 0.001showes statistical difference between the two methods and this outcome had similarity with V.P Myneedu et al findings. (9)

7.2 The proportion of MDR case

From the total 49 patents which were positive 44 cases had valid LPA result ,40 0f them were susceptible for both rifampicin and isoniazid, 01 cases was resistance for both rifampicin and isoniazid the other 03 cases had INH mono resistance. The patient who was resistance for Rifampicin and Isoniazid (MDR) was smear negative in both spot –extra spot (same day approach) and spot-morning-spot (conventional approach).

8. Limitation

Lack of literature on the detection of MDR tuberculosis on same day sputum smear approach.

9. Conclusions

The performance of same day smear microscopy for the diagnosis of pulmonary tuberculosis in the case of pulmonary tuberculosis suspect has lower performance to the current standard method and it is associated with significant missed diagnosis. This has indirect impact on the number cases of MDR.

10. Recommendations

- The federal ministry of health should promote the use of spot–morning-spot sputum collection approach (conventional smear microscopy) for the diagnosis of pulmonary TB.
- The concerned bodies should design a way to provide laboratories nearby in the community in order to reduce patient cost during multiple visit and minimize defaulting patients

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Annex I

This informed consent was obtained after reading /let them read the information sheet to the study participants. This information sheet was read by nurses, health officers or medical doctors at OPD at the time of patients visiting to the Hospital.

Information sheet

You are invited to participate in the study. This study involves the performance of the same day sputum smear microscopy for the detection of pulmonary and MDR tuberculosis among patients visiting St, peter TB specialized hospital. The aim of this study was to assess the performance of same day sputum smear for detection of pulmonary and MDR tuberculosis. And to give recommendation to concerned bodies so as to take appropriate measures before implementing this new strategy

Purpose: performance of the same day sputum smear microscopy for the detection of pulmonary and MDR tuberculosis among patients visiting St, peter TB specialized hospital.

a. **Duration**: the duration of this study deepened on the availability of study subjects 2-3 months.
b. **Procedure to be carried out**: the procedure is easy and simple ;first you was be asked few questions and then you are asked to provide sputum serial sputum specimens
c. **Risk and discomfort**: There would be minor discomfort during collection of multiple sputum sample and sample was collected by trained health professional's instruction according to SOP. appropriate medical care would be provided to you if needed
d. **Expected benefits**: The information gained from Yours and others study participant was help to consider prevention strategy for tuberculosis infection that remains a major public health problem in Ethiopia, if you are positive for TB/MDR-TB appropriate medical care would be provided to you
e. **Confidentiality:** we respect your privacy and confidentiality. Any information that identifies you was not be shared with anyone else outside the study team. If a research article or publication comes from this study, you were not be identified by name. The information we collect from you as part of the study was be kept in locked file cabinet, or

be protected by a password on the computer only accessible to personnel involved in the study.

f. **Voluntary participation and withdrawal from the study:** The participation is completely voluntary and you have the right not to participate in the study. You can stop participating in study at any time after giving your consent .This decision was not affect in any way yours current or future medical care in the health facility.

   Contact information: if you have any question about the study you can contact the following investigators and the ethical committee for further information.

   Nibret Mekuria: 0913788091

   Kassu Desta: 0911 10 70 99

   School of laboratory sciences department (office):0112755170

Thank you for your assistance.

Annex II

Consent form for study participant (English version)

I_____ I have been requested to participate in this study which involves collection of four consecutive sputum specimen from me and in which I will answer few question. The purpose of this study and sample collection procedure has been explained for me .I have also read the information sheet (or it has been read to me); I have understood that this study is about tuberculosis /MDR-Which is major problem causing morbidity and mortality in our country .I have asked some questions and clarification has been given to me .I have given my consent on behalf of myself to participate in study and I hereby confirm my agreement with my signature.

Signature _____ Date_____

Thank you for your participation in this important study.

N.B: If you want to request additional information about the study, you can contact as by 0913788091/ nibretmekuria@gmail.com

Annex III

Amharic Version of Consent Form

<u>ለጥናቱ ተሳታፊዎች የተዘጋጀ የፍቃደነት መግለጫ ቅፅ (ኮንሰንት)</u>

እኔ..... አራት ተከታታይ የአክታ ናሙና መሰጠት እንዲሁም ጥቂት ጥያዎች መመለስ በሚያካትተው በዚህ ጥናት እንድሳተፍ ጥያቄ ቀርቦልኛል።ጥናቱ ስለሚሰጠውም ጥቅም እንዲሁም የናሙና አሰጣጥ ሂደቱ ላይ ገለጻ ተደርጎልኛል።የመረጃ መሰጫ ቅፅ አንብቤ / ተነቦልኝ ተረድቻለሁ።ጥናቱ በሀገራችን ላይ ከፍተኛ ጉዳት እያደሰ ስለሚገኘው የሳንባ በሽታ እንዲሁም መድሀኒት የተላመደ የሳንባ በሽታ መሆኑን ተረድቻለሁ።የነበሩኝ ቀጥቂት ጥያቄዎች በሚገባ ተብራርቶልኛል። ስለዚህ በጥናቱ ለመሳተፍ ፍቃደኛ መሆኔን በፊርማዬ አረጋግጣለሁ።

ፊርማ---------- ቀን------/------/------

በዚህ ጠቃሚ ጥናት ስለተባበራችሁ አመሰግናለሁ።

ስለጥናቱ ተጨማሪ መረጃ ማግኘት ከፈለጉ 0913788091 መደወል ይችላሉ።

Annex IV

Section 1 Patient Identification and demographic questions

Lab No_____

Sex ----------

Age----------

Date of sample collection _____ (dd/mm/yyyy)

Total no of sample received _____

Result:

a) Completed_____ b) Incomplete _____c) excluded_____

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

Section 2 clinical assessment

HIV status positive_____negative_____

Cough >than 2weeks YES_____NO_____

Weight loss YES_____NO_____

Night sweating YES_____NO_____

Family history of TB YES_____NO_____

Chest pain YES_____NO_____

Hemoptysis YES_____NO_____

Fever YES_____NO_____Fatigue YES_____NO_____

Section 2 Gross appearance of sputum

Haemoptysis____ Purulent _____ Mucopurulent_____ Saliva _____

AFB Result

By principal investigator:

FM microscopy spot_____ extra spot_____morning_____spot

ZN microscopy spot_____ extra spot_____morning_____spot

Date and signature of laboratory technician _____

Comment:

Annex V

Laboratory procedure

Sputum collection Procedure

Give the patient confidence by explaining to him/her the reason for sputum collection

Instruct the patient to rinse his/her mouth with water before producing the specimen. It helps to remove food and any contaminating bacteria in the mouth

Instruct the patient to take two deep breaths, holding the breath for a few seconds after each inhalation and then exhaling slowly. Ask him/her to breathe in a third time and then forcefully blow the air out. Ask him/her to breathe in again and then cough. This should produce a specimen from deep in the lungs. Ask the patient to hold the sputum container close to the lips and to spit into it gently after a productive cough. Sputum is frequently thick and mucoid, but it may be fluid, with chunks of dead tissue from a lesion in the lung. The color may be a dull white or a dull light green. Bloody specimens was red or brown. Thin, clear saliva or nasopharyngeal discharge is not sputum and is of little diagnostic value for tuberculosis.

If the sputum is insufficient encourage the patient to cough again until a satisfactory specimen is obtained. Remember that many patients cannot produce sputum from deep in the respiratory track in a few minutes. Give him/her sufficient time to produce an expectoration, which s/he feels, is produced by a deep cough.

If there is no expectoration, consider the container used and dispose of it in the appropriate manner.

Check that the container is securely closed and label the container (not the lid) clearly

Wash hands with soap and water.

Give the patient a new sputum container and make sure that s/he understands that a specimen must be produced as soon as s/he wakes up in the morning.

Demonstrate to the patient how the container should be securely closed.

Instruct the patient to bring the specimen back to the health center or laboratory

Ziehl Neelsen methods: Procedure

Prepare smear as describes; allow air to dry.

Heat fix smear either on an electrics slide warmer at 65 to 75 ^{o}c for at least 2 hours or pass slide through Bunsen burner flame as for other bacteriological smear. Don't over heat.

Cover smear with a 2x 3 cm piece of filter paper to holds the stain on the slide and to minimize precipitation of dye crystal on to the smear.

Flood the paper strip with carbon fuchsine.

Heat the slides to steaming with Bunsen burner or an staining electric racks, let stands 5 minutes if the smear dries, add more stain add more stain but don't re heat.

Use forceps to remove paper strips from slides and to place them in discard containers wash slides with water (use tap water or water from reservoir bottles)

Flood smear with acid alcohol, allow to distain for 2 minutes

Wash smear with again with water and drain

Floods slides with methylene blue and counter stain for 1 to 2 minutes

Rinse with water, drain and air dry .do not blot

Examine smears under oil immersion objectives lens of the microscope

Examination of smears

The smear should be searched in an orderly manner by making a series of three parallel or nine sweeps the length of smears or nine parallel sweeps the width of the smears.

Reporting of smear results

Smear result should be quantified to be meaningful.

No AFB seen report

None…….. No AFB seen

1-2/300 fields……..±

1-9/100 fields……. …….1^{+}

1-9 /10 field's……2^{+}

1-9 /fields……..3^{+}

Annex VI

WHO Grading of Sputum Microscopy Results

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

Table 8. IUATLD/WHO recommended grading of sputum microscopy

Annex VII

Procedure used for preparation of reagents and media used for sputum processing and culture

1. **Preparation of N-acetyl L-cysteine- Sodium hydro oxide for sputum processing**

NaOH is toxic, both for contaminants and also for tubercle bacilli; therefore, strict adherence to the indicated timings is required.

Reagents: NALC-NaOH: 4% and Phosphate buffer 0.067M, pH 6.8

Procedure:

Step1-Weight 4g in 100 ml distill water

Step2- Weight 2.97 g in 100 ml distill water

Step 3- Mix step 1 &2

Step 4- Add 0.5g NALC

2. **Sputum processing**

Step 1- transfer the sputum (at least 2 ml, not more than 5 ml) in to a centrifuge

Step 2- add equal volumes of NALC-NaOH solution

Step 3- tighten cap of container and vortex slowly

Step 4-shake intermittently to aid homogenization and decontamination

Step 5-invert each bottle to ensure that NaOH solution contacts all the sides and inner portions of caps

Step 6-keep at 20 ^{o}c -25^{o}c for 15 min for decontamination

Step 7-fill the tube with phosphate buffer up to 50 ml mark on the tube

Step 8- vortex

Step 9- centrifuge at 3000g for 15 min

Step 10-carfully pours off the supernatant in to a discarded can containing 5 % phenol or other germicide

Step 11-inoculate deposit on to two slopes of L-J medium labeled with the ID number

Step12-use a pipette inoculate each slope with 3 to 4 drops

Step 13-smear on a slide with the ID number for microscope examination

3. Preparation of egg-based LJ media

LJ medium containing glycerol favors the growth of M. tuberculosis while LJ medium without glycerol but containing pyruvate encourages the growth of M. bovis. Both should be used in countries or regions where patients may be infected with either organism. And LJ medium prepared according to EHNRI standards.

Ingredients:

A) **Mineral salt solution**:

-Potassium dihyderogen phosphate anhydrous (KH2PO4) ---2.4g

-Magnesium sulphate (MgSO47H2O) 0.2g

-Magnesium citrate 0.6g

-Asparagines …….3.6g

Glycerol (reagent grade)……...12ml

-Distilled water……..600 ml

NB: Dissolve the ingredients in the distilled water by heating, autoclave at 121^{oc} for 30 minutes to sterilize. Cool to room temperature. This solution keeps indefinitely and may be stored in suitable amounts in the refrigerator.

B) **Malachite green solution:**

-Malachite green dye……2.0g

-Sterile distilled water …….100ml

NB: Using aseptic techniques dissolve the dye in sterile distilled water by placing the solution in the incubator for 1-2 hours. This solution will not store indefinitely and may precipitate or change to a less-deeply colored solution. In either case discard and prepare a fresh solution.

c). Homogenized whole eggs

Scrubbing thoroughly with a hand brush in warm water and a plain alkaline soap cleans fresh hens' eggs, not more than seven days old. Let the eggs soak for 30 minutes in the soap solution. Rinse eggs thoroughly in running water and soak them in 70% ethanol for 15 minutes. Before handling the clean dry eggs scrub the hands and wash them. Crack the eggs with a sterile knife into a sterile flask and beat them with a sterile egg whisk or in a sterile blender.

d). Preparation of complete medium

The following ingredients are aseptically pooled in a large, sterile flask and mixed well:

Mineral salt solution…….600 ml

Malachite green solution …….20 ml

Homogenized eggs (20-25 eggs, depending on size….1000ml

Finally the complete egg medium is distributed in 6-8ml volumes in sterile 14ml or 28ml McCartney bottles or in 20ml volumes in 20 x 150mm screw-capped test tubes, and the tops are securely fastened.

e). **Coagulation of the medium**

Before loading, heat the inspissator to 80^{oc} to quicken the build-up of the temperature. Place the bottles in a slanted position in the inspissator and coagulate the medium for 45 minutes at 80 ^{oc}-85^{oc} (since the medium has been prepared with sterile precautions this heating is to solidify the medium, not to sterilize it). Heating for a second or third time has a detrimental effect on the quality of the medium.

The quality of egg media deteriorates when coagulation is done at too high a temperature or for too long. Discoloration of the coagulated medium may be due to excessive temperature. The appearance of little holes or bubbles on the surface of the medium also indicates faulty coagulation procedures. Poor quality media should be discarded

f). Sterility cheek: After inspissations, the whole media batch or a representative sample of culture bottles should be incubated at 35^{oc}-37^{oc} for 24 hours as a check of sterility.

j).Storage: the LJ medium should be dated and stored in the refrigerator and can keep for several weeks if the caps are tightly closed to prevent drying out of the medium. For optimal isolation from specimens, LJ medium should not be older than 4 weeks.

3.1. **Quality Control**

3.1. **Sensitivity of plain egg based medium.**

Serious problems affecting the sensitivity of culture medium, i.e. its capacity to sustain consistent growth of tubercle bacilli, can be detected by seeding a 1/10.000 dilution of a suspension of Mycobacterium tuberculosis calibrated to McFarland No 1. (Equivalent to a bacterial suspension containing 1 mg/ml of tubercle bacilli)

- Prepare a McFarland No 1 suspension with a M. tuberculosis reference strain.

- Dilute the suspension with 10-fold dilutions to the 10^{-4} dilution.
- 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 10^{-4} diluted suspension.
- Incubate at 36°C+/- 1°C
- 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.

This register allows the identification and the elimination of deficient media batches. In the case of egg-based media, 20 days of incubation are usually enough to determine whether the sensitivity of the batch is satisfactory. If it is not, negative culture results obtained with tubes inoculated with the deficient medium will be invalidated and these cultures will be repeated. Media batches that are not homogeneous or contaminated, those that were exposed to high temperatures of inspissations as well as those showing low sensitivity, should never be used and should be discarded without delay.

3.2. **Reading: Solid media**:

- Make sure that cultures are checked at regular intervals:
- At 3 days of incubation to detect and to register early contamination
- Weekly to detect growth as early as possible.
- Confirm that new specimens have been requested in those cases when the smear positive specimens turn out to be culture negative or when all inoculated tubes/vials are contaminated.

3.3. Determination of the contamination rate: The contamination rate is a valuable indicator of the efficiency of procedures used for specimen processing. It is calculated as the percentage of contaminated tubes among all inoculated tubes or vials and not as the percentage of patients.

It should be within the range 2-4% and not exceed 5%, if the Petroff decontamination method is used. When available, computer databases should be preferred to hard copies forms to register and monitor results of positive patients and culture quality indicators

4. **Waste management and other safety precautions-**

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

Annex VIII

Consent form for parents/guardians (English version)

This study involves the performance of the same day sputum smear microscopy for the detection of pulmonary and MDR tuberculosis among presumptive cases visiting St, peter TB specialized hospital. The objective of this study was to assess the performance of same day sputum smear for detection of pulmonary and MDR tuberculosis

I have read the information above, or it has been read to me. I have been given the opportunity to ask questions and my questions have been answered to my satisfaction. I voluntarily consent that my child participates in this study provided he/she gives assent. To give his/her sputum sample and be a participant in this study and understand that I have the right to withdraw my child from the study at any time.

Name of participant and signature Date

_____ ____ /____ /____ (dd/mm/yy) _____

Thank you for your participation in this important study.

N.B: If you want to request additional information about the study, you can contact as by 0913788091 or nibretmekuria@gmail.com

Annex IX Assent form for children aged 12-17 years

This study involves the performance of the same day sputum smear microscopy for the detection of pulmonary and MDR tuberculosis among presumptive cases visiting St, peter TB specialized hospital. The objective of this study was to assess the performance of same day sputum smear for detection of pulmonary and MDR tuberculosis.

I have read the information above, or it has been read to me. I have been given the opportunity to ask questions and my questions have been answered to my satisfaction. I voluntarily assent that I would participate in this study provided my parents/guardians give their consent. To give my sputum sample and be a participant in this study and understand that I have the right to withdraw from the study at any time.

Name of participant, and signature Date

_____ ____ /____ /____ (dd/mm/yy)

Thank you for your participation in this important study.

N.B: If you want to request additional information about the study, you can contact as by 0913788091 or nibretmekuria@gmail.com

Declaration

I, the undersigned, declare that this MSc thesis is my original work, has not been presented for a degree in Addis Ababa University or any other universities. I also declare that all sources of materials used for the thesis have been duly acknowledged.

Name of the candidate: Nibret Mekuria (BSc) Signature_____

Place: Addis Ababa University Department of Medical Laboratory Sciences, Ethiopia

Date of submission_____/_____/_____

This thesis was submitted with my approval as university advisor.

Name of advisor: Kassu desta (MSc, PhD candidate) Signature _____

Place: Addis Ababa University, Department of Medical Laboratory Sciences, Ethiopia

Date of submission_____/_____/_____

Name of advisor: Muluwork Getahun (MSc) Signature _____

Place: Ethiopian public health institute, National reference TB Laboratory, Ethiopia

Date of submission_____/_____/_____