Evaluation of Alternative Diagnostic Methods for the Diagnosis of Smear Negative Pulmonary Tuberculosis: A cross-sectional comparative study at Jimma University specialized hospital, Jimma, Southwest Ethiopia

By

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A Thesis Submitted to Department of Medical Laboratory Sciences and Pathology, College of Health Sciences, Jimma University; in Partial Fulfilment of the Requirements for the Degree of Masters of Science in Medical Microbiology

April, 2015

Jimma, Ethiopia

Jimma University

College of Health Sciences

Department of Medical Laboratory Science and Pathology

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Abstract

Background: *Smear-negative pulmonary tuberculosis (SNPTB) is an increasing problem worldwide. Its diagnosis remains a challenge especially in developing countries like Ethiopia, where majority of SNPTB has been diagnosed only on the basis of clinical and chest radiographic findings which lead to high rate of misdiagnosis. Even though culture is the gold standard, results may take 2-8 weeks, and delay in initiation of treatment may allow further transmission of disease. Little is known about the effect of bleach concentration for the detection of acid fast bacilli by fluorescent microscopy and GeneXpert MTB/RIF assy. Thus, this study is intended primarily to determine effect of simple bleach concentration method for the diagnosis of smear-negative tuberculosis by fluorescent microscopy and GeneXpert MTB/RIF.*

Objective: *To evaluate the diagnostic yield of different methods (GeneXpert and fluorescent microscopy) for the detection of M. tuberculosis in smear negative sputum samples*

Methods: *A cross-sectional comparative study was conducted at Jimma University Specialized Hospital from February 14 to August 1, 2014. A total of two hundred and two patients suspected of having SNPTB were enrolled. Two sputum specimens (spot and morning specimen) were collected from each suspect. The spot sputum was analysed for culture (L-J media& MGIT 960) and direct GeneXpert test. The morning sputum was processed for direct and indirect GeneXpert test (bleach treated sediment) as well as fluorescent microscopy (FM). Half of the morning sputum was treated with 5% bleach and centrifuged at 3000g for 15 minutes. The concentrated sediment was used for Xpert and FM. Data was analysed by using SPSS version 20.0. The sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of the assays with 95%CI was calculated by using culture as reference method.*

Result: *Complete data were available for 185 samples. The detection rate of direct GeneXpert was 5.4 % (10/185) on spot and 6.5% (12/185) on morning sputum. Similarly indirect Gene Xpert detected M. tuberculosis in 11.4% (21/185) and LED-FM in 7.6% (14/185) of the suspected cases. L-J media supported the growth of*

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mycobacteria in 4.9% (9/185) and that of MGIT 960 in 8.6% (16/185) of the cases. Based on the gold standard method (combination of L-J and/or MGIT 960 media), 16(8.6%) cases were confirmed as smear negative tuberculosis. Direct GeneXpert had sensitivity of 50.0%, specificity of 97.6%, PPV of 66.7%, and NPV of 95.4%. While on bleach treated sputum, GeneXpert had sensitivity of 56.2%, specificity of 92.9%, PPV of 42.9% and NPV of 95.7%. LED-FM on bleach treated specimen had 25% sensitivity, 94.1% specificity, 28.6% PPV and 92.9% NPV. The sensitivity of direct GeneXpert on both spot and morning sputum was equal (50.0%) and GeneXpert on bleach treated sputum had 4.9% incremental yield than the direct one. All GeneXpert positive cases were sensitive for rifampicin.

Conclusions and recommendation*: GeneXpert on bleach treated sputum had highest detection yield. A significant proportion of suspected cases missed on conventional Ziehl-Neelsen method were detected on LED-FM. Bleach concentration method for GeneXpert had relatively higher sensitivity than the direct one and can potentially improve the diagnosis of SNPTB suspects. The use of only one sputum sample could be sufficient for TB diagnosis with Gene Xpert MTB/RIF.*

Key words*: Smear negative pulmonary tuberculosis, Gene Xpert MTB/RIF, Fluorescence microscopy, Bleach pre-treatment.*

Table of contents

Contents   **Pages**

List of Tables

List of Figures

Acknowledgement

Abbreviations and Acronyms

AFB   Acid Fast Bacilli

ART   Antiretroviral Therapy

BD   Becton Dickinson

CI   Confidence Interval

DOT   Directly Observed Treatment

DST   Drug Susceptibility Testing

EFY   Ethiopian Fiscal Year

EPTB   Extra Pulmonary TB

FNA   Fine Needle Aspiration

HIV   Human Immunodeficiency Virus

HPF   High Power Field

IQR   Inter Quartile Range

IUATLD   International Union against Tuberculosis and Lung Disease

IUC-JU   Institutional University Cooperation programme-Jimma University

LJ   Lowenstein Jensen

MDR-TB   Multidrug Resistant TB

MOTT   Mycobacteria Other Than Tuberculosis

MTC   Mycobacterium Tuberculosis Complex

NALC   N-Acetyl L-Cysteine

NPV   Negative Predictive Value

NTM   Non-Tuberculous mycobacteria

PCR   Polymerase Chain Reaction

PPV   Positive Predictive Value

PTB   Pulmonary TB

RIF   Rifampicin

ROS   Reactive Oxygen Species

Rpoβ   Gene Encoding For The ß-Subunit of the DNA-Dependent RNA
            Polymerase of *Mycobacterium Tuberculosis*

RRDR   Rifampicin Resistance Determining Region

SR   Sample Reagent

TB   Tuberculosis

WHO   World Health Organization

XDR-TB   Extensively Drug Resistant- TB

ZN   Ziehl-Neelsen

1. Introduction

1.1. Background Information

Tuberculosis (TB) is a chronic granulomatous disease caused by infection with some species of mycobacteria. It typically affects the lungs (pulmonary TB) but can affect other sites as well (extra pulmonary TB) depending on the portal of entry of the infection. It is caused by members of *Mycobacterium tuberculosis* complex *(*MTB complex)*,* a group of mycobacteria with a fairly large non motile rod-shaped, aerobic, acid fast, facultative intracellular parasite usually of macrophages. It has a slow generation time, 18-24 hours and it takes between three to eight weeks to view visible colonies on solid medium (1, 2).

The cellular envelope of *M. tuberculosis* consists of a plasma membrane and a highly unusual cell wall. The plasma membrane consists of a classical bilayer structure. The distinctive features of the mycobacterial cell walls include the lipoarabinomannan (LAM), lipomannan, mycolyl arabinogalactan, phosphatidylmyoinositol, sulfatide, cord factor, and other acylated trehaloses, phenolic glycolipids, and other lipoligosaccharides. Many of these have been shown to be involved in the virulence and pathogenesis of this bacillus. LAM, a predominant component of the cell wall, is a virulence factor for *M .tuberculosis*, which activates macrophages and scavenges reactive oxygen species (ROS) (3-5).

1.1.1. Etiology

TB is an infectious disease caused by *M. tuberculosis* complex (MTC) bacteria which has an endemic character and worldwide distribution. The MTC comprises closely related species responsible for strictly human and zoonotic tuberculosis. The complex consists of seven species including *M. tuberculosis, M. africanum, M. bovis*, *M. Canetti, M. pinnipedii, M.microti,* and *M.caprae* (6). Despite the different species tropisms, the MTC is characterized by 99.9% or greater similarity at the nucleotide level and possess identical 16SrRNA sequence(7).

Mycobacteria are slim, slow growing organisms that are 1-10μm long. They are rod shaped, acid fast, aerobic or micro-aerophilic, non-spore forming, non-motile, non-capsulated, lipid rich bacteria. They contain mycolic acids, and are complex, long chain fatty acids. They have

a cell envelope with high lipid content. This accounts for the difficulty in staining them with conventional techniques (8).

1.1.2. Transmission

In about 95 % of cases, TB is an airborne disease, transmitted by small airborne droplets, called droplet nuclei that are expelled when persons who have pulmonary TB (PTB) sneeze, cough, speak or sing. These tiny droplets can remain airborne for minutes to hours after spitting. The risk of infection is depend on several factors such as the infectiousness of the source case, the closeness of contact, the bacillary load inhaled, and the immune status of the potential host (9).

The presence of extensive pulmonary lesions such as cavities is the most important individual human factor in determining the infectious power. Since extensive pulmonary lesions are associated not only with an important concentration of oxygen that allows active bacillary multiplication but also with a rapid pathway to the external environment. The amount of bacilli released into the atmosphere under these conditions is enough to produce the transmission from person to person (10).

The main source of infection is the patient with pulmonary tuberculosis. Such patients may have pulmonary cavities that are rich in bacilli. Patients with cavitary PTB are almost always smear positives and are the main source of infection in the transmission of tuberculosis. The number of infectious droplets projected into the atmosphere by a patient is very high when coughing or sneezing (11).

Bovine tuberculosis (BTB) is caused by *M. Bovis*, a Mycobacterium highly similar to *M. tuberculosis*. The main host of *M. bovis* is cattle but it also affects many other mammalians including man. In human, it is the most frequent cause of zoonotic TB which is clinically indistinguishable from TB caused by *M. tuberculosis*. Before milk pasteurization, *M. bovis* was an important cause of human TB especially intestinal TB in children (12).

1.1.3. Pathogenesis and Immunity

M. tuberculosis complex usually enters the alveolar passages of exposed humans and animals in an aerosol droplet where its first contact is thought to be with resident macrophages i.e. alveolar macrophages. In addition dendritic cells play a very important role in the early stages of infection since they are much better antigen presenters than are macrophages and presumably play a key role in activating T cells with specific *M. tuberculosis* antigens (13).

In contrast with most phagocytised bacteria, *M. tuberculosis* prevents fusion of the phagosomes with lysosomes. This is due to the Waxy nature of cellular envelope and by arresting the maturation of the phagocytic vacuole. It also prevents killing by reactive oxygen species (ROS) formed in the macrophage since glycolipids and sulfolipids in the cell wall decrease the effects of oxidative cytotoxic mechanism. And also Mycobacterial ingestion is mediated via the chemokine (CR1) and (CR3) receptors, which do not stimulate microbicidal oxidative responses(1, 14).

When the infected macrophage calls for help,circulating macrophage, lymphocytes, neutrophils and dendritic cells are attracted to site of infection and form multi-nucleated giant cells of fused macrophages called "Granuloma".Infected macrophages spread to the local lymph nodes as well as into the blood stream and other tissues (bone marrow, spleen, kidneys, CNS) to cause Miliary tuberculosis(15).

M.tuberculosis known to be the most successful pathogen for a long period in human history. It does not possess the classic bacterial virulence factors such as toxins, capsules and fimbriae. Its success is achieved by its ability to persist in the hostile intracellular environment of infected macrophages (16).

The intracellular bacteria will stimulate both helper (CD4+) T cells and Cytotoxic (CD8+) T cells. Activated CD4+ cells release interferon gamma (INF gamma) which activate Macrophage in which activated macrophages engulf and kill mycobacterium. On the other wing cytotoxic T cell can lyses the cells with replicating bacteria. As a result if the bacteria are small in number it can be killed with minimal tissue damage but if is present in great number,

intense cellular immune response results in tissue necrosis by cytokines toxicity, production of hydrolytic enzyme and reactive oxygen intermediates (17, 18).

Localized collection of activated macrophage (granuloma) prevents further spreading of bacteria. The bacteria remain dormant in this stage or can be reactivated years later, when the patient immunity is wanes as the result of different factors like old age, immune compromising diseases like HIV, malnourished and chemotherapy (16).

1.1.4. Clinical manifestations

The most common clinical manifestation of TB is pulmonary distress which is insidious at onset. Patients typically have nonspecific complaints of malaise, fever, weight loss, cough, shortness of breath, chest pain and night sweats. Sputum may be bloody if cavitary and purulent. Uncontrolled cytokine release is responsible for many of the symptoms and signs of tuberculosis such as fever and wasting.

Tissue destruction is caused by cytokines, among which tumor necrosis factor α (TNF-α) appears to play an important role. This cytokine is also responsible for the cachexia (loss of appetite, loss of weight, muscular wasting) which are associated with tuberculosis (19).

As the cellular processes occur, tuberculosis may develop differently in each patient. According to immune status of the patient, it has different stages that include primary progressive disease, latency, reactivation and extra pulmonary disease. Each stage has different clinical manifestations (20).

Active tuberculosis develops in only 5% to 10% of persons exposed to *M tuberculosis.* When a patient progresses to active tuberculosis, early signs and symptoms are often nonspecific. Manifestations often include progressive fatigue, malaise, weight loss, and a low-grade fever accompanied by chills and night sweats. A cough eventually develops in most patients. Although the cough may initially be non-productive, it advances to a productive cough of purulent sputum (21).

Primary infection with *M. tuberculosis* leads to clinical disease in only~10% of individuals. In the remaining cases, the immune response arrests further growth of *M. tuberculosis.* The

pathogen is completely eradicated in only~10% of people, while the immune response in the remaining~90% of individuals only succeeds in containment of infection as some bacilli escape killing by blunting the microbicidal mechanisms of immune cells and remain in dynamic interaction between replicating bacilli and host immune system (dormant or latent) state (22).

In immune compromised individuals such as elderly, malnourished, or HIV-infected individuals, there will be reactivation of the latent state leading to multiplication of the bacteria in the macrophage to progress to active pulmonary state and sometimes disseminate to different organs like lymph node, kidney, bone, or joint, meninges, skin/soft tissues, liver/spleen or other organs to cause extra pulmonary tuberculosis (23).

Studies prior to the HIV epidemic estimated that there were 1.22 cases of smear negative and extra pulmonary TB for each smear positive case. Patients with smear negative pulmonary TB was found to be less infectious and to have a lower mortality, but a significant proportion (50%−71%) progressed to active disease justifying treatment. The coming of HIV has changed many of these parameters. Countries affected by both HIV and TB have experienced a disproportionate increase in smear negative disease. While apparently remaining less infectious than smear positive cases, HIV positive patients with smear negative pulmonary TB are generally more immune compromised, have more adverse drug reactions, and suffer higher mortality rates on treatment. Clinical decision making has also been complicated because HIV co-infection broadens the differential diagnoses of smear negative pulmonary TB to include diseases such as Pneumocystis carinii pneumonia (PCP), pulmonary Kaposi's sarcoma, and Gram negative bacteraemia (24, 25).

The proportion of SNPTB in Ethiopia is also higher than smear positives i.e. 34.5% of cases notified in 2005 EFY were smear negative pulmonary TB while 33.4% were smear positive pulmonary TB. The currently used diagnosis methods in Ethiopia (Jimma) for this large proportion of cases is based on clinical diagnosis and chest X-radiography which are nonspecific which leads to delays in diagnosis which may delay initiation of treatment, and further TB transmission may occur (26).

1.1.5. Diagnosis

A detailed history and physical examination, tuberculin skin test, AFB staining for acid-fast bacilli, radiologic examination, culture and PCR would be instrumental in arriving at the diagnosis of *M. tuberculosis*. Tests for the diagnosis of tuberculosis vary in sensitivity, specificity, speed, operational characteristics and cost. Patients with persistent cough lasting longer than two weeks in addition to the other signs should be assessed for TB.

1.1.5.1.Chest Radiography (CXR)

Often, the diagnosis is first entertained when the chest radiograph of a patient being evaluated for respiratory symptoms is abnormal. Radiographic findings suggesting TB include upper lobe infiltrates, cavitary infiltrates, and hilar or paratracheal adenopathy. In many patients with primary progressive disease and those with HIV infection, radiographic findings are more subtle and can include lower lobe infiltrates or a Miliary pattern (27).

1.1.5.2.Sputum smear microscopy

The conventional Ziehl-Neelsen (ZN) method on direct smears for acid fast bacilli (AFB) is widely used and plays a vital role in the diagnosis of TB since it is fast, inexpensive, and specific for *Mycobacterium tuberculosis*, but it has low sensitivity ranging from 43% to 55% depending on the bacilli load in the sputum sample. The other limitation of this method is that it could not differentiate between *M.tuberculosis* and *non-tuberculous mycobacteria.* But many reports have suggested that treatment of the sputum sample with sodium hypochlorite (NaOCl) will significantly increase the sensitivity of smear microscopy (28-30).

Sputum smear examination for acid fast bacilli (AFB) can diagnose up to 50–60% of cases of pulmonary tuberculosis in well-equipped laboratories. In low income countries like Ethiopia, poor access to high microscopy services contributes to even lower rates of AFB detection. Furthermore, in countries with high prevalence of both pulmonary tuberculosis and HIV infection, the detection rate is even lower owing to the paucibacillary nature of pulmonary tuberculosis in paediatric and patients with HIV infection. In the absence of positive sputum smears for AFB, at primary care level, most cases of pulmonary tuberculosis are diagnosed on the basis of clinical and radiological indicators (31).

Although the classic Ziehl-Neelsen method is still used, numerous reports have indicated that the fluorescent microscopic technique is much more sensitive. Light emitting diode (LED) microscopy is a newly introduced diagnostic tool to complement the conventional microscopy. It is recommended for centres with high case load as it saves time and improves sensitivity. In fluorescence microscopy, the number of organisms observed in equal areas of duplicate preparations has been reported to average 3.65 times the number seen with the Ziehl-Neelsen stain. This suggests that, overall, fluorescence microscopy is more sensitive than conventional microscopy, and has almost similar specificity (32).

1.1.5.3.Culture

In the isolation of mycobacteria by culture, the ideal medium should be able to support rapid and dense growth, and allow the determination of its characteristic features, e.g. Colony, morphology, growth rate and pigment production. Most of mycobacterial culture media fall in to egg potato base media and agar base media. The most popular egg based media is the Lowenstein-Jensen buffered egg potato medium. Among the agar based media, Middlebrooks 7H-10, Middlebrook 7H-11, and Dubose oleic albumin agar are recommended. The advantages of egg based media are the long shelf life and the low cost of preparation. Egg media require heat for solidification, which, along with the presence of albumin, inactivates certain anti tuberculous. agents (33).

Mycobacteria are slow growing and hence culture is not routinely done in all laboratories. The rapid radiometric culture system or BACTEC-460 which is automated has been accepted for the culture isolation of mycobacteria using an enriched Middlebrooks 7H12 containing ^{14}C labelled palmitic acid. The use of the radiometric method has significantly improved the recovery rates of mycobacteria from respiratory secretions and other specimen sources. However, BACTEC 460 TB has a number of well-known disadvantages: (I) it requires the use of radioactive reagents (^{14}C palmitic acid), (ii) it is labour-intensive in the handling of vials and maintenance of the instrument, and (iii) there is a potential risk of cross contamination of the cultures (34, 35).

Another automated culture method is BACTEC MGIT 960 the one which works by fluorescent detection system. This media is enriched with supplement containing oleic acid, albumin,

dextrose, catalase (BBL MGIT OADC) and antibiotic mixture of Polymyxin B, amphotericin B, Nalidixic acid, trimethoprim and Azlocillin (BBL MIGT PANTA) (36).

The BACTEC MGIT 960 system involves culture tube containing a fluorescent sensor that detects the concentration of oxygen in the medium. The fluorescent material in the medium is oxygen quenched but while the mycobacterium is growing there will be consumption of oxygen as a result there will be fluorescence signal. After a certain level of fluorescence the machine indicates the presence of growth (37).

1.1.5.4.Nucleic acid amplification test

Rapid, sensitive and improved diagnostic tools are needed for tuberculosis (TB) case detection including smear-negative disease often associated with HIV. This is to overcome the impact of length of time required by classical diagnostic tests as well as to expanded capacity of diagnosing multidrug-resistant tuberculosis (MDR-TB) since this is global priority for TB care and control (38, 39). To achieve this, nucleic acid amplification assays that can be used directly on clinical specimens are mandatory. Of these assays GeneXpert MTB/Rif is the newly developed diagnostic tool.

Test principle of GeneXpert MTB/Rif assay: Xpert MTB/RIF is an automated molecular test for detection of *Mycobacterium tuberculosis* (MTB) and resistance to rifampin (RIF). It uses real-time polymerase chain reaction (PCR) assay to amplify an MTBC specific sequence of the *rpoβ* gene, which is probed with molecular beacons for mutations within the rifampin resistance determining region (RRDR). Testing is carried out on the MTB/RIF test platform (Cepheid ,Sunnyvale, CA, United States), which integrates sample processing and PCR in a disposable plastic cartridge containing all reagents required for bacterial lysis, nucleic acid extraction, amplification, and amplicon detection. The only manual step is the addition of a bactericidal buffer to sputum before transferring a defined volume to the cartridge. The MTB/RIF cartridge is then inserted into the GeneXpert device, which provides results within 2 hours (40, 41).

1.1.5.5.Immunological techniques

Immunodiagnostic tools employed in TB diagnostics measure nonspecific mediators of inflammation secreted by innate and adaptive immune cells, aspects of the T-cell-mediated immune response to *M. tuberculosis* antigens or the detection of specific antibodies against these antigens by serological tests.

Tuberculin skin test (TST)

Tuberculin Skin Test (TST) is one of the most common tests to determine if someone has been exposed to and become infected with the TB bacteria. TST relies on principles of delayed hypersensitivity to recruit memory T cells to the site of an intradermal injection of purified protein derivative (PPD) of *Mycobacterium bovis.* However, vaccination with the BCG vaccine can also lead to a reaction at the TST site ,which limits the test's usefulness in vaccinated children or people repeatedly tested because of high risks of exposure (such as healthcare workers).

Interferon gamma release assay (IGRA)

Interferon-Gamma Release Assays (IGRAs) are whole-blood tests that can aid in diagnosing *Mycobacterium tuberculosis* infection. It measures a person's immune reactivity to *M. tuberculosis.* White blood cells from most persons that have been infected with *M. tuberculosis* releases interferon-gamma (IFN-g) when mixed with antigens derived from *M. tuberculosis*. It shows an excellent specificity, but until now they do not help differentiate latent tuberculosis infection (LTBI) from active tuberculosis disease.

1.1.6. Treatment

Effective regimens for the treatment of TB must contain multiple drugs. The course of drug therapy usually lasts from 6-9 months. Most commonly used first line drugs are Rifampin (RIF), Isoniazid (INH), Pyrazinamide (PZA), Ethambutol (EMB) and Streptomycin (SM). And there are also second line anti-TB drugs like Aminoglycosides (kanamycin, amikacin, capromycin,clarithromycin),Fluroquinolones(ofloxacin,ciprofloxacin,levofloxacin,moxifloxaci

n), Cycloserine, Thioamides (ethinoamide, prothionamide) and Paraminosalicylic acid (PAS) (1).

Updated first-line and second-line anti Tuberculosis drug groups includes five groups of drugs including (a). First-line oral anti-TB agents consisting of Isoniazid, Rifampicin, Ethambutol and Pyrazinamide; (b) Injectable anti-TB agent including Streptomycin, Kanamycin, Amikacin and Capreomycin; (c) Fluoroquinolones consisting: Ofloxacin, Levofloxacin, Moxifloxacin and Gatifloxacin; (d).Oral bacteriostatic second-line anti-TB agents: consisting of Ethionamide, Prothionamide, Cycloserine and P-aminosalicylic acid; and (e) Anti-tuberculosis agents with unclear efficacy (not recommended by WHO for routine use in MDR-TB patients): includes Clofazimine, Amoxicillin/clavulanate, Clarithromycin and Linezolid (42).

The major problem in the treatment of tuberculosis is the emergence of drug resistance to anti-TB drugs that can occur mainly due to man-made like when, (a) patients do not complete their full course of treatment, (b) health-care providers prescribe the wrong treatment, the wrong dose, or length of time for taking the drugs and (c) supply of drugs is not always available (43).

1.2. Statement of the problem

Tuberculosis remains a major global health problem. According to WHO 2014 TB report, there were an estimated 11 million prevalent, 9 million incident cases and 1.5 million people died from the disease globally in 2013. The case detection rate for all forms of TB was 64%. An estimated 1.1 million (13%) of the 9 million people who developed TB were HIV positive. The highest proportion (78%) of TB cases co-infected with HIV worldwide was in the African region. The majority of TB cases worldwide were in the South-East Asia, African, and Western Pacific regions (29%, 27% and 19%) respectively. In Ethiopia, the TB case detection rate of new all forms was only 62% (44). A major challenge observed over the past years is the low Case detection rate.

Smear-negative pulmonary tuberculosis (SNPTB) is an increasing clinical and epidemiological problem worldwide (45). HIV infection has been associated with an increased incidence of SNPTB and a higher mortality rate among patients with SNPTB (46, 47). In Ethiopia, more than 34% of TB cases had SNPTB and the proportion of smear negative TB case increased to 60% in HIV infected patients (48).

Although patients with sputum smear–negative TB are less infectious than patients who are sputum smear–positive, overall, 17% of TB transmission events were attributable to source patients with sputum smear-negative, culture-positive cases (49). Moreover, a longer health-service delay in the diagnosis of smear-negative than smear-positive pulmonary tuberculosis has been reported. Such delays in diagnosis may delay initiation of treatment, and further TB transmission may occur (25, 50).

In developing countries, the majority of SNPTB cases have been diagnosed only on the basis of clinical and chest radiographic findings and the misdiagnosis rates have been estimated as high as 35% to 52% (51). It has been reported that the number of unfavorable treatment outcome is high, and this in turn may lead to development of multi drug resistant (MDR) tuberculosis.

Chest X-ray (CXR) remains a component of the WHO diagnostic algorithm for the diagnosis of sputum smear negative PTB in low resource settings like Ethiopia. However, in regions of high HIV sero prevalence, many patients with culture confirmed TB have 'atypical' or normal

CXR features (52). This has questioned the role of CXR for the clinical diagnostic of smear-negative PTB in resource poor settings.

Zihel-Neelsen (ZN) microscopy has been the most widely used means of tuberculosis diagnosis in resource limited countries. However, Zihel-Neelsen (ZN) microscopy was found to be highly specific in areas with a high prevalence of TB but with varying sensitivity (20 - 80%). On the other hand, fluorescence microscopy (FM), both conventional and LED using Auramine-O, has showed increased detection of TB bacilli by 10%. But conventional fluorescence microscopy use has been limited by the high cost of mercury vapor light sources due to short life span, the need for regular maintenance and the requirement for a dark room. As a result in 2011, WHO has recommended for the replacement of conventional FM by LED microscopy and as an alternative for ZN microscopy (53).

The conventional diagnosis for multi-drug-resistant tuberculosis (MDR-TB) relies on bacterial culture, which is a slow and labour-intensive process. Since culture result requires 3-8weeks until the results are available, patients may not gate appropriate treatments on time and drug resistance strains may be amplified (54, 55). Thus, improved and timely diagnosis of tuberculosis (TB) in smear-negative pulmonary specimen is a priority area in TB control program.

WHO strongly recommend, Xpert MTB/RIF to be used as the initial diagnostic test in individuals suspected of having MDR-TB or HIV-associated TB. But WHO conditionally recommends the use of Xpert MTB/RIF for diagnosis of smear-negative specimens in settings where MDR-TB or HIV is of lesser concern (38).Mutations in *rpoB* and phenotypic resistance to RIF are highly correlated in 95 to 98% of cases; therefore, Xpert's high sensitivity for detecting these genotypic variations means that the technique has a high predictive value for the rapid diagnosis of multidrug resistance (56). Consequently, this may assist clinicians to provide proper initial treatment of patients with potential MDR TB and thus minimize its spread. Thus, this study primarily aimed to evaluate the diagnostic accuracy of Xpert MTB/RIF and fluorescent microscopy for the diagnosis of smear-negative and detection of rifampicin resistance in Ethiopia with high prevalent of drug resistance TB

One study in Ethiopia has indicated that 82.6% smear negative pulmonary tuberculosis suspects were etiologically unexplained by culture and they recommended a need to develop the most effective approach for the diagnosis of smear negative pulmonary tuberculosis in the Ethiopian setting (57).

In December 2010, the World Health Organization (WHO) endorsed the new diagnostic tool called Gene Xpert MTB/RIF assay to TB laboratory (38). The newly developed Xpert MTB/RIF assay utilizes real-time PCR technology for diagnosis of TB and detection of rifampicin resistance. The assay is conducted within a simple, almost fully automated cartridge-based system. The GeneXpert MTB/RIF purifies and concentrates *Mycobacterium tuberculosis* bacilli from sputum samples, isolates genomic material from the captured bacteria and subsequently amplifies the genomic DNA by PCR for detection (58)

Results are obtained from unprocessed sputum samples in 2 hours, with minimal biohazard and very little technical training (59). But sensitivity of this assay is low for SNPTB as compared to the smear positive pulmonary tuberculosis. This indicates a need for simple and efficient way to enhance the sensitivity of the GeneXpert MTB/RIF assay and fluorescent microscopy for detection of *Mycobacterium tuberculosis* in smear negative pulmonary samples. Therefore, we aimed to evaluate the diagnostic yield of simple centrifugation method before GeneXpert testing and fluorescent microscopy in minimally equipped laboratories in resource-poor settings for the diagnosis of SNPTB.

Several improvements have been suggested to increase the yield of the microscopic detection: a serial sputum specimen examination, fluorescent microscopy with auramine or Rhodamine staining and chemical fluidization of the sputum with concentration by sedimentation or centrifugation . Sodium hypochlorite (NaOCl) or bleach has been used for over a century in this application. The concentrations of 2-5% of NaOCl digest the sputum products and they inactivate the mycobacteria without altering their structures, so that even when they are killed, they can still be stained and observed. This provides a greater safety for laboratory use. Further centrifugation or sedimentation concentrates the acid fast bacilli (AFB) in the mixture and it increases the rate of the positivity (60).

Fluorescence microscopy increases the probability of detecting acid fast bacilli, especially if the sputum contains few bacteria, and hence improves the sensitivity of microscopy. The use of fluorescence microscopy in resource-constrained settings is limited by high investment and maintenance costs. It is four to five times more expensive than light microscopy. Therefore for economic reasons, fluorescence microscopy is currently recommended only in district laboratories that process more than 30 smears per day (61).

Fluorescence microscopy is credited with increased sensitivity and lower work effort, but there is concern that specificity may be lower. The most important advantage of the fluorescence technique is that slides can be examined at a lower magnification, thus allowing the examination of a much larger area per unit of time. There is insufficient evidence to determine the value of fluorescence microscopy in bleach treated sputum specimens for smear negative pulmonary tuberculosis (32).

Little is known about the effect of bleach concentration for the detection of acid fast bacilli by fluorescent microscopy and GeneXpert MTB/RIF assy. Thus, this study is intended primarily to determine effect of simple bleach concentration method for the diagnosis of smear-negative tuberculosis by fluorescent microscopy and GeneXpert MTB/RIF assay as compared to the gold standard which is culture.

1.3. Justification of the study

Nationally, more than 34% of all reported cases are smear negative pulmonary tuberculosis. Despite the presence of different diagnostic methods, limited information is available on the diagnostic accuracy of alternative diagnostic methods (GeneXpert, fluorescence microscopy, bleach processing of the specimen and culture) available for diagnosis of smear negative pulmonary tuberculosis in Ethiopia.

Although public health efforts so far have generally focused on the most infectious forms of the disease i.e. sputum smear positive tuberculosis, efforts are also needed for the potential new test capable of identifying sputum smear negative tuberculosis that would detect all cases, not just those that contribute to transmission.

Despite its advantage in enhancing sensitivity of conventional smear microscopy, no information is available on the added diagnostic value of bleach treatment of specimen for GeneXpert, fluorescence microscopy for the diagnosis of smear negative tuberculosis in Ethiopia.

Therefore, this study will help the stakeholders to formulate possible recommendation whether to consider bleach treatment of specimen for GeneXpert, and fluorescence microscopy for the diagnosis of smear negative tuberculosis. Besides, the study will help for national/regional implementation of GeneXpert and fluorescence microscopy for the diagnosis of smear negative pulmonary tuberculosis.

2. Literature review

TB can be diagnosed using different methods using smear microscopy, bacteriological, FNA cytology and histopathology (in case of EPTB), radiological and molecular diagnostic methods. In many developing countries, direct sputum smear microscopy is the primary tool for the laboratory diagnosis and monitoring the treatment of pulmonary tuberculosis using a conventional light microscope (62).

A significant rise in the number of TB cases reported from sub-Saharan Africa has been observed following the expanding Human immunodeficiency virus (HIV) epidemic. HIV is the strongest risk factor for developing tuberculosis disease. TB is responsible for more than a quarter of deaths among people living with HIV. The risk of developing TB is between 20 and 37 times greater in people living with HIV than among those who do not have HIV infection (63, 64).

Ethiopia adopted the 1996 of the World Health Organization (WHO) recommended strategy known as DOTS since 1997. To build on the achievements of DOTS and address the remaining challenges, the stop TB strategy was launched by WHO in 2006 to help achieve the millennium development goals for TB in 2015. Ethiopia also adopted this strategy to achieve the national 70% TB detection target. Ethiopia has surpassed the Millennium Development Goal (MDG) target of TB detection rate of 70% as per the 2011 TB prevalence survey (72%).

In 2005 EFY(2012/13), the TB case detection rate was 58.9%, which was below the detection rate estimated in 2011 TB prevalence survey as well as below target set 82.7% of all forms case detection for the year (26).The limited diagnostic capacity of the tests used in the country remains a challenge for improving case detection rate (65, 66).

According to 2014 WHO report, Ethiopia ranks 10th among the 22 high burden countries (HBC) and is member of the 27 high MDR-TB countries with a total of 130,614 cases (all forms) notified. This performance was below that observed in EFY 2004, when 145,367 cases (all forms) were reported (26).

Table 1. Latest estimates of tuberculosis in Ethiopia, 2013

Parameters	Figures
Population	94 101,000
Global rank	10th
Incidence (all cases /100,000 pop/year)	224
Prevalence(all forms/100,000pop/year)	211
TB mortality (all cases/100,000 pop/year)	32
New cases of multidrug resistant (%)	1.6
Previously treated TB cases with MDR TB (%)	12
Case notification (all cases %)	62
Estimated MDR-TB cases	1400
Treatment success new smear-positive (%)	91

Source: Global Tuberculosis Report 2014

In clinical evaluation studies, the sensitivity of GeneXpert in patients with smear-negative pulmonary TB was reported to be low,57–83%, as compared to 99–100% in patients with smear-positive pulmonary TB (38).

An evaluation of the accuracy of Xpert in Montreal, Canada, for the detection of pulmonary tuberculosis on induced sputum samples, using mycobacterial cultures as the reference standard, Xpert had a pooled sensitivity of 46% (95% confidence interval [CI], 26%-67%) and specificity of 100% (95% CI, 99%-100%) for detection of Mycobacterium tuberculosis. But Sensitivity was 86% (95% CI, 42%-100%) in the subjects with smear positive results There is

(67) also a potential reduction in time to diagnosis and treatment initiation(a median 12 days) by using Xpert before culture results issued (68).

A study conducted in Ibadan, Nigeria, on epidemiology of smear negative tuberculosis, revealed that 8.9% were smear positive while 11.3% of the specimens processed were culture positive. However, 2.6% of the smear negative specimens were culture positive while 21.4% of the smear positive specimens were negative for culture. The majority, 86.8% were smear and culture negative while 8.9% were positive for both tests. The findings of 2.6% smear negative but culture positive specimens in this study reveals that culture of specimens and other alternative diagnostic tools in addition to smear microscopy from suspected cases is necessary as a confirmatory test (69).

Another study conducted in Iran on clinical and laboratory diagnosis of the patients with sputum smear-negative pulmonary tuberculosis, 14.8% of smear negative suspects were culture positive (70).

In adults thought to have TB, with or without HIV infection, Xpert MTB/RIF is sensitive and specific. As compared with smear microscopy, Xpert MTB/RIF substantially increases TB detection by 23% (95% CI 15% to 32%) among culture confirmed cases. As an initial test replacing smear microscopy, Xpert MTB/RIF pooled sensitivity is 89% (95% (CI) 85% to92%). But as an add-on test following a negative smear microscopy result, Xpert MTB/RIF pooled sensitivity was 67% (95% CI 60% to 74%) (71). This shows that Xpert MTB/RIF have higher sensitivity for TB detection in smear positive than smear negative patients.

A study conducted in at five trial sites in Lima, Peru; Baku, Azerbaijan; Cape Town and Durban, South Africa; and Mumbai, India on rapid molecular detection of tuberculosis and rifampicin resistance, among culture positive patients, a single, direct MTB/RIF test identified 551 of 561 patients with smear positive tuberculosis (98.2%) and 124 of 171 with smear negative tuberculosis (72.5%). The test was specific in 604 of 609 patients without tuberculosis (99.2%). Among patients with smear negative, culture positive tuberculosis, the addition of a second MTB/RIF test increased sensitivity by 12.6 percentage points and a third by 5.1 percentage points, to a total of 90.2% (59).

A study conducted in Cape Town, south Africa on screening for HIV-associated Tuberculosis and Rifampicin resistance before ART using the Xpert MTB/RIF Assay showed that, the sensitivity of the Xpert MTB/RIF assay for smear-negative TB was substantially lower than for smear-positive disease and was dependent on the number of sputum samples, with sensitivities of 43.4% and 62.3% from one and two samples, respectively (72).

A study in Lima, Peru to determine incremental yield of bronchial washing for diagnosing smear negative pulmonary tuberculosis, the incremental diagnostic yield of acid fast bacilli smear and culture of bronchial washing specimens over sputum culture was 44% (95%CI 25; 65) (73).

One study, in Malawi, included follow-up data (7 years) and reported that patients with smear-negative pulmonary tuberculosis had a significantly higher risk of death than patients with smear-positive tuberculosis, with a hazard ratio of 2·2 (47).

A study conducted in Uganda revealed that 17.1% of smear negative were Xpert positive, while 42.5% had CXR suggestive of TB ($P = 0.0018$), of whom only 29.4% were Xpert positive this shows that the majority of the sputum smear negative patients did not have TB on single Xpert testing. CXR gave an overestimate of sputum smear negative TB cases (74).

Another study conducted in Mulago Hospital, Kampala, Uganda fluorescence microscopy (FM) identified 11% more smear positive patients than conventional light microscopy (49% vs. 38%). However, positive FM results were less likely than positive conventional microscopy (CM) results to be confirmed by culture when smears were read as either "scanty" (54% vs.90%) or 1+ (82% vs. 91%). Compared to CM, FM sensitivity was higher (72% vs.64%, p=0.005) and specificity lower (81% vs. 96%) (75).

A study conducted to determine the diagnostic accuracy of bleach microscopy, overall, examination of bleach processed versus direct smears led to small increases in sensitivity (for bleach centrifugation, 6% (95% confidence interval CI = 3% to 10%, P = 0.001]; and small decreases in specificity (for bleach centrifugation, −3% (95% CI = −4% to −1%, P = 0.004). As a recommendation, further research should focus on alternative approaches to optimizing smear microscopy, such as use of light emitting diode fluorescence microscopy (76).

A study done in Gambella Regional State, Ethiopia, to assess impact of DOTS strategy on tuberculosis case finding and treatment outcome,40.9% were smear positive pulmonary TB, 31.6% were smear negative pulmonary TB and 27.5% had extra pulmonary TB (77).

Another study conducted in five public health centres (Mojo, Adama, Geda, Dhera and Wolenchiti) providing ART services for residents in Adama town and adjacent rural and suburban districts of Oromia Region of Ethiopia, among bacteriologically confirmed cases, 22.6% were smear-positive, 70.1% were Xpert-positive, and 89.8% were culture positive. Xpert MTB/RIF increased the TB detection rate by 64 cases (47.4%) compared with smear microscopy (67).

A TB working group has estimated that, rapid, accurate and widely available diagnostic test for tuberculosis with a sensitivity of ≥85% for smear positive and smear negative cases, and specificity of 97%, could save ~400,000 lives annually (78).

Out of the 130,614 cases reported in EFY 2005, much of it (34.5%) were smear negative pulmonary TB,33.4% were smear positive pulmonary TB, and 32.1% were extra pulmonary TB. This shows that SNPTB accounts a high burden (26).

Most studies conducted globally and nationally indicated that, SNPTB is the most common manifestation of tuberculosis. It remains diagnostic challenge due to lack of fast and accurate diagnostic methods in a high TB burden setting. The low sensitivity and specificity of conventional methods in detecting tubercle bacilli in clinical specimens makes the diagnosis of tuberculosis in general and SNPTB in particular, a major challenge in developing countries like Ethiopia. To best of our knowledge no study so far was done on diagnostic accuracy of alternative diagnostic methods especially GeneXpert for the diagnosis of SNPTB in Ethiopia.

3. objectives

3.1. General objective

To evaluate the diagnostic yield of alternative methods for the detection of *M. tuberculosis* in smear negative pulmonary samples

3.2. Specific objectives

- To determine the incremental diagnostic yield of GeneXpert after bleach treatment and simple centrifuge, that can be available in microscopic centre as compared to untreated sputum specimen
- To determine the test accuracy of LED-Fluorescent microscopy in bleach concentrated sputum specimen
- To compare the diagnostic yield of spot versus morning sputum with GeneXpert testing

4. Materials and Methods

4.1. Study setting

The study was conducted at Jimma University Specialized Hospital, Mycobacteriology Research Centre, Jimma zone, South West, Ethiopia. Jimma town is the main city of Jimma zone and located 350 kilometres away from Addis Ababa in the south west direction. The Zone is found in Oromiya Region with a total population of 2,788,390 according to 2007 national census report. It covers an area of 199316.18 KM^2 and an average altitude of about 2180m above sea level. The zone has warm and humid climate with a mean annual maximum temperature of 33°C and a mean annual minimum temperature of 10°C. It lies in the climatic zone locally known as 'Woyna Daga' which is considered ideal for agriculture as well as human settlement (79).

Jimma town has two governmental hospitals and three health centres which provides health care services for Jimma. Jimma University Specialized Hospital (JUSH) is one of the oldest public hospitals in the country. It was established in 1930 E.C during the Italian occupation for the service of their soldiers. It became the only teaching and referral hospital in the southwestern part of the country. It provides services for approximately 9,000 inpatient and 80,000 outpatient attendances in a year. It has a bed capacity of 450 and a total of more than 750 staffs of both supportive and professionals (80).

The Mycobacteriology Research center was established as part of IUC-JU collaborative research project between Jimma University and consortium of Flemish Universities from Belgium in November 2010. The laboratory activities are mainly focused on basic research, training and service in the field of Mycobacteriology. It is involved in the provision of service to the patients as a part of national Mycobacteriology laboratory network. The laboratory is equipped with Conventional bright field Microscopy, Fluorescent Microscopy, Culture to detect *M. tuberculosis* using solid media (Lowenstein-Jensen), and BACTEC MGIT 960 TB detection system, Drug susceptibility testing (DST) on both solid media (Lowenstein-Jensen) and liquid media using BACTEC MGIT 960 TB detection system and GeneXpert MTB/RIF (81).

22

4.2.   Study Period

This study was conducted from February 14, 2014 to August 1, 2014.

4.3.   Study design

A comparative cross-sectional laboratory based study was conducted.

4.4.   Source population

Patients clinically suspected for pulmonary tuberculosis and visiting the Jimma University Specialized Hospital for AFB smear microscopy were our source population.

4.5.   Study population

Patients who became three times AFB smear negative on direct Ziehl-Neelsen staining and suspected for smear negative pulmonary tuberculosis.

4.6.   Inclusion and exclusion criteria
4.6.1.   Inclusion criteria

- Patients with age greater than or equal to 18 years
- Three times AFB smear negative
- Cough for at least 2 weeks
- Unintentional loss of weight or failure to gain weight

4.6.2.   Exclusion criteria

- Suspects unable to produce adequate sputum
- Patients who are critically ill
- Patients taking anti-tuberculosis treatment or prophylaxis
- HIV positive cases

4.7. Sample size Determination

To determine the minimum sample size of the study, single population proportion formula was used

$$n = \frac{(z \, \alpha/2)^2 \, (p) \, (1-p)}{d^2} = 216$$

Where the assumptions were as follows

- n=sample size
- P= 0.17 prevalence of smear negative culture positive TB (57)
- α =critical value at 95% confidence interval of certainty (1.96)
- d=margin of sampling error to be tolerated which is 5%

By considering 5% contamination rate on culture media (Lowenstein-Jensen), the final sample size was 227

4.8. Sampling Technique

Consecutive pulmonary TB suspected patients visiting Jimma University Specialized hospital for AFB smear microscopy and those who fulfil our inclusion criteria were enrolled until the required sample size was met.

4.9. Study variables
4.9.1. Dependent variables (outcome variables)

- Accuracy of Alternative Diagnostic Methods
- Smear negative pulmonary tuberculosis

4.9.2. Independent variables

- Age
- Gender
- Gross sputum appearance
- Simple bleach concentration

- Duration of cough
- Fever
- Weight loss
- Loss of appetite
- Type of sputum
- Contact history with coughing person

- Night sweat
- Chest pain
- Shortness of breath
- Haemoptysis
- Antibiotic treatment

4.10. Participant recruitment and data collection

Spot-morning-spot sputum specimen was collected from patient suspected for pulmonary tuberculosis, after clear instruction was given for all patients on how to produce sputum specimen. Specimens lacking any purulent/mucoid material were rejected and the patient was asked to try again. For all patients the first spot and the morning sputum specimens were maintained in the refrigerator at Mycobacteriology Research Centre until the three times smear result was available.

Those pulmonary TB suspects who had three times negative resulted on smear microscopy were enrolled after obtaining written informed consent. Pre-tested questionnaire was used to collect participants' socio-demographic and clinical information. The sputum specimens (first spot and morning specimen) collected from each suspect was treated with N-acetyl L-cysteine (NALC) powder and diluted with normal saline then each was divided in to two aliquots. The first aliquot of spot specimen was used for culture (L-J media& MGIT 960) and the other part for direct GeneXpert. The first aliquot of morning specimen was used for the second direct GeneXpert testing and the second aliquot was treated with 5% bleach followed by centrifugation for Xpert testing as indicated in figure-1 below. Culture positive results were confirmed for *Mycobacterium tuberculosis* complex by SD BIO LINE TB AgMPT64(Standard Diagnostics, Yongin, South Korea) rapid immunochromatographic method.
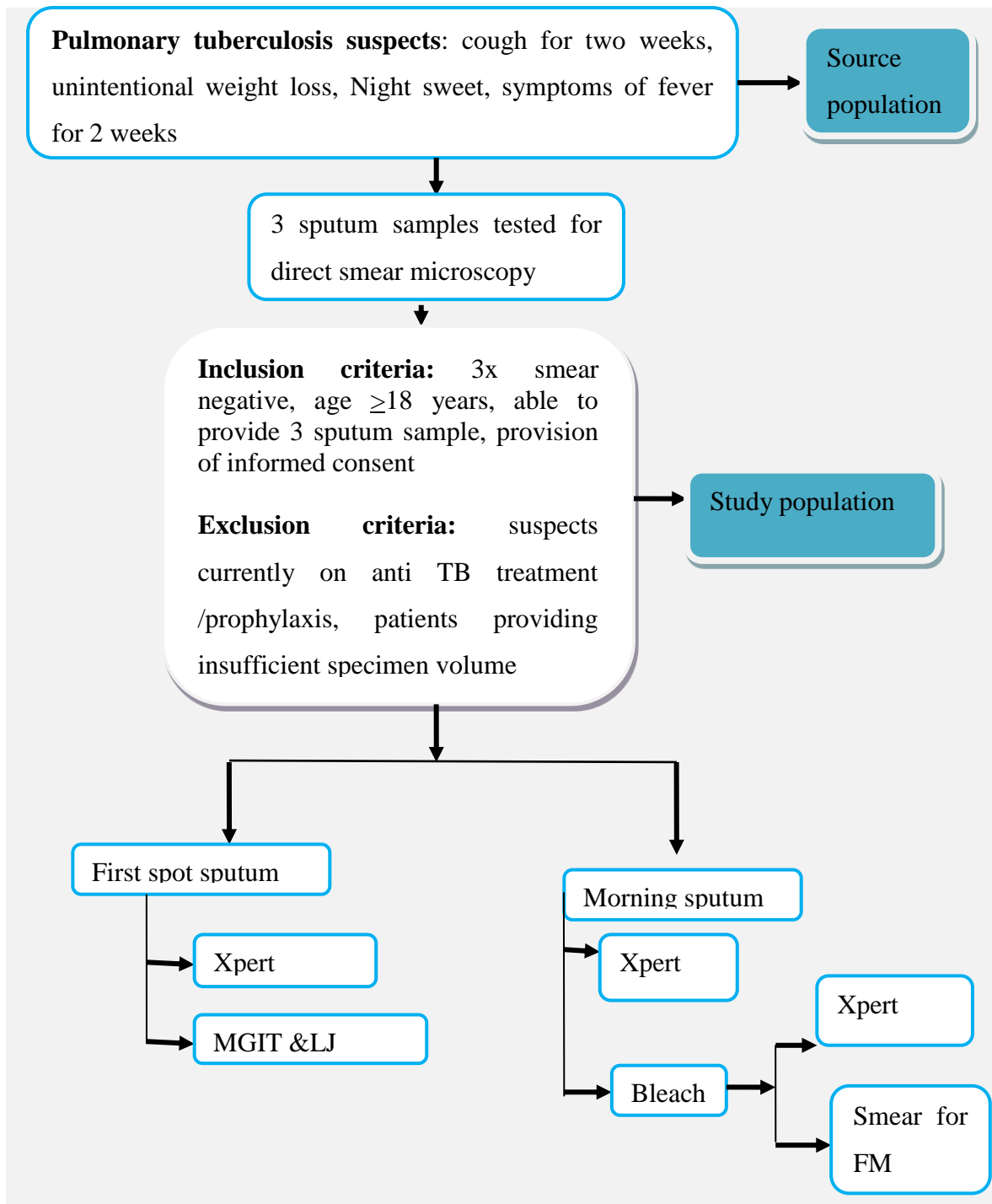
Figure 1: Flow chart of Showing study subject recruitment and laboratory work flow at JUMRC, Jimma, southwest Ethiopia from February 14 to August 1, 2014. All the lab works were done at Mycobacteriology Research Centre.

4.10.1. Sputum smear Microscopy

Direct smear was prepared on the spot of specimen collection by using clean slide. Standard Ziehl-Neelsen staining procedures were applied (53). 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 (HPFs) (82).

For this particular study, smears were considered positive if a patient with at least one initial sputum smear examinations becomes positive for AFB by direct microscopy from the three sputum samples (Spot-Morning-Spot). Those who have sputum smear negative (having symptoms suggestive of TB with at least 3 initial smear examinations negative for AFB by direct microscopy) and who consented were enrolled in this study.

4.10.2. Light Emitting Diode -Fluorescent microscopy

Smear was prepared from bleach treated sediment by using clean frosted slide. Smeared slides were stained 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 (83).

4.10.3. Sputum culture

All 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 (pH 6.8) was

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 at 4^0C. After decanting the supernatant, the pellet was re-suspended by 1 ml of phosphate buffer (*p*H 6.8) and was used for inoculation on both L-J media and MGIT tube (84).

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 colony morphology and by microscopic examination of the colonies for acid-fast bacilli (AFB).For liquid culture (MGIT 960), 0.5 ml of the sample sediment was inoculated into 7ml MGIT 960 vials supplemented with OADC and PANTA as described by the manufacturer. All inoculated MGIT vials were incubated in the MGIT 960 instrument. All positive MGIT vials were confirmed for acid fast bacilli by ZN staining and inoculating on blood agar plates.

4.10.4. SD Bio line MTP64 antigen test

One hundred micro litter of sample taken from liquid culture processed by sputum specimen was applied directly to the sample well. For solid culture (LJ), 3-4 colonies were suspended in 200µl of extraction buffer prior to test. 100µl of suspended solid culture was added in to the sample well. As the test begins to work, purple colour was seen across the result window in the centre of the test device, SD Bioline TB AG MPT64 rapid test, (Standard Diagnostics, Yongin, South Korea) and the result was interpreted within 15 minutes of sample addition.

4.10.5. Direct GeneXpert MTB/RIF assay

The GeneXpert assay was performed as described in annex-I. Sample reagent was added to sputum specimen in a 2:1 ratio in sterile falcon tube. The solution was vortexed for 15 seconds and then left to settle for 15 min, with vortexing for 15 seconds halfway through. Using the sterile transfer pipette provided with the kit, the liquefied sample was measured until the meniscus is above the minimum mark (corresponding around 2ml) and transferred to cartridge. The Xpert MTB/RIF cartridge barcode was scanned and loaded to GeneXpert instrument as indicated in Annex-I: Laboratory procedures.

4.10.6. Bleach concentration for GeneXpert MTB/RIF assay

The second part of the morning specimen was transferred to a conical centrifuge tube (10ml; SARSTEDT) and treated with an equal volume of 5% bleach solution. Then the tubes were vortexed for 30 seconds and left on the table top for 10-15 minutes at room temperature with shaking for 30 seconds in every five minutes. Then phosphate buffered saline (PBS) was added. The tube containing the specimen and bleach was centrifuge at 3000rpm for 15 minutes using a simple centrifuge (a centrifuge that is available in any health centre). After centrifugation the supernatant was decanted carefully and the sediment was re-suspended with 1ml of phosphate buffered saline (pH=6.8) and used for GeneXpert MTB/RIF assay and fluorescent staining.

For GeneXpert test, sample reagent was added to sputum sediment in a 3:1 ratio. The mixture was shacked vigorously for 15 seconds. Finally, the procedure described for direct Xpert testing was followed as described above (59).

4.11. Data analysis

Data were analysed by using SPSS version 20.0. Simple descriptive statistics like frequency distribution was used to characterise the study population. The reference standard was a positive culture for *M. tuberculosis complex.* The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) of the GeneXpert MTB/RIF assay and fluorescent microscopy, LJ-media and MGIT 960 with 95%CI was calculated by using culture(LJ and/or MGIT) as reference method.

4.12. Quality assurance

To ensure data quality, the collected data were checked out for the completeness, accuracy and clarity by the principal investigator and supervisors. Data checking was done daily after data collection and amendments were done before the next data collection. Data clean up and cross check was done before analysis.

Internal quality control procedures were implemented for all laboratory procedures used to generate the data. Smears from a known positive and a known negative sputum specimen were included with each run and batch of staining for quality control of Ziehl-Neelsen and fluorescent staining reagents. To check weather culture media supports growth, known ATCC strain of H37Rv was used.   Every new batch of LJ media and/or MGIT media were controlled for growth, by inoculating known control strain (ATCC strain of $H_{37}Rv$). Randomly selected slants of LJ media or MGIT tube inoculated with sterile distilled water incubated for the negative controls.

4.13. Ethical consideration

Ethical clearance was secured first from Jimma University ethical review committee. Written letter from the department of medical laboratory science and pathology was obtained and submitted to Jimma University Specialized Hospital. All patients were requested for oral and written informed consent prior to enrolment to the study. The purpose of the study was clearly described to the study participants including the benefits and risks of the study. Participant's involvement in the study was on voluntary basis; participants who were unwilling to participate in the study and those who wish to quit their participation at any stage were informed to do so without any restriction. Any information concerning the patients was kept confidential and the specimen collected from the patients was only analysed for the intended purposes. Results of laboratory investigations were reported back to the physicians for treatment initiation or decision making as early as available.

4.14. Dissemination of study findings

The findings of the study will be submitted and presented to Department of Medical Laboratory Science and Pathology as part of MSc thesis defence. The finding of this study will be disseminated to different stakeholders at different levels including Jimma University specialized hospital. Besides, the findings will also be presented in different scientific meetings and workshops. Finally the paper will be submitted to journals for publication.

4.15. Operational Definitions

Pulmonary tuberculosis suspects: had two or more of cough for two weeks, unintentional weight loss of body weight, or symptoms of fever for two weeks

Smear positive tuberculosis: A patient with one initial sputum smear examinations positive for AFB on Ziehl-Neelsen staining

Smear negative pulmonary tuberculosis suspects: Those patients suspected for pulmonary tuberculosis and three times AFB smear negative

Smear-Negative Pulmonary Tuberculosis:

- A patient with three times AFB smear-negative sputum which is culture-positive for *Mycobacterium tuberculosis* on MGIT 960 OR
- A patient with three times AFB smear-negative sputum which is culture-positive for *Mycobacterium tuberculosis* on L-J media

4.16. Initial validation study on bleach pre-treatment for GeneXpert

Before enrolling study participants in the study, an experiment was done to determine the effect of 5% bleach on GeneXpert PCR. The protocol was based on pre-treatment of sputum sample with 5% bleach (Chora Gas and chemical products factory, Ethiopia). Our assumption was that the bleach (sodium hypochlorite) solution can digest and liquefy mucus and debris in sputum. This makes the tuberculous bacilli to be released, which can be further concentrated by centrifugation.

The protocol was first evaluated by spiking of smear negative sputum specimen with known concentration of *M. tuberculosis* strain $H_{37}Rv$. Briefly, *M. tuberculosis* strain $H_{37}Rv$ obtained from the American Type Culture Collection (ATCC) (Manassas, VA) was cultured and quantified. Using a sterile wire loop, 3–5 well-isolated colonies were emulsified in 3 ml of sterile physiological saline (0.9%w/v). In a good light the bacterial suspension's turbidity was matched with that of McFarland No 0.5 standard (approximately 1×10^8 CFU/mL). McFarland 0.5% comparable *M.tuberculosis* cells were spiked into 2 ml of liquefied smear negative sputum samples and split into two (1ml each). The first sample was treated with 5% bleach and the other left untreated. Bleach-treated and untreated samples were analysed identically by Gene Xpert MTB/RIF assay (as recommended by manufacturer instruction).

The second experiment was done on known smear positive sputum specimens (approximately 2ml each). The sputum samples were kept at room temperature for 24 hours to allow liquefaction. This enables us to split the sample into two equal parts (approximately 1ml each). As in the case of spiked sputum, the first sample was treated with 5% bleach and the other left untreated. Bleach-treated and untreated samples were tested identically by GeneXpert MTB/RIF assay.
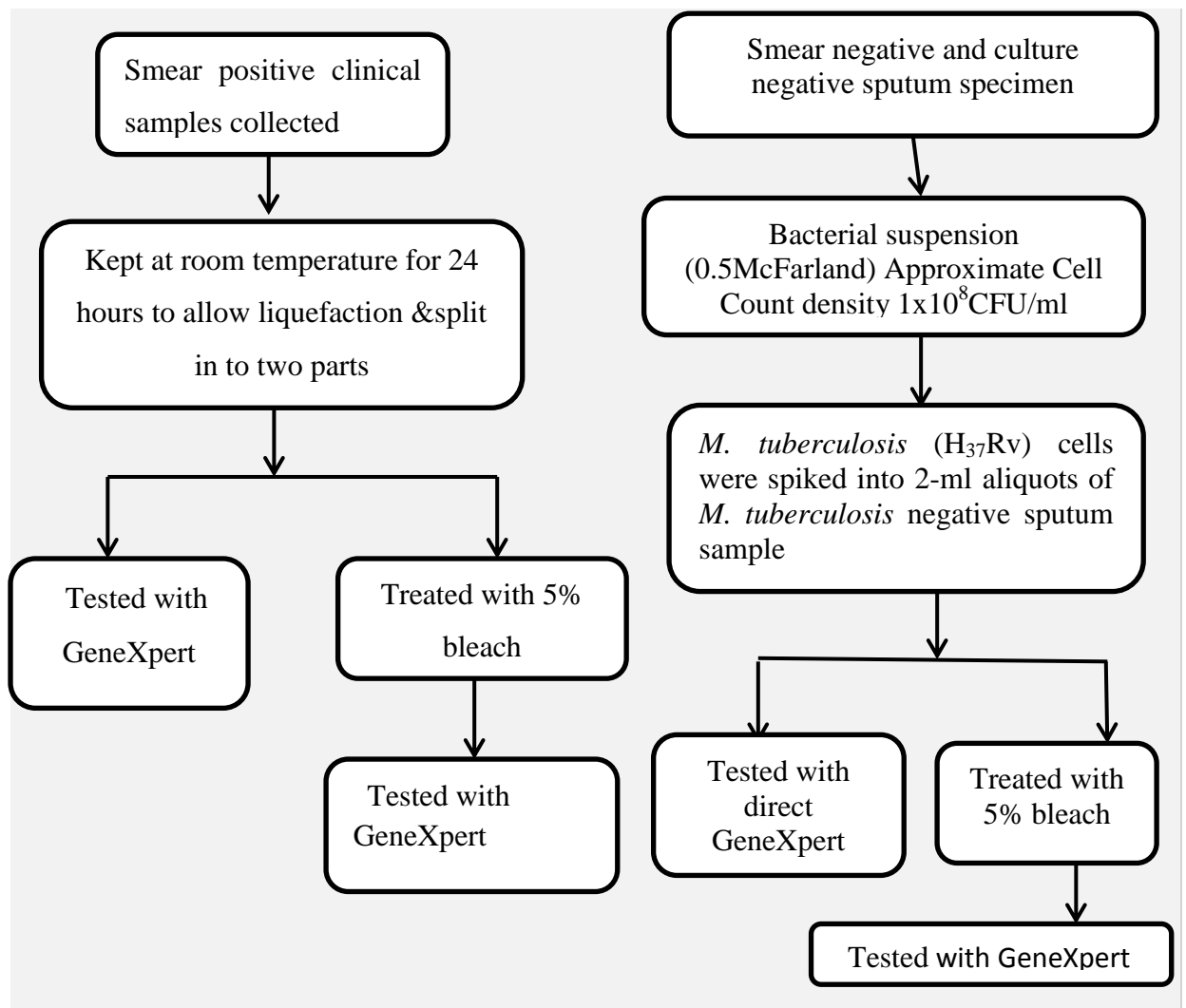
Figure 2: Showing work flow for testing of bleach treating of sputum for GeneXpert MTB/RIF Diagnosis system on known smear positive sputum specimens and spiked smear negative sputum specimen

5. Result

5.1. Socio Demographic and clinical Characteristics of Study Subjects

A total of 202 smear negative patients clinically suspected for tuberculosis were enrolled in this study from February 14 to August 1,2014. Sputum specimen was collected from 202 patients to perform smear microscopy by ZN staining, GeneXpert MTB/TB assay (both direct and concentrated method), light emitting diode (LED) fluorescent microscopy and culture (Lowensten-Jensen media and MGIT 960). Analysis

was done on 185 cases. seventeen cases were excluded because of contamination on solid and/or liquid culture system and non-reportable result on GeneXpert diagnostic system as shown in **Figure 4**. Out of 185 suspects, males accounted for 114(61.6%) and females were 71(38.4%). Their age ranged from 18-75 years with median age of 38 years. Most study subjects were in the age group of 18-24 years (25.9%) followed by 55-64 years(18.4%) and 25-34 years (16.8%) as shown in (**Table 2**).

Table 2. Socio demographic Characteristics of clinically suspected cases for smear negative Tuberculosis at JUMRC Jimma, southwest Ethiopia from February 14 to August 1; 2014 (n=185)

Variable		Frequency (%)	Mean and SD
Sex	Male	114(61.6)	
	Female	71(38.4)	
	Total	185(100.0)	
Age category	18-24	48(25.9)	
	25-34	31(16.8)	
	35-44	24(13.0)	
	45-54	30(16.2)	40(SD$\pm$17)
	55-64	34(18.4)	
	65-74	15(8.1)	
	>75	3(1.6)	
	Total	185(100.0)	
Address	Urban	94(50.8)	
	Rural	91(49.2%)	
	Total	185(100.0)	
Occupation	Self-worker	31(16.8)	
	Government's employee	21(11.4)	
	Farmer	49(26.5)	
	House wife	38(20.5)	
	Daily Laborer	1(0.5)	
	Student	35(18.9)	
	Merchant	2(1.1)	
	Other	8(4.3)	
	Total	185(100.0)	
Educational Level	Primary school	37(20.0)	
	Secondary school a	20(10.8)	

	preparatory	
	Can read and write	22(11.9)
	College/university	39(21.1)
	Uneducated	67(36.2)
	Total	185(100.0)
	Married	100(54.1)
	Single	57(30.8)
Marital status	Divorced	16(8.6)
	Widowed	12(6.5)
	Total	185(100.0)

Clinical symptoms that patients experienced during the onset of the illness include cough 96.2% (with majority having duration of cough greater than 4 weeks) followed by chest pain (72.4%), night sweating (70.8%), loss of appetite (69.7%), weight loss (60.0%), fever (40.5%) and hemoptysis (13.5%). A previous history of anti-tuberculosis treatment was reported in (27.0%) of patients.

Among 16(8.6%) culture (LJ and MGIT 960) proven cases of smear negative pulmonary tuberculosis cases, 11(68.8%) were males and 5 (31.2%) were females. The highest percentage of smear negative pulmonary tuberculosis cases were observed between the age range of 18-24 (56.2%) years followed by 25-34(18.8%) years. The result of chi square association test showed that there is no statistically significant difference (p-value >0.05) in distribution of culture proven smear negative pulmonary tuberculosis cases and sex , address , marital status, contact history, and anti-biotic use. But age category and history of anti TB treatment have statistically significant association with SNPTB infection (P-value< 0.05) as shown in (**Table 3**).

Table 3. Socio Demographic and clinical variables distribution of culture positive cases compared with negative cases at JUMRC, Jimma, southwest Ethiopia from February 14 to August 1; 2014,(n=185)

Variable		SNPTB (n=16)		χ^2 - test
		Yes n (%)	No n (%)	P-Value
Sex	M	11(9.6)	103(90.4)	0.54
	F	5(7.0)	66(93.0)	
Age in Years	18-24	9(18.8)	39(82.1)	0.01*

	25-34	3(9.7)	28(90.3)	
	35-44	1(4.2)	23(95.8)	
	45-54	2(6.7)	28(93.3)	
	55-64	0(0)	34(100.0)	
	65-74	1(6.7)	14(93.3)	
	≥75	0(0)	3(100.0)	
Address	Urban	10(10.6)	84(89.4)	0.328
	Rural	6(6.6)	85(93.4)	
Marital Status	Married	9(9.0%)	91(91.0%)	0.457*
	Single	7(12.3%)	50(87.7%)	
	Divorced	0(0.0%)	16(100.0%)	
	Windowed	0(0.0%)	12(100.0%)	
Contact History	Yes	5(13.2%)	33(86.8%)	0.328*
	No	11(7.5%)	136(92.5%)	
Anti-biotic use	Yes	9(8.5%)	97(91.5%)	0.929
	No	7(8.9%)	72(91.1%)	
History of anti TB Treatment	Yes	0(0.0%)	50(100.0%)	0.007*
	No	16(11.9%)	119(88.1%)	

*Fisher's exact test

5.2. Gross appearance of Sputum

Gross sputum was described as purulent in 61.6% (114/185), muco- purulent in 27.6% (51/185), bloody stained in 6.5% (12/185) and saliva (when patients unable to produce good sputum and if they come with saliva for the second times) in 4.3 %(8/185) of the cases among first spot sample. A similar trend was observed in the morning sputum samples also i.e. 53.5 %(99/185) purulent, 36.8% (68/185) muco- purulent and 9.7 %(18/185) were bloody stained.

Xpert positivity rate was highest in sputum with bloody stained appearance in all the three Xpert tests i.e. direct Xpert on spot sputum and morning and bleach treated sputum 5.3% (6/114), 16.7% (3/18) and 33.3 %(6/18) respectively and least in muco purulent sputum in all the three Xpert testes as shown in table 8 below. There is statistically significant association observed between Xpert detection rate and gross appearance of sputum (p-value< 0.05) similarly Culture positivity rate was highest in sputum with bloody stained appearance 33.3% (4/12) cases and least in muco purulent sputum 2.0 %(1/51).

36

LED-FM positivity rate was 7.1 %(7/99) in purulent, 8.8 %(6/68) in muco purulent and 5.6%% (1/18) in bloody stained of the cases. There is no statistically significant association observed between LED-FM detection rate and gross appearance of sputum (p-value> 0.05) (**Table 4**).

Table 4. Correlation between gross appearance of sputum with, GeneXpert, culture and FM staining at JUMRC, Jimma, southwest Ethiopia; February 14 to August 1, 2014 (N=185)

Tests	Gross appearance	Frequency (%)	Positive	Negative	χ^2 - test P-Value
Direct Gene Xpert on spot	Purulent	114(61.6%)	6(5.3%)	108(94.7%)	0.039*
	Muco purulent	51(27.6%)	1(2.0%)	50(98.0%)	
	Bloody stained	12(6.5%)	3(25.0%)	9(75.0%)	
	Saliva	8(4.3%)	0(0.0%)	8(100.0%)	
Culture on spot	Purulent	114(61.6%)	10(8.8%)	104(91.2%)	0.011*
	Muco purulent	51(27.6%)	1(2.0%)	50(98.0%)	
	Bloody stained	12(6.5%)	4(33.3%)	8(66.7%)	
	Saliva	8(4.3%)	1(12.5%)	7(87.5%)	
Direct GeneXpert on Morning	Purulent	99(53.5%)	6(6.1%)	93(93.9%)	0.039*
	Muco purulent	68(36.8%)	3(4.4%)	65(95.6%)	
	Bloody stained	18(9.7%)	3(16.7%)	15(83.3%)	
Bleach treated GeneXpert on Morning	Purulent	99(53.5%)	12(12.1)	87(87.9%)	
	Muco purulent	68(36.8%)	3(4.4%)	65(95.6%)	
	Bloody stained	18(9.7%)	6(33.3%)	12(66.7%)	0.004*
LED-FM on Bleach treated Morning	Purulent	99(53.5%)	7(7.1%)	92(92.9%)	0.864
	Muco purulent	68(36.8%)	6(8.8%)	62(91.2%)	
	Bloody stained	18(9.7%)	1(5.6%)	17(94.4%)	

*Fisher's exact test

5.3. Diagnostic Methods

4.16.1. Detection rate

Sputum was collected from 185 suspected patients who become negative for three times sputum smear microscopy. Of 185 sputum samples, MTB was detected in 6.5% (12/185%) of cases by direct Gene Xpert and 11.4% (21/185) of the cases on bleach concentrated Gene Xpert. Acid fast bacilli were observed in 7.6% (14/185) of bleach

concentrated smear on FM. Of the 185 sputum samples cultured on LJ and MGIT media, 9(4.9%) were positive on LJ and 16 (8.6%) on MGIT 960 media for *Mycobacterium tuberculosis complex* (MTBC) (**Figure 3**).
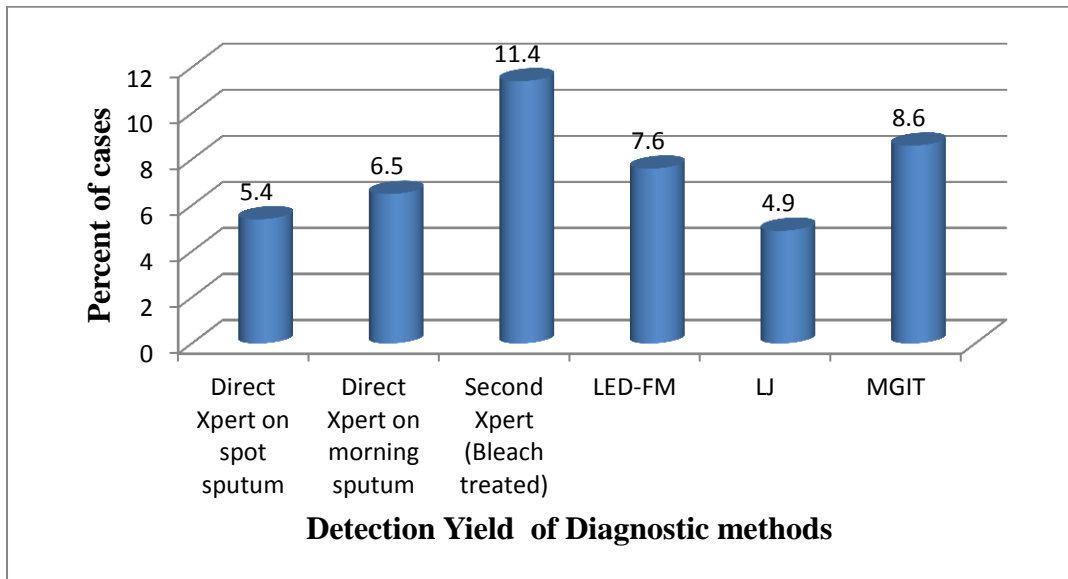
Figure 3: Percent of positive result for smear negative tuberculosis by different methods at JUMRC, southwest Ethiopia, from February 14 to August 1, 2014

4.16.2. Sputum Culture

Among 16 culture positive samples on LJ and/or MGIT, 9(4.9%) have shown growth on LJ media, and all of 16 (8.6%) showed growth on MGIT. In the present study, 2.7% (5/185) culture isolates were phenotypically identified as Non-Tuberculous Mycobacteria (NTM) /MOTT (Mycobacteria Other Than Tuberculosis) using ρ-nitro benzoic acid (PNBA) inhibition assay and SD Bioline TB AG MPT64 rapid test (Standard Diagnostics, Yongin, South Korea). Of these one is positive on bleach treated Gene Xpert).

4.16.3. Light Emitting Diode -Fluorescent Microscopy

Among 185 sputum samples, AFB was detected in 7.6% (14/185) of bleach concentrated smear on light emitting diode -fluorescent microscopy (LED-FM) which was missed with conventional bright field microscope. Of these, 4 cases were culture

positive and 10 were culture negative on LJ and/or MGIT. Twelve culture Positive cases were not detected by this method.

4.16.4. GeneXpert MTB/RIF Diagnostic Method

The positivity rate by direct GeneXpert MTB/RIF diagnostic method was 5.4% (10/185) on first spot sputum and 6.5% (12/185) on the morning sputum. While the positivity rate get almost doubled 11.4% (21/185) after bleach treatment and centrifugation **Figure 5**. All of the cases with smear negative PTB confirmed on GeneXpert were susceptible to Rifampicin.
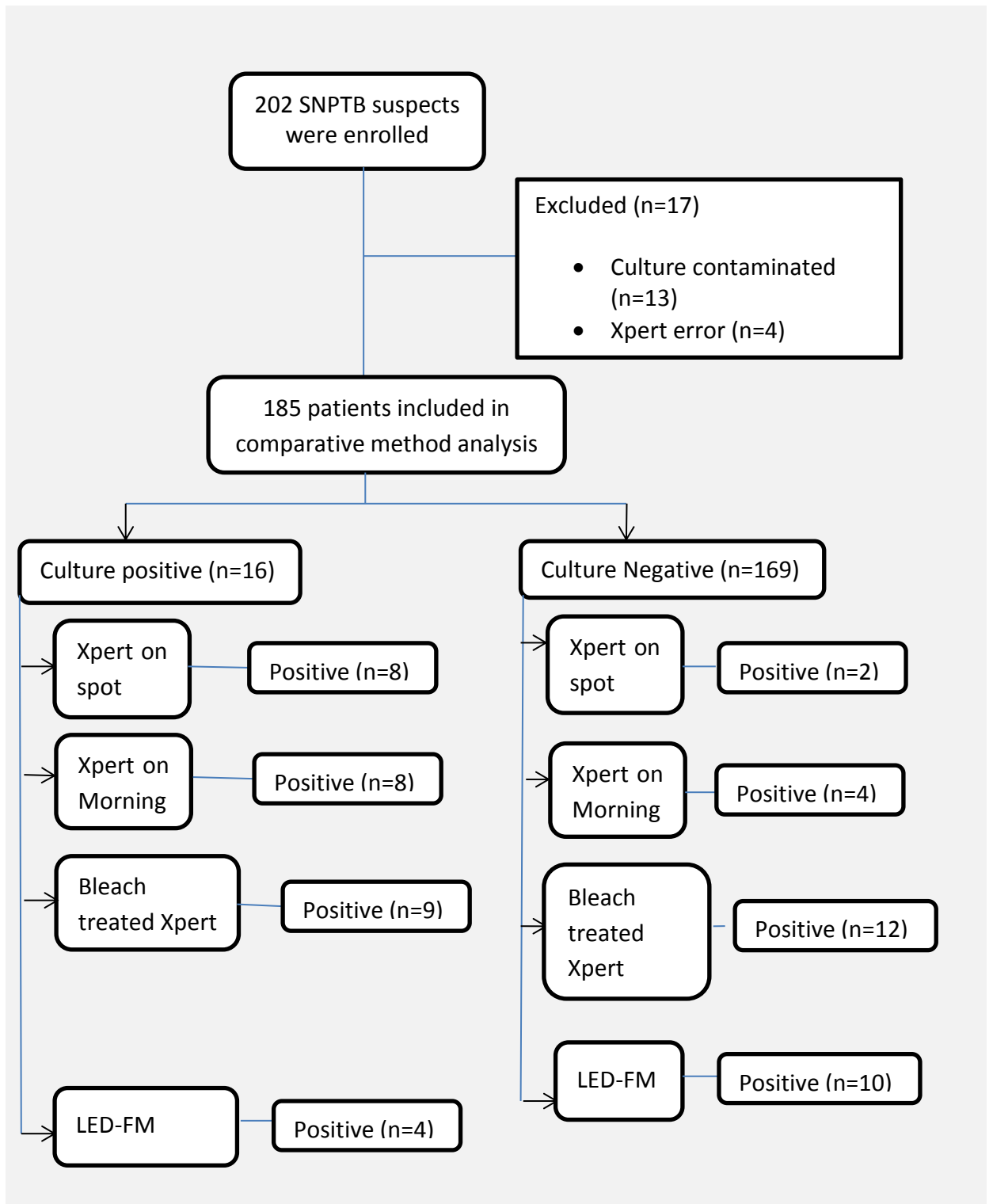
Figure 4: Enrollment and Outcomes of patients with suspected SNPTB for diagnostic methods in JUMRC Jimma, southwest Ethiopia from February 14 to August 1; 2014 (n=185).

4.16.5. Comparison of direct versus bleach pre-treated GeneXpert

The positivity rate by direct GeneXpert MTB/RIF diagnostic method was 6.5% (12/185) while the positivity rate get almost doubled 11.4% (21/185) after bleach treatment and centrifugation. Using Bleach treatment and centrifugation 9 extra cases on morning samples were detected with an incremental yield of 4.9% (9/185) **(Figure 5)** All positive cases on direct GeneXpert test were also detected by indirect GeneXpert (bleach concentrated GeneXpert test).
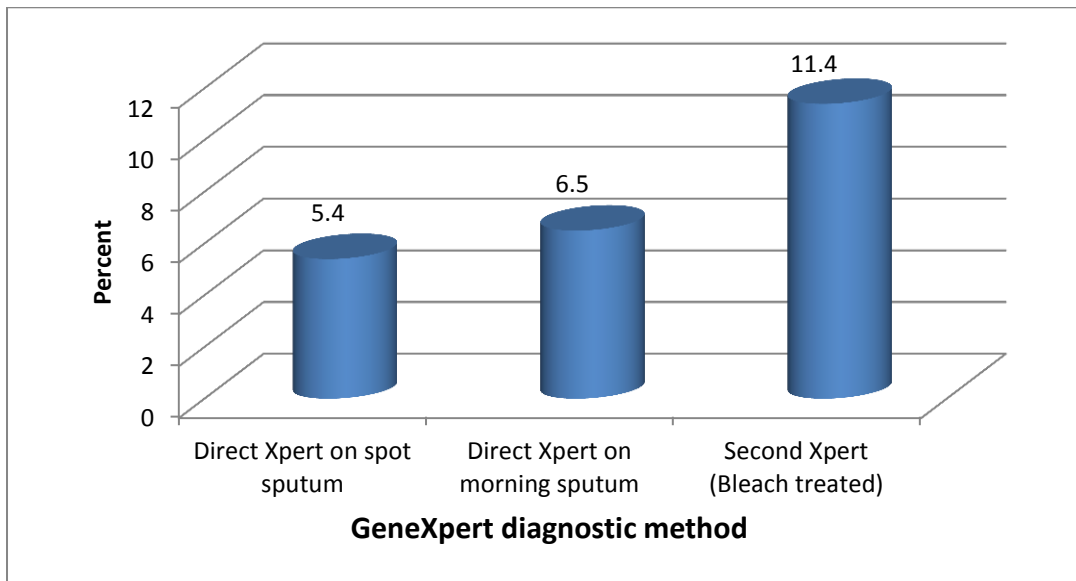
Figure 5: Comparison of Direct and Bleach concentration GeneXpert MTB/RIF Diagnostic system on spot and morning sputum for the diagnosis of smear negative tuberculosis at JUMRC ,Jimma, southwest Ethiopia from February 14 to August 1; 2014 (n=185)

Out of 12 specimens positive by direct GeneXpert, 8(66.7%) were positive on BACTEC MGIT 960. The sensitivity, specificity, positive and negative predictive values of direct GeneXpert on morning sputum was 50% with 95% CI [25.5-74.5], 97.6% [95.3-100], 66.7% [39.9-93.3%] and 95.4% [92.2-98.5], respectively when compared with the reference method. Out of 16 culture proven cases, 9(56.2%) were also positive on bleach treated GeneXpert method. The bleach treated GeneXpert method had sensitivity of 56.2% [95%CI 31.9-80.5], specificity of 92.9% [95%CI 89.0-

96.8], PPV of 42.9% [95%CI21.7-64.0] and NPV of 95.7% [95%CI 92.6-98.8] (**Table 5**).

Table 5. Comparisons of direct and bleach treated Gene Xpert with culture (LJ&MGIT) among clinically suspected SNPTB cases at JUMRC, Jimma, South west Ethiopia, February 14 to August1, 2014(n=185)

| | | Culture (MGIT 960 and/or LJ) | | |
		Positive	**Negative**	**Total**
Direct Xpert	**Positive**	8(66.7%)	4(33.3%)	12(100.0%)
	Negative	8(46.2%)	165(53.8%)	173(100.0%)
Bleach Treated Xpert	**Positive**	9(42.9%)	12(57.1%)	21(100.0%)
	Negative	7(4.3%)	157(95.7%)	164(100.0%)

4.16.6. Diagnostic test accuracy

Among the different alternative methods tested in this study, BACTEC MGIT 960 had the highest sensitivity (100%) while LED-FM had the lowest (25%) sensitivity. The specificity of LJ and BACTEC MGIT 960 was high (100%). Direct Gene Xpert on spot and morning sputum samples has equal sensitivity (50.0%) and comparable specificity (98.82%, and 97.63%, respectively). Positive predictive value (PPV) of LJ and BACTEC MGIT 960 was highest (100%) and that of LED-FM and GeneXpert on bleach treated sputum was 28.57% and 42.86% respectively. Negative predictive value (NPV) of BACTEC MGIT 960 was highest (100%) and that of LED-FM (92.98) was the lowest. BACTEC MGIT 960 had highest (100%) test efficiency while LED-FM (88.11%) had the lowest test efficiency (**Table 6**).

Table 6. Diagnostic accuracy of Direct GeneXpert, Bleach treated GeneXpert, and LED- FM against the reference method (LJ and/or MGIT) at JUMRC, Jimma South west Ethiopia; from February 14 to August 1, 2014 (n=185).

		Direct Xpert *		Direct Xpert**		Bleach Xpert		LED-FM	
		Pos	Neg	Pos	Neg	Pos	Neg	Pos	Neg
Culture	Pos (n=16)	8	8	8	8	9	7	4	12
	Neg(n=169)	2	167	4	165	12	157	10	159
Sensitivity [95%CI]		50%[25 -74]		50%[25-74]		56.2%[32-80]		25%[4-46]	
Specificity [95%CI]		98.8%[97 -100]		98%[95-100]		92.9%[89-97]		94.1%[91-98]	
PPV[95%CI]		80%[55-100]		66.7%[40-93]		42.9%[22-64]		28.6%[5-52]	
NPV[95%CI]		95.4%[92 -98]		95.4%[92-98]		95.7%[93-99]		92.9%[89-97]	

* Direct Xpert on spot sputum, ** Direct Xpert on morning sputum

4.16.7. Culture contamination rate versus GeneXpert error rate

The overall contamination rate was 6.4% (13/202) in the BACTEC MGIT 960 system and 10 (4.9%) on the LJ medium. Ten (4.9%) samples were found to be contaminated in both media. GeneXpert error rate in this particular study was 1.9 % (4/202). Of these 1 was on direct and 3 were on bleach treated sputum samples. All GeneXpert errors were found to be error code 2008 i.e. syringe pressure reading exceeds the protocol limit. The difference between contamination rate and GeneXpert error rate was statistically significant with a p-value of less than 0.05 (**Table 7**).

Table 7. Culture contamination rate (MGIT and/or LJ-Medium) and GeneXpert error rate among SNPTB suspects at JUMRC, Jimma, southwest Ethiopia; February 14 to August 1, 2014 (n=202)

Diagnostic method	Number of unreportable samples (n/N)	Percent (%)
LJ media	10/202	4.9%
BACTEC MGIT 960	13/202	6.4%
Both media (LJ+MGIT)	10/202	4.9%
GeneXpert	4/202	1.9%

Initial validation result

The test result showed that in both spiked smear negative sputum samples and smear positive clinical samples, almost all cases were positive for MTB complex by direct Gene Xpert and bleach treated Gene Xpert except one negative and one error in smear positive clinical samples and spiked smear negative sputum samples respectively on bleach treated Gene Xpert. There was no statistical significant difference on Ct value of probe A between direct GeneXpert and bleach treated GeneXpert in both spiked smear negative sputum samples and smear positive clinical samples on paired samples T-test statistics as shown in the table 2 below. This shows that treating with 5% bleach has no interfering effect on the PCR of Gene Xpert MTB/RIF assay (**Table 8**).

Table 8. Paired samples T-test of Gene Xpert MTB/RIF test on Spiked Smear Negative Sputum samples and Smear positive Clinical samples

TB lab Code	Sputum Appearance	Direct GeneXpert		Bleach treated GeneXpert		Paired T-Test P value
		Result	C_t value*	Result	C_t value*	
	Spiked Smear Negative Sputum samples					
3830	Purulent	Positive	13.4	Positive	16.6	0.080
3854	Muco purulent/Bloody	Positive	13.8	Positive	15.7	
3830	Purulent	Positive	13.7	Positive	20.3	
3864	Muco purulent	Positive	14.9	Positive	15.6	
3852	Purulent	Positive	12.9	Positive	12.5	
3830	Purulent	Positive	15.6	Positive	17.4	
3854	Purulent	Positive	15.6	Positive	16.3	
3839	Muco purulent	Positive	19.7	Positive	19.1	
3846	Muco purulent	Positive	9.7	Positive	10.9	
3846	Highly mucoid	Positive	10.4	Positive	26.5	
	Smear positive Clinical samples(sputum)					
3558	Muco Purulent	Positive	15.8	Positive	20.5	0.460
3560	Purulent	Positive	16.2	Positive	14.3	
3577	Muco Purulent	Positive	10.6	Positive	10.9	
3608	Muco Purulent	Positive	13.5	Positive	12.7	
3612	Purulent	Positive	12.7	Positive	16.6	
3669	Purulent	Positive	10.0	Positive	9.0	
3778	Muco Purulent	Positive	12.6	Positive	15.2	
3788	Muco Purulent	Positive	13.9	Positive	18.4	

3836	Purulent	Positive	15.7	Positive	11.0

* Ct value of Probe A

* Ct value of Probe A

6. Discussion

To best of our knowledge this study is the first done in Ethiopia on evaluation of GeneXpert diagnostic method for the diagnosis of smear negative pulmonary tuberculosis patients visiting JUSH.

Smear-negative pulmonary tuberculosis (SNPTB) is an increasing clinical and epidemiological problem worldwide due to HIV pandemicity (45). Ethiopia ranked 10th among the 22 countries world-wide with a high burden of TB having , more than 34% of TB cases had smear negative pulmonary tuberculosis and the proportion of smear negative TB case increased to 60% in HIV infected patients (48).

In developing countries like Ethiopia, the detection of *Mycobacterium tuberculosis* is challenging due to the limitations of conventional diagnostic techniques (ZN staining and culture). Sputum-smear microscopy especially in smear negative cases has limited sensitivity owing to paucibacillary nature of the specimens.

Manipulation of both solid and liquid culture requires the highest biosafety measures in the TB laboratory, and results are inevitably delayed due to the slow growth of mycobacteria, demanding huge laboratory infrastructure and human resources (85). The existing limitations of the conventional methods have contributed to the difficulties in managing patients with smear negative tuberculosis. It is therefore essential to have a reliable diagnostic method that is rapid, easy and accurate diagnostic method for early detection and management of TB patients.

In the present study, the added value of recently WHO endorsed Gene Xpert MTB/RIF diagnostic system and LED-FM in comparison with other diagnostic methods for the diagnosis of smear negative tuberculosis was assessed. Even though Zihel-Neelsen (ZN) microscopy has been the most widely used means of tuberculosis diagnosis in resource limited countries, WHO has recommended for the replacement of it and conventional FM by LED- FM due to its 10% higher sensitivity and workload advantages in which a large number of specimens are processed daily (53).

46

The World Health Organization (WHO) conditionally recommends GeneXpert as a follow-on test to smear microscopy in further testing of smear negative specimens in settings where MDR or HIV are of lesser concern (85). In Ethiopia, where TB and MDR-TB is highly prevalent, the effectiveness of GeneXpert for diagnosing smear negative TB and/or detection of drug resistance has not been conclusively demonstrated.

In the current study, among culture–positive sputum samples, the overall sensitivity of direct GeneXpert was 50.0%. But this finding was much lower than what WHO declared for these groups (72.5%) (59), and other published studies reported sensitivities ranging from 68%-75.3% (56, 86, 87) (sensitivity,68.0%,71.7% ,75.3%). There were 8 culture positive cases which were negative on direct Gene Xpert in this study. The reason for false negative Xpert test results may be due to paucibacillary nature of smear negative sputum specimen since culture needs lower bacilli load than the GeneXpert, and presence of PCR inhibitors which may inhibit have affected the result.

The specificity of direct Gene Xpert on first spot and morning sputum was (98.8% and 97.6% respectively). This finding was found to be similar to that found in the multi-centre study conducted to validate the Xpert test reported (99.2% specificity) (59) and another study (100.0% specificity) (56). Four culture-negative cases were positive by direct Gene Xpert, indicating nonviable bacilli due to a decontamination process which may lead to the death of the majority, if not all, of the tubercle bacilli or the patient may produce dead bacilli for being culture negative.

Even though conventional ZN microscopy has played an important role in the diagnosis of TB in resource poor settings, Xpert detected MTB in (6.5%) 12/185 of cases missed by smear microscopy. In agreement with other studies (88), (89), Xpert has higher sensitivity than smear microscopy. There was no difference in the sensitivity of Xpert on first spot sputum and on morning sputum samples. But the addition of a second MTB/RIF test increased sensitivity by 6.2 percentages on the same specimen. This finding was in line with study by Boehme CC *et.al.* have 12.6 percentage increase by doing second test (59).

47

Fluorescence microscopy (FM) has not been implemented widely in TB endemic settings due to its expensiveness and more advanced technical skills requirements and little evaluation has been done in smear negative pulmonary patients. We evaluated diagnostic performance of LED-FM compared with conventional Mycobacterial culture by using bleach treated sputum samples. Our assumption is that the bleach (sodium hypochlorite) which is cheap, easily available, safe agent solution can digest and liquefy mucus and debris in sputum. This makes the tuberculous bacilli to be released, which can be further concentrated by centrifugation. The detection yield of LED-FM was 14/185(7.6%). of cases missed by conventional smear microscopy with Ziehl-Neelsen staining. This finding is similar with findings of Albert H. *et al* (90) between 5.6 and 9.4% more sensitive than ZN microscopy but much lower than Bonnet M *et al (91)* (with detection yields of 20.3 %) and 11% more smear positive patients than conventional light microscopy reported by Cattamanchi A *et al* (75).

The sensitivity of LED-FM in our study was 25.0% [95%CI 3.8-46.2] which is much lower than published studies by Bonnet M *et al* (sensitivity of 73.2%) and Cuevas LE *et al* (sensitivity 72.8%) (91, 92) although Adults with cough ≥2 weeks were enrolled consecutively. This variation might be due to methodological variation and paucibacillary nature of smear negative sputum specimen. There were 12 culture positive cases which were negative on LED-FM in this study. The reason for false negative LED-FM test results may be due to low bacilli load of bleach treated sputum specimens since we have performed smears after resuspention of the sediment before LED-FM test, which may have affected the result.

The specificity 94.1% [90.5-97.6%] of the LED-FM in the current study was found to be almost similar to published studies by Bonnet M *et al* (96.7%) and Singh NP *et al* (94.1%) *(91, 93)*. Ten culture-negative cases were positive by LED-FM. This variation may be due to the low culture recovery rate in which dividing the first spot in to two parts and low bacilli load of spot specimen, the viability of the bacilli or if cellular debris/other artifacts may also stain auramine as a result considered as AFB positive.

The detection yield of Gene Xpert on bleach treated sputum was 21/185(11.4%) which is almost twice higher than the direct Gene Xpert 12/185(6.5%). The sensitivity of Gene

Xpert on bleach treated sputum in this study was 56.2% [95%CI 31.9-80.5] as compared to the direct one which is 50.0% [95%CI 25.5-74.5]. Although the detection yield is higher, its point sensitivity lays in 95%CI of the direct gene Xpert i.e. 50.0% [25.5-74.5]. This indicates that the difference is not statistically significant. Seven culture positive cases were negative on Gene Xpert and 12 cases which are positive on Gene Xpert were culture negative. Still the higher positivity on bleach treated Gene Xpert which is culture negative may be due to compromised recovery rate in mycobacterial culture. It needs further explanation/investigation why these 12 Gene Xpert-positive cases were culture-negative. When we compare the incremental yield of Gene Xpert on bleach treated sputum with that of the direct one, it is higher 11.4% (21/185) than the direct 6.5% (12/185).

In this study, all of the patients with smear negative PTB confirmed on Xpert tested were susceptible to Rifampicin, indicating no primary MDR-TB among new smear-negative cases. The same finding was reported by Desta K. *et al*,(94) that no MDR was found among SNPTB cases in Addis Ababa. This might be due to low bacilli load of cavities as a result the probability of spontaneous mutation less likely to happen in turn selecting drug resistant mutant strains is less common. Drug resistance studies performed in south western Ethiopia, Jimma have reported similarly low rates (2.2%) using growth based susceptibility testing,(95) and resistance as has a WHO report released in 2014 that showed MDR-TB rates of 1.6% in Ethiopia among new PTB cases. But another unpublished data in the same area a little bit higher on FNA showed that 4.7% of Xpert positive cases were Rifampicin resistant although these are different group of population (personal communication).

The overall culture confirmed smear negative cases in our study were (8.6%).This finding was comparable with study conducted in Northern Ethiopian Prisons with overall prevalence of smear-negative culture positive pulmonary TB cases was 5.5% (97). But this finding was much lower than study conducted in southeast Iran found 14.8% (70) and St. Peter specialized hospital Addis Ababa Ethiopia which was 17.4%.(57). But much higher than study conducted in Ibadan, Nigeria, on epidemiology of smear negative tuberculosis, revealed that 2.6% of the smear negative specimens

were culture positive (69). This difference might be seasonal variation in which the studies are being conducted, variation in methods and sample size.

WHO recommends that, all mycobacterial isolates should be speciated at least to the level of *M. tuberculosis* complex versus NTM (98). The prevalence of *non-tuberculous mycobacteria* in our study was 2.7%. Similarly Abebe G *et al* (99) in the same study area reported 2.1% and slightly lower recovery rate (1.2%) of NTM by Desta K. *et al (57)* in Addis Ababa has been reported. These five of *non-tuberculous mycobacteria* (NTM) were miss-diagnosed as if they were tuberculous without identification and gene Xpert MTB/RIF. Thus, this study supports that culture and Gene Xpert are helpful in differentiating MTBC from NTM in order to effectively treat and control mycobacterial infections.

The overall Xpert error rate in this particular study was 1.9 % (4/202) which is by far lower than the contamination rate on culture which was 6.4% (13/202). But our finding of non-reportable results mainly through Syringe pressure reading exceeds the protocol limit (error 2008) is almost comparable with a multicenter implementation study a number of sites reported an error rates of 2.4% during the early phase of rolling out Xpert (100). The error rate was higher on bleach treated sputum which is 3 as compared to direct sputum which is one .This might be due to the sputum being over concentrated while centrifugation so that derbies and the sodium hypochlorite chemical becomes source of sample to be viscous and becomes source of error for the PCR reaction.

In this study, the contamination rate was 6.4% in the BACTEC MGIT 960 system, almost comparable to the result described in previous report (101) (8.1%) and 4% by Diraa.O.*et.al (102).* But much lower than by Lee *et al (103)* (15.1%).

Contamination rates of 4.9% in LJ media and 6.4% in MGIT 960 media was observed in our study. The rate of contamination on both LJ and MGIT 960 media was 4.9%. A contamination rate of LJ that varied from 10.1*(103)* to 21.1% (101) was reported in previous studies. In general, liquid culture media are implicated in higher contamination rate than solid media due to their more enriched nature supporting growth of other microorganisms.

Limitation of the study

- Dividing the spot sample in to two parts may compromise the culture recovery rate on both LJ and MGIT 960 culture system.
- Due to limited resources we didn't do culture on both spot and morning sputum samples for comparing LED-FM and each Gene Xpert MTB/RIF tests .
- We didn't follow the treatment outcomes of smear-negative pulmonary TB cases found to be negative for mycobacterial culture but clinical suspicion of TB treated with anti-TB drugs based on physician's decision. There might be some culture negative cases that may get treatment and responds well which are more likely true TB cases.

7. Conclusion and Recommendations

7.1. Conclusion

Among suspected cases, 8.6% were TB confirmed cases based on culture result (combination of LJ and/or MGIT 960). LED-FM detected AFB in 7.6% (14/185) of smear negative pulmonary TB suspects. It has a lowest sensitivity (25%) but good specificity (94%).

 Bleach pre-treatment of sputum sample increased the sensitivity of GeneXpert as compared to direct GeneXpert test. The specificity was comparable for direct and indirect (bleach pre-treated) GeneXpert test. About half of GeneXpert positive cases on bleach treated sputum specimen were culture negative. The additional incremental yield of the second GeneXpert test was insignificant.

Among the culture system used, the MGIT 960 TB detection system had the higher recovery rate than the LJ medium. All of the patients with smear negative PTB confirmed on Xpert tested were susceptible to Rifampicin, indicating no new MDR-TB among smear-negative cases.

7.2. Recommendation

Based on the finding of this study the following recommendations were forwarded.

➤ Bleach pre-treatment followed by concentration of sputum for GeneXpert assay may potentially aid the diagnosis of smear negative pulmonary tuberculosis though further studies are needed with large number of culture positive SNPTB patients.

➤ Cases tested positive by Xpert MTB/RIF on the first spot and morning sputum give similar result, suggesting that the use of only one sputum sample could be sufficient for TB diagnosis in this context.

➤ Auramine staining for LED-FM can supplement and should be considered for the diagnosis of pulmonary TB suspects in addition to conventional ZN method.

➤ Testing of all SNPTB suspects with GeneXpert may not be cost effective. Thus further study is warranted to identify potential predictors for GeneXpert positivity among SNPTB patients.

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Annexes

Annex-I: Laboratory procedures

Ziehl Neelsen staining

Reagents

- o Carbol fuchsin.............. 1%
- o Acid alcohol....................3%
- o Methylene blue............. 0.1%

1. Select a new, unscratched slide and label the slide with a Laboratory Serial number.
2. Make a smear from sputum samples.
3. Let the smear air-dry for 15-30 minutes
4. Fix the smear by passing the slide over the flame 3-5 times for 3-4 seconds each time
5. Place the fixed slide on the staining rack with the smeared side facing upwards.
6. Pour filtered 1% Carbol fuchsin over the slide so as to cover the entire slide
7. Heat the slide underneath until vapours start rising. Do not let Carbol fuchsin to boil or the slide to dry. Continue the process up to five minutes.
8. Gently rinse the slide with tap water to remove the excess Carbol fuchsin stain. At this point, the smear on the slide looks red in colour.
9. Decolorize the stained slide by pouring 3% acid alcohol on the slide and leaving the acid for 3 minutes.
10. Lightly wash away the free stain. Tip the slide to drain off the water.
11. If the slide is still red, reapply 3% acid alcohol for 1 minute and rinse gently with tap water.
12. Counter stain the slide by pouring 0.1% methylene blue solution onto the slide and let it stand for one minute.
13. Gently rinse the slide with tap water and tip the slide to drain off the water
14. Place the slide in the slide tray and allow it to dry.

15. Examine the slide under a microscope using 10 x lenses to select the suitable area of the slide and examine under 100 x lenses using a drop of immersion oil.

Sputum Smear Fluorescence Microscopy

Mycobacteria retain the primary stain i.e. auramine-O solution even after exposure to decolorizing with acid alcohol, hence the term "acid-fast". A counter stain is employed to highlight the stained organisms for easier recognition. Potassium permanganate is used as counter-stain and it helps prevent non-specific fluorescence.

Reagents

- o Auramine-O solution----------------------0.1%
- o Acid alcohol-------------------------------0.5%
- o Potassium permanganate-----------------0.5%

Procedures

1. Select a new, unscratched slide and label the slide with a Laboratory Serial number.
2. Make a smear from bleach treated sputum samples by using albumin fixative
3. Let the smear air-dry for 15-30 minutes
4. Place the slides on a staining rack, with the smeared side facing up, the slides not touching each other
5. Flood the slides with freshly filtered 0.1%auramine-O. Let stand for 20 minutes
6. Wash well with running water, taking care to control the flow of water so as to prevent washing away the smear
7. Decolorize by covering completely with 0.5% acid-alcohol for 3 minutes
8. Wash well with running water, as before to wash away the acid alcohol
9. Counterstain with 0.5% potassium permanganate for 1minute
10. Wash as before with water and slope the slides to air dry
11. Examine the slide under a microscope using 20x, or 40 x lens

Preparation of Lowenstein-Jensen medium
Salt solution:

1. Dissolve 37.2gm of salt solution (readymade) in 600ml of distilled water
2. Add 20ml of glycerol and mix well.
3. Autoclave for15 minutes at 121°C, cool to room temperature

Beaten egg

1. Use only fresh eggs preferably not more than three days old.
2. Using a soft brush and soap and with soda solution clean the outside of the egg's wall. Leave the eggs in 5% soap and soda solution for 30 minutes.
3. Place in running tap water till the water is perfectly clear. Drain and dry the eggs.
4. Just before breaking, clean the outside of the eggs with a piece of sterile gauze dipped in alcohol.
5. Break the eggs into a sterile flask aseptically.
6. Close with a rubber stopper and shake vigorously until well homogenized.
7. Filter through two or three layers of sterile gauze stretched over a sterile funnel and collect the filtrate in a sterile container.
8. Measure 1000ml of homogenised egg and transfer to flask containing 600ml of salt solution
9. Mix all components gently to avoid bubbles to homogeneity
10. Dispense in 6-8 ml in sterile McCartney bottles or 16x125 mm screw-capped tubes.
11. Inspissate for 55 minutes at 82°C to 85°C.

Sterility check and Performance Testing for LJ- Media

After inspissation, the whole media batch or a representative sample of culture bottles should be incubated at 35°-37°C for 14 hours as a check of sterility. And some culture bottles will be inoculated with a laboratory strain H37Rv ATCC #27294 and incubated in the incubator.

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.

Sputum processing for culture

1. Add NaOH-NALC-sodium citrate solution in a volume equal to the quantity of specimen in 50mlfalcon tubes. All specimens should be digested and decontaminated by the sodium hydroxide and N-acetyl-L-cysteine (NaOH/NALC) method, with final concentrations of 1% for NaOH. NALC liquefies sputum sample and facilitates the decontaminating action of NaOH.

2. Vortex the tubes lightly for about 15-30 seconds and allowed to stand for 15–20 minutes.

3. When specimens are completely liquefied, add phosphate buffer (*p*H 6.8) up to the top ring in the centrifuge tube and mixed well.

4. Centrifuge the tube at the speed of 3000 g for 15minutes.

5. Carefully decant the supernatant into a container containing a mycobactericidal disinfectant.

6. Resuspend the sediment with 1-1.5ml of phosphate buffer (*p*H 6.8) and inoculate on MGIT and L-J media as indicated below

Inoculation to L-J medium

1. Inoculate 0.2ml of thoroughly mixed and decontaminated specimen onto Lowenstein Jensen medium.

2. Incubate culture of the specimen at 37^0C for 3-8 weeks.

3. Check the media for evidence of bacterial growth daily for the first week and weekly for the rest of the time until 8 weeks.

4. Look for colony morphology, growth rate and preference of media which are some of the parameters used for phenotypic characterization of the isolates

MGIT medium

The MGIT (Mycobacteria Growth Indicator Tube) consists of liquid broth medium that is known to yield better recovery and faster growth of mycobacteria. The MGIT contains 7.0 ml of modified Middle brook 7H9 broth base. An enrichment, MGIT OADC (Oleic acid, Albumin, Dextrose and Catalase) or MGIT 960 Growth Supplement, is added to make the medium complete. This Growth Supplement is

essential for growth of many mycobacteria, especially those belonging to *M. tuberculosis* complex. Addition of the MGIT PANTA is necessary to suppress contamination.

Principles of the Procedure

A fluorescent compound is embedded in silicone on the bottom of 16 x 100 mm round-bottom tubes. The fluorescent compound is sensitive to the presence of oxygen dissolved in the broth. Initially, the large amount of dissolved oxygen quenches emissions from the compound and little fluorescence can be detected. Later, actively respiring microorganisms consume the oxygen and allow the fluorescence to be observed using a 365nm UV trans illuminator. Growth can also be detected by the presence of a non-homogeneous turbidity or small grains or flakes in the culture medium (84).

Reagents

The BBL MGIT Mycobacteria Growth Indicator Tube contains: 110µl of fluorescent indicator and 4 ml of broth. The indicator contains Tris 4, 7-diphenyl-1, 10-phenanthroline ruthenium chloride pentahydrate in a silicone rubber base. The tubes are flushed with 10% CO_2 and capped with polypropylene caps.

Approximate Formula per L Purified Water

- Modified Middlebrook 7H9 Broth base ....5.9 g
- Casein peptone ....1.25 g

BBL MGIT OADC contains 15 ml Middlebrook OADC enrichment.

Approximate Formula per Litre Purified Water

- Bovine albumin 50.0g
- Dextrose 20.0g
- Catalase... 0.03g
- Oleic acid ....0.6 g

The BBL MGIT PANTA vial contains a lyophilized mixture of antimicrobial agents.

Approximate Formula per Vial Lyophilized PANTA

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

Storage of Reagents:

BBL MGIT Mycobacteria Growth Indicator Tubes − On receipt, store at 2 to 25°C (35 to 77°F). DO NOT FREEZE. Minimize exposure to light. Broth should appear clear and colorless. **BBL MGIT** OADC - On receipt, store in the dark at 2 to 8°C. Avoid freezing or overheating. Do not open until ready to use. Minimize exposure to light.

BBL MGIT PANTA Antibiotic Mixture - On receipt, store lyophilized vials at 2 to 8°C. Once reconstituted, the **PANTA** mixture may be used within 72 hrs provided it is stored at 2 to 8°C, or up to 6 months if stored at -20°C or colder. Once thawed, the **PANTA** mixture must be used immediately. Discard unused portion.

Inoculation of MGIT Tubes:

1. Label the MGIT tube with specimen number.
2. Reconstitute MGIT PANTA with 15.0 ml MGIT growth supplement. Mix until completely dissolved.
3. Add 0.8 ml of this enrichment to each MGIT tube. The enrichment with reconstituted PANTA should be added to the MGIT medium prior to inoculation of specimen in MGIT tube.
4. Add 0.5 ml of the concentrated specimen suspension prepared above.
5. Immediately recap the tube tightly and mix by inverting the tube several times.
6. Tubes should be incubated at 37°C.

Incubation Temperature: All inoculated MGIT (7mL) tubes should be entered in the BACTEC MGIT 960 instrument after scanning each tube. The instrument maintains 37ºC + 1ºC temperature. Since the optimum temperature for growth of *M. tuberculosis* is 37ºC, make sure the temperature is close to 37ºC.

Length of incubation: MGIT tubes should be incubated until the instrument flags them positive. After a maximum of six (6) weeks, the instrument flags the tubes negative if there is no growth.

Detection of positive growth

The instrument signals a tube positive for growth, and an indicator green light shows the exact location of the positive tube in the drawer of the instrument. At this point, the tube should be removed and scanned outside the instrument. The tube should be observed visually. Mycobacterial growth appears granular and not very turbid while contaminating bacterial growth appears very turbid. Growth, especially of the *M. tuberculosis* complex, settles at the bottom of the tube.

Work-up of Positive Cultures

AFB smears from a positive MGIT tube

Once a MGIT tube is positive by fluorescence or by visual observation, prepare a smear and stain with carbol fuchsin stain.

Procedure

- Use a clean frosted slide.
- Mix the broth by vortexing and then by using a sterile pipette, remove and aliquot.
- Place 1-2 drops on the slide and spread over a small area (approx. 1½ x 1 cm).
- Let the smear air dry.
- Heat-fix the smear by passing it over a flame a few times
- Stain the smear with Ziehl-Neelsen

- Place a drop of oil on the stained and completely dried smear and screen under a low power objective to locate stained bacteria. Switch to an oil immersion objective lens for detailed observation.
- If the broth appears turbid or contaminated, irrespective of AFB smear results, subculture on a blood agar to rule out the presence of contaminating bacteria.
- If the smear is negative for AFB and the tube does not appear to be contaminated, i.e. broth is clear, re-enter the tube into the instrument for further monitoring. Repeat AFB smears after 1-3 days.

Detection of contamination

Observe all fluorescent positive MGIT tubes visually for turbidity and to make an AFB smear. If a MGIT tube broth is heavily turbid, contamination is suspected even if the AFB smear is positive. Contamination may be confirmed by the following method:

- Make a smear and stain with Ziehl-Neelsen stain. Presence of non-acid-fast contaminating bacteria on smear confirms contamination.
- Sub-culture a loopful of the broth on blood agar. Several specimens (4) may be carefully inoculated on a plate (small streak for each specimen, properly labelled). Divide the plate and identify specimen number by a marker. Incubate these subcultures at 35ºC+1ºC and observe after 24-48 hours. If contaminating growth appears, confirm again by ZN stain.
- If contamination is confirmed with negative AFB smear from the broth, discard the specimen and report as contaminated. If contamination is confirmed with positive AFB smear from the broth, repeat the isolation procedure

Isolation of mycobacteria from contaminated or mixed cultures

The contaminated broth may be reprocessed to recover mycobacteria as follows:

- ✓ Transfer the entire MGIT broth into a 50 ml centrifuge tube i.e. falcon tube.
- ✓ Add an equal quantity of 4% sterile NaOH solution.
- ✓ Mix well and let stand for 15-20 minutes, mixing and inverting the tube periodically.

- ✓ Add phosphate buffer at a pH of 6.8 after 15-20 minutes up to 45 ml mark. Mix well.
- ✓ Centrifuge at 3000x g for 15 minutes.
- ✓ Pour off the supernatant fluid.
- ✓ Re-suspend the sediment in 1 ml of buffer and mix well.
- ✓ Inoculate 0.5 ml into a fresh MGIT tube supplemented with MGIT growth supplement/PANTA.
- ✓ Place inoculated tubes in the instrument of MGIT 960 and follow for observation of growth.

Quality Control

Quality control of AFB smears staining

Prepare smears from positive cultures of *M. tuberculosis* (H37Rv, ATCC #27294)

Quality control (QC) testing of MGIT medium

Every new lot of MGIT medium and every new lot of the enrichment should be quality control tested by the user upon receipt and before it is used routinely.

QC strains

Cultures: The following mycobacterial culture is recommended for quality control testing.

- ▪ *M. tuberculosis,* H37Rv ATCC 27294

GeneXpert MTB/RIF Dx System

Principle: The Xpert MTB/RIF test for use with the Cepheid GeneXpert System is a semi-quantitative nested real-time PCR *in-vitro* diagnostic test for: 1) the detection of *Mycobacterium tuberculosis* complex DNA in sputum samples or concentrated sediments prepared from induced or expectorated sputa that are either acid-fast bacilli

(AFB) smear positive or negative; and 2) the detection of rifampin resistance associated mutations of the *rpoB* gene in samples from patients at risk for rifampin resistance.

The system consists of an instrument, personal computer, barcode scanner, and preloaded software for running tests on collected samples and viewing the results. The system requires the use of single-use disposable GeneXpert cartridges that hold the PCR reagents and host the PCR process.

Procedure

1. Collect the sputum specimen in leak-proof collection container.
2. For each of the samples; add Sample Reagent 2:1 (v/v) to sample, replace the lid, and shake vigorously 10 - 20 times.
3. Incubate for 15 minutes at room temperature.
4. At one point between 5 and 10 minutes of the incubation again shake the specimen vigorously 10 – 20 times.
5. Using the sterile transfer pipette provided, aspirate the liquefied sample into the transfer pipette until the meniscus is above the minimum mark.
6. Open the cartridge lid.
7. Transfer sample into the open port of the Xpert MTB/RIF cartridge. Dispense slowly to minimize the risk of aerosol formation.
8. Close the cartridge lid.
9. Turn on the computer and the GeneXpert Dx instrument
10. Scan the barcode on the Xpert MTB/RIF cartridge.
11. Click Start Test. In the dialog box that appears, type your password.
12. Open the instrument module door with the blinking green light and load the cartridge.
13. Wait until the system releases the door lock at the end of the run, then open the module door and remove the cartridge.
14. Record the result

GeneXpert MTB/RIF assay (bleach processed sputum sample)

1. Transfer the specimen into conical centrifuge test tubes (10ml ;SARSTEDT)

2. Add equal volume of 5% bleach solution in to the tube containing specimen

3. Vortex the tube for about 15-30 seconds and allowed to stand for 15–20 minutes at room temperature

4. Add distilled water/phosphate buffer saline (PBS) up to the 8mlof the centrifuge tube and mix well.

5. Centrifuge the tube for 15minutes at 3000 *rpm* by centrifuge available in smear microscopy health centres

6. Discard the supernatant carefully and resuspended the sediment in 1ml of distilled water/ phosphate buffer saline (PBS)

7. Transfer at least 0.5 mL of the total resuspention pellet to a conical, screw-capped tube for the Xpert MTB/RIF using a sterile transfer pipette. Alternatively, the entire sample may be processed in the original tube.

8. Add 1.5 mL of Xpert MTB/RIF Sample Reagent (SR) to 0.5 mL of resuspended sediment sample using a sterile transfer pipette and shake vigorously 10 – 20 times.

9. Incubate the specimen for 15 minutes at room temperature. At one point between 5 and 10 minutes of the incubation, again shake the specimen vigorously 10 – 20 times. Samples should be liquefied with no visible clumps of sputum.

Preparing the Cartridge

1. Using the sterile transfer pipette provided, aspirate the liquefied sample into the transfer pipette until the meniscus is above the minimum mark.

2. Open the cartridge lid. Transfer sample into the open port of the Xpert MTB/RIF cartridge. Dispense slowly to minimize the risk of aerosol formation.

3. Close the cartridge lid. Make sure the lid snaps firmly into place. Remaining liquefied sample may be kept for up to 12 hours at 2 – 8 ℃ should repeat testing be required

Starting the Test

1. Turn on the computer, and then turn on the GeneXpert Dx instrument.
2. On the Windows R desktop, double-click the GeneXpert Dx shortcut icon.
3. Log on to the GeneXpert Dx System software using your user name and password.
4. In the GeneXpert Dx System window, click Create Test. The Scan Cartridge Barcode dialog box appears.
5. Scan the barcode on the Xpert MTB/RIF cartridge. The Create Test window appears. Using the barcode information, the software automatically fills the boxes for the following fields: Select Assay, Reagent Lot ID, Cartridge serial number, and Expiration Date.
6. In the Sample ID box, scan or type the sample ID. Make sure you type the correct sample ID. The sample ID is associated with the test results and is shown in the "View Results" window and all the reports.
7. Click Start Test. In the dialog box that appears, type your password.
8. Open the instrument module door with the blinking green light and load the cartridge.
9. Close the door. The test starts and the green light stops blinking. When the test is finished, the light turns off.
10. Wait until the system releases the door lock at the end of the run, then open the module door and remove the cartridge.
11. Dispose of used cartridges in the appropriate specimen waste containers.

Test of effect of bleach method for Gene Xpert test

Materials Required

- 50ml volum falcon tube
- Conical centrifuge test tubes (10ml; SARSTEDT)
- Sterile McCartney bottles/screw caped tubes
- Pipettes (2-5ml)
- Sterile wire loop
- Sterile physiological saline (0.9% NaCl)
- 5% household bleach (Sodium hyphochlorite ,NaClO)
- 0.5 McFarland standard solution
- Well isolated colonies of H37Rv # ATCC 27294 strain on Lowestein - Jensen (LJ) media
- *M.tuberculosis*-negative sputum samples

Procedures

The laboratory *M.tuberculosis* strain $H_{37}Rv$ # ATCC 27294 obtained from the American Type Culture Collection (ATCC) (Manassas, VA) was cultured and quantified. Sputum samples were collected from patients not suspected of tuberculosis. 0.5 McFarland comparable *M.tuberculosis* cells were spiked into 2 ml aliquots of *M.tuberculosis*- negative sputum samples to final concentrations 2.5×10^7 CFU/ml and was tested with the Xpert MTB/RIF assay according to the manufacturer's instructions.

Preparation of McFarland turbidity standard number 0.5

The McFarland standard was intended to standardize the mycobacterium suspension at a known concentration of bacilli. The bacterial suspension with turbidity similar to McFarland Number 0.5 was assumed to be approximately 1×10^8 CFU/mL. It was prepared by adding of 0.5ml of 1.175% (wt/vol) barium chloride dihydrate ($BaCl_2.2H_2O$) solution to 99.5ml of 1% (vol/vol) H_2SO_4. The turbidity standard was then aliquoted into test tubes identical to those used to prepare the inoculum suspension.

Preparation of suspension of H$_{37}$Rv # ATCC 27294control strain

The laboratory *M.tuberculosis* strain H$_{37}$Rv# ATCC 27294 obtained from the American Type Culture Collection (ATCC) (Manassas, VA) was cultured and using a sterile wire loop, 3–5 well isolated colonies were emulsified in 3-4 ml of sterile physiological saline (0.9%w/v). In a good light the suspension's turbidity was matched with that of the turbidity standard (0.5 McFarland turbidity standard prepared above).

Bacterial cell suspension (0.5McFarland turbidity) was spiked into 2ml aliquots of *M.tuberculosis*- negative sputum samples Each of the samples containing bacterial suspension were split into two (1ml each). The first sample was treated with 5% bleach and the other left untreated. Bleach-treated and untreated samples were analysed identically by GeneXpert (as recommended by manufacturer instruction), and ΔCt values were calculated. On the other hand, around 2ml *M. tuberculosis* positive sputum sample (without spiking) were collected and split into two (1ml each).). The first sample was treated with 5% bleach and the other left untreated. And GeneXpert was tested for both groups and Ct values for probe A were compared between two groups.

1. Emulsify 3–5 well isolated colonies Using a sterile wire loop, in 3-4 ml of sterile physiological saline
2. In a good light match the turbidity of the suspension to the turbidity standard (0.5 McFarland turbidity standard)having approximate Cell Count density cells 1×10^{8}CFU/ml
3. Prepare around 2ml aliquots of *M.tuberculosis*- negative sputum samples into falcon tube of 50 ml capacity
4. Bacterial cell suspension (0.5McFarland turbidity) will be spiked into 2ml aliquots of *M.tuberculosis*-negative sputum samples
5. Divide each of the samples containing bacterial suspension into two equal parts (1ml each)
6. Treat the first sample with 5% bleach and run GeneXpert
7. Analyse the second part identically by direct GeneXpert (as recommended by manufacturer instruction),
8. Compare for Ct values between the two methods

Annex II: Information sheet

A. English Version

Title of the project: Alternative Diagnostic Methods for the Diagnosis of Smear Negative Pulmonary Tuberculosis: A cross-sectional comparative study at Jimma University specialized hospital, Jimma, Southwest Ethiopia

Name of Principal Investigator: Dossegnaw Aragaw

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

Name of sponsor: Jimma University and VLIR-OUS project, Jimma University

This information sheet is prepared for pulmonary tuberculosis suspects 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 is the most common infectious disease worldwide, especially in a developing world like our country, Ethiopia. So this study is designed "Alternative Diagnostic Methods for the Diagnosis of Smear Negative Pulmonary Tuberculosis: A cross-sectional comparative study at Jimma University specialized hospital, Jimma, Southwest 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.

- Your medical history will be reviewed
- You will provide us a 10 minutes interview
- We will take sputum

- The collected sample will be processed in Mycobacteriology laboratory ,Jimma University
- Molecular, microscopical and bacteriological culture studies will be done on the sample and preserved for further analysis related with the current study.

Risks and discomforts

There is no risk and discomfort in participating in this study. During all sample collection we will follow Standard operational procedures.

Benefits and Compensation

By participating in this study, there will not be direct financial benefit. If you are AFB positive, 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. Dossegnaw Aragaw, Phone No +251 913 97 06 83, Email: dos.aragaw@gmail.com

Dr. Gemeda Abebe, Phone No +251 911 99 12 85, Email: gemeda.abebe@ju.edu.et

Mr. Mulualem Tadesse, Phone No +251913162624, Email:
mulualemt.tadesse@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

Unkaa Odeeffannoo

Maqaa Projektii:-Adeemsa qorannoo adda addaa warra dhukkuba sombaa ismiir negativii ta'aniif gegeefamu

Maqaa Qu'ataa : Doosanyaaw Araagaaw

> Dhaabbataa: Yuuniversitii Jimma, Muummee Barnoota meediikal Laaboraatorii Saayinsii fi Paatoolojii.

Maqaa Spoonsaraa: Yuuniversitii Jimmaa fi Pirojectii VLIR –OUS

Unkaan odeeffannoon kuni kan qophaa'e shakkamtoota dhukkuba sombaa ta'anii warroota qorannoo kana irratti hirmaataniif yommuu ta'u ,isaanis walqabatee wantoota qorannoon kun qabatuuf fedhii keessaniin qofa kan hirmaattan ta'uu isaa kabajaanan isinii ibsa.

Faayidaa Qorannoo: dhibeen sombaa dhukkuba yeroo amma Addunyaa keenya sodaachisaa jiruudha. Keessattu biyyoota guddataa jiran kanneen akka Itiyoophiyaa irratti daran cimaa jira.dhibeen kunis afuuraan namarra gara namaatti kan tamsa'uudha. Kanaafuu, qorannoon kunis filannoo addaa shakkamtoota ismiir negativii dhibee sombaatiif ta'a.

> ❖ Adeemsa qorannoo

>> ➢ kaardiin dhukkubsataa ni ilaallama

> - daqiiqa kudhaniif waliin turra
> - akkitaa ni kennitu
> - Akkitaan fudhatame Yuuniversitii Jimmaatti, Institiyuutii Maayikoobaacteriyoolojitti yommuu hojjamu ,qorannoon hojjatamuus : molecular maayikrooskoppii fi culture tu hojjetama.

Yaaddoo fi miidhaa: Akkitaa kennuu fi yeroo jedhametti hayyamamaa ta'uun yaaddoo fi rakkoo isin irraan ga'u hin jiru.

Faayidaa hirmaannaa: Qorannoo kana irratti hirmaachuun keessaniif faayidaan qarshiidhaan argattan jiraachuu baatuus, dhibeen sombaati fi HIV'n sin keessatti yoo argame yaaliidhaaf gara Kilinikaa TB fi ART ni ergamtu.

Iccitii:- odeeffannoon qorannoo kana irra argamu hundaafuu iccitiin ni eeggama. Odeeffannoon qorannoo kana irraa argamu ,maqaa keessan kan hin qabannee fi lakkoofsota qorannoof kennamaniin kaa'amanii kan bakka bu'an ta'a.

Fedhii hirmaachuu: Qorannoo kana irratti kan hirmaattan fedhiin qofa yommuu ta'u ,yoo hin barbaannee ta'e yeroo barbaaddanitti adda kutuu ni dandeessu. Filannoon keessan ammas ta'ee fulduraaf maamiltummaa keessan irratti fi faayidaa argattanii walitti dhufeenya hin qabu.Qorannoo kana wajjin wal qabatee wanta ifa isiniif hin taane ammas ta'ee fulduraaf gaafachuu ni dandeessu.

Gaaffillee keessaniif

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Obboo Mulaalem Taaddasaa: Bilbila: +251913162624 e-mail.
mulualemt.tadesse@gmail.com

milkaa'ina qoranoo kanaaf gaaffilee armaan gaditti tarreeffaman iif deebii sirrii laachuudhaan akka nu gargaartan kabajaan isin gaafanna

C.   የአማርኛ ግልባጮ

የጥናቱ ርዕስ፦ ስሚር ኔሌቲቭ የሚባላዉን የሳንባ ቲቢን ለመመርመር አማራጭ የምርመራ ዘዲዎች ያላቸዉ ፋይዳ

የዋና ተመራማሪ ስም፦ ዶሰኛዉ አራጋዉ

የድርጅቱ ስም፦-ጅጋ ዩኒቨርሲቲ / የህክምና ላቦራቶሪ ሳይንስ እና ፓቶሎጅ ትምህርት ክፍል

ይህ የመረጃ ቅፅ የተዘጋጀዉ ከላይ በተጠቀሰዉ ጥናት ለሚሳተፉ ስሚርኔጌቲቭ የሚባላዉን የሳንባ ቲቢን ለመመርመር አማራጭ የምርመራ ዘዲዎች ያላቸዉን ፋይዳ ሲሆን በአጠቃላይ በጥናቱ ዉስጥ ልናካሂዳቸዉ ስለፈለግናቸዉ ጉዳዮች እና ስለጥናቱ ጠቅላላ ማብራርያ ይሰጣል:: በመሆኑም በጥናቱ የሚሳተፉት በራስዎ ፍላጎት ብቻ መሆኑን በትህትና እንገልፃለን::

ስለ ጥናቱ በጥቂቱ

የሳንባ ነቀርሳ በሽታ በአለማችን ላይ በከፍተኛ ደረጃ ላይ ይገኛል፡ በተለይም እንደኢትዮጵያ ባሉ ታዳጊ አገሮች ጉዳቱ ከፍተኛ ነዉ:: በሽታዉ በአብዛኛዉ በትንፋሽ ከሠዉ ወደ ሰዉ የሚተላለፍ ነዉ:: ስለሆነም የዚህ ጥናት ዋና አላማ ስሚርኔጌቲቭ የሚባላዉን የሳንባ ቲቢን ለመመርመር አማራጭ የምርመራ ዘዲዎች ያላቸዉን ፋይዳ ማጥናት ይሆናል::

የጥናቱ ሂደት ዝርዝር

በጥናቱ ለመሳተፍ ከተስማሙ የሚከተሉትን መረጃዎችና ናሙና እንወስዳለን:

- የህክምና መዝገብዎ ይታያል ::
- እንዲሁም ከራስዎ አንደበት የ10 ደቂቃ ቃለመጠየቅ ይደረግሎታል::
- የአክታ ናሙና እንወስዳለን::
- የአክታ ምርመራ ይደረጋል::
- የተሰበሰበዉ የአክታ ናሙና በጅጋ ዩኒቨርሲቲ የባዮቴክኖሎጂ ተቀም ማይኮባቴርዮሎጂ ላቦራቶሪ ከጥናቱ ጋር የተያያዙ የሞለኪዩላር እና ካልቸር ምርመራዎች ይካሄዳሉ::

ስጋትና ጉዳት

በአጠቃላይ ከላይ የተጠቀሰዉን ናሙና በሚወሰድበት ጊዜ ሊያጋጥም የሚችል የህመም ስሜት አይኖርም::

ሊያስገኙቸዉ የሚችሉት ጥቅሞች እና የካሳ ክፍያ

በዚህ ጥናት ውስጥ በመሳተፍዎ በጥሬ ገንዘብ የሚደረግ የካሳ ክፍያ አይኖርም:: በተጨማሪም የሳንባ ነቀርሳ ከተገኘ ለበለጠ ህክምና እና አንክብካቤ ወደ የሳንባ ነቀርሳ እና ኤርቲ ክሊኒክ ይላካሉ::

የጥናቱ ምስጢራዊነት

ማንኛውም በጥናቱ የሚገኙ መረጃዎች በምስጢር ይጠበቃሉ:: የጥናቱ መረጃዎች በሙሉ የሚቀመጡት ከእርሶ ስም ጋር ሳይሆን ለጥናቱ ተብሎ በሚሰጠው ስውር ቁጥር ሲሆን ጥናቱን ከሚያስኬዱት ባለሙያዎች በስተቀር ማንም ሊያወቅ አይችልም:; የእርስዎን ማንነት በሚገልጥ መልኩ የተዘጋጀውን መረጃ በፈርማዎ የተረጋገጠ ፍቃድ ሳናገኝ ይፋ አናደርግም:: ይህ ጥናት ሳይንሳዊ መረጃ እንደመሆኑ መጠን በወረቀት ታትሞ ቢወጣ ወይንም በሚድያ ቢነገር የእርስዎ ስም በምንም መልኩ አይጠቀስም::

ያለ መቀበል ወይም ጥሎ የመውጣት መብት

በዚህ ጥናት ውስጥ የሚኖሯት ተሳትፎ ሙሉበሙሉ ፈቃደኝነት ላይ የተመሰረተ ይሆናል:: በማንኛውም ጊዜ ይህንን ጥናት የማቋረጥ መብትዎ ሙሉበሙሉ የተጠበቀ ነው:: በጥናቱ ባለመሳተፍዎ ወይም ከጥናት በመገልገያ ምክንያት በአሁኑ ወይም የወደፊት የህክምና እርዳታ ላይ ተፅዕኖ አይኖረውም: ከዚህ በፊት ሲያገኙ ከነበሩት ጥቅሞች አንዳች ነገር አይጎሉብትም:: ጥናቱን የሚያካናውነው አካል ወይም ድጋፍ ስጭ አካል ከእራሶ ጥቅም ሲባል በጥናቱ እንዳይሳተፉ ሊከለከል ይችላል::

ጥያቄ ካለወት

ስለ ጥናቱ ማንኛውንም ጥያቄ ወይም እርስፃ በዚህ ጥናት ውስጥ ለሚኖሯት ድርሻ፣ አሳሳቢ ጉዳት ወይም ቅሬታ ካለዎት የሚከተሉትን ስልኮች ወይም ኢሜል አድራሻ በመጠቀም የጥናቱን ባለቤቶች ማነጋገር ይችላሉ::

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82

Annex III: Patient consent form

A. English Version

Participant Code Number_____

Participant full 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 "Alternative Diagnostic Methods for the Diagnosis of Smear Negative Pulmonary Tuberculosis: A cross-sectional comparative study at Jimma University specialized hospital, Jimma, Southwest Ethiopia." I have been informed that medical history and sputum 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.

Patient Name _____ signature _____ Date_____

Investigator name _____signature _____Date_____

A. Afan Oromo version

Lakkoofsa hirmaataaf kenname ………………

Maqaa hirmaataa……………

Yommuun qorannoo kana irratti hirmaadhu afaan naaf galuun natti himameera ykn naaf ibsameera.Faayidaa qorannoo kanaatis<<alternative diagnostic method for the diagnosis of smear negative pulmonary tuberculosis>> naaf galeera. Waa'ee dhibee sombaa akkan gaafatamuu fi akkitaan 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………

B. የአማርኛ ግልባጭ

የተሳታፊው ልዩ መለያ ቁጥር _____

የተሳታፊው ስም _____

እኔ ስሜ ከላይ የተጠቀሰው ተሳታፊ ስሜር ኔጌቲቭ የሚባለዉን የሳንባ ቲቢን ለመመርመር አማራጭ የምርመራ ዘዴዎች ያላቸዉን ፋይዳ ለማወቅ በታሰበው ምርምር ላይ በሚገባኝ ቋንቋ በቂ መረጃ አግኝቻለሁ፡፡ የህክምና መረጃና የአከታ ናሙና ምንም አይነት ጉዳት በማያደርስ መልኩ እንደሚወሰድ ተረድቻለሁ፡፡ በተጨማሪም የሚወሰዱ ማናቸውም መረጃዎች በሚስጥር እንደሚያዘ ተነግሮኛል፡፡ እንዲሁም የምጠየቀውን መረጃ ያለማስጠትና በጥናቱ ያለማሳተፍ ከጥናቱ በማናቸውም ወቅት ራሴን ማግለል እንደምችል የተገለፀልኝ ሲሆን ይህንንም በማድረጌ ወደፊትም ሆነ አሁን የማገኛቸውን የህክምና ግልጋሎቶች እንደማይጎዱልኝ ተነግሮኛል፡፡

እስማማለሁ _____   አልስማማም _____

በመሆኑም ለዚህ ምርምር ለመሳተፍ ወስኛለሁ ፡፡

የታካሚ/ የተሳታፊ ስም _____ ፊርማ ----------   ቀን ----------------

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

Annex IV: Questionnaire

A. English Version

Data collection tool: To assess the diagnostic accuracy of Alternative Diagnostic Methods for the Diagnosis of Smear Negative Pulmonary Tuberculosis: A cross-sectional comparative study at Jimma University specialized hospital, Jimma, Southwest Ethiopia

	Variables	Response category
1	Identification of the patient	1. Patient card No_____ 2. Lab Code: _____
2	Age	
3	Sex	1. Male 2. Female
4	Address	2. Urban 2. Rural
5	Occupation	1. Farmer 5. Merchant 2. Self-worker 6. House wife 3. Daily labour 7. Student 4. Gover't employee 8.Others
6	Educational Level	1. Primary school 2. Secondary school & above 3. can read and write 4. College/university 5. . Uneducated
7	Marital status	1. Married 3. Widowed 2. Single 4. Divorced
8	Cough	1. Yes 2. No
9	If yes to Qn.8 is yes, duration of cough	1. <2weeks 2. 2-4weeks 3. >4weeks
10	Chest pain	1. Yes 2. No
11	Shortness of breath	1. Yes 2. No
12	Night sweating	1. Yes 2. No
13	Weight loss	1. Yes 2. No

14	Loss of appetite	1. Yes	2. No		
15	Fever	1. Yes	2. No		
16	Weakness	1. Yes	2. No		
17	Haemoptysis	1. Yes	2. No		
18	TB Contact history in the past 2years	1. Yes	2. No		
19	Broad spectrum antibiotic treatment in the last 2weeks	1. Yes	2. No		
20	Current anti-TB treatment	1. Yes	2. No		
21	History of anti-TB treatment	1. Yes	2. No		
22	If yes, for Question 21 , did you complete the treatment	1. Yes	2. No		
23	HIV sero-status	1. Non-reactive	2. Reactive	3. Unknown	
24	Gross specimen appearance	1. Purulent 2. Muco-purulent	3. Bloody stained 4. Saliva		
25	AFB Result	1. Positive	2. Negative		

B. Afaan Oromo version

SEENSA

Kaayyoon qorannoo kanaa inni guddaan shakkamtoota dhukkuba sombaa smiir nagativii ta'an addaan baasuudhaaf faayidaa qorannoowwan filachuun qaban beekuuf yoo ta'u , gaaffiin afaani kuni kan qophaa'e dhukkuba sombaa wajjin wal qabatee gaaffilee tokko tokkoo kaasuudhafi.

Qo'annoon kun tajaajilaa fi qulqullina qorannoo dhukkuba sombaa fooyyeessuuf faayidaa guddaa qaba.

Qo'annoo kana irratti hirmaachuun duratti fedhii qabaachuu keessan ibsuu qabdu.kanaaf fedhii hirmaannaa ni qabdu taanan ,qo'annoo kanaaf kan oolu akkee akka kennitan kabajaan isin gaafanna. kanarratti dabaluudhaan akkeen fudhatame qo'annoof akka itti fayyadamnu bartanii fedhii keessan akka nuuf ibsitan kabajaan cimsine isin gaafanna.Qorannoo kana rratti jarmiin dhukkuba kana fidu yoo isinirratti argame

qoricha sirrii ta'e tolaan argattu. Kanatti aansuudhaan gaaffilee dhukkuba sombaa kanan wal qabatan isin gaafanna. Deebii fi yaadni kennitanis iccitiin ni eeggama. Qorannoo kana irratti yoona hirmaattan yaada keessan kennuu dhiisuu fi yeroo barbaaddan addaa kutuuf mirgi keessan kan eeggameedha. Kanaafuu, yaadden armaan olitti ka'e kana ilaalcha keessa galchuudhaan , fedhii gaarii qabachuu keessan mallattoon akka nuuf ibsitan kabajaan isin gaafanna.

Qorannoo kana irratti hirmaachuuf fedhii qabduu?   A. eeyyee   B. hin qabu

Maqaa hirmaataa………   mallattoo……..   guyyaa………….

Maqaa qo'ataa………   mallattoo…………   guyyaa………….

shakkamtoota dhukkuba sombaa smiir nagativii ta'an qoratee   addaa baasuudhaaf faayidaa qorannoowwan filachuun qaban beekuuf kan qophaa'e

Ajaja: gaaffilee gaafatamtaniif deebii filannoowwan tarreeffamanitti maraa

Lakk	Gaaffii	Deebii
1	Lakk. Dhukkubsataan ittiin adda bahu	Lakk. Kaardii dhukkubsataa……. Koodii laboratorii…..
2	Umurii	
3	Saala	A.dhiira b.durba
4	Teessoo	A. Magaalaa B. Baadiyyaa
5	Hojii	A. Qote bulaa B.hojjetaa dhuunfaa C.dafqaan bulaa D.hojjetaa mootummaa E.daldalaa F.barataa G.hojjettu manaa H. Kan biraa yoo jiraate ……
6	Sadarkaa barumsaa	A. Barumsa hin eegallee B. Sad.1ffaa C. Sad.2ffaa D. Kollejjii ykn yuuniversitii E.hin barannee F. Dubbisuu fi barreessu kan hin dadeenye
7	Fuudhaafi heeruma	A. Fuudhera(heerumeera) B. Hin fuune(hin hheerumne) C. Abbaaykn haati manaa kan du'e ykn duute D.kan hike

8	Isin ni qufaasisaa	A. Eeyyee B. Hin qufaasisu
9	Gaaffii 8ffaan yoo eeyyee ta'ee, guyyaa hagamiif	A. Torbee lamaa gad B. Torbee 2-4 C. Torbee afur tureera
10	Dhukkubbi laphee qabdu?	A. Eeyyee B. Na hin dhukkubu
11	Hargansuu ni dadhabduu?	A. Eeyyee B. Hin dadhabu
12	Halkan ni dafqituu	A. Eeyyee B. Hin dafqu
13	Ulfaatinni qaama keessanii gad bu'eera	A. Eeyyee B. Lakki
14	Fedhiin nyaataa keessanii hir'ateeraa?	A. Eeyyee B. Hin hir'annee
15	Ho'inni qaama keessanii ol ka'eera	A. Eeyyee B. Lakki
16	Isin ni dadhabsiisaa?	A. Eeyyee B. Hin dadhabsiisu
17	Akkeen tuftan dhiiga qabaa?	A. Eeyyee B. Hin qabu
18	Nama dhukkuba sombaa qabu wajjin jiraattanii beektuu?	A. Eeyyee B. Hin geenye
19	Dhukkuba keessaniif qoricha fudhattanii beektuu?	A.eeyyee B. Fudhadhee hin beeku
20	Yeroo ammaa qoricha dhukkuba sombaa fudhataa jirtuu?	A. Eeyyee B. Fudhataa hin jiru
21	Qoricha dhukkuba sombaa fudhattanii beektuu?	A. Eeyyee B. Lakki
22	Deebiin gaaffii 21ffaa eeyyee yoo ta'e qoricha ajajame sirnaan fudhattanii xummurtanii?	A.Eeyyee B. hin xummurre
23	HIV qoratamtee beektaa?	A.eeyyee B. hin qoratamne
24	Qoratamtee yoo ta'e bu'aansaa maal ture?	A.HIV qaba B.HIV hin qabu
25	Waligalatti akkeen waan fakkaatu	A. purulent B.muco purulent C.dhiigaan wal makaa D. Gorora

C. የአማርኛ ግልባጭ

መግቢያ

የዚህ የጥናት ምርምር ዋና አላማ ስሚር ኔጌቲቭ የሚባለዉን የሳንባ ቲቢን ለመመርመር አማራጭ የምርመራ ዘዴዎች ያላቸዉን ፋይዳ ለማወቅ ሲሆን ይህ ቃለ መጠይቅ የተዘጋጀዉ ከቲቢ ጋርና ከመሳሪያዉ ጋር የተያያዙ እንዳንድ ጥያቄዎች ለመዳሰስ ነዉ። ይህ ጥናት የቲቢን ምርምራ አገልግሎትና ጥራት ለማሻሻል ትልቅ ፋይዳ አለዉ። በዚህ ጥናት ለመሳተፍ ከመወስንዎ በፊት ፍቃደኖ መሆንዎን መጠለጽ አለባቸዉ።

ፍቃደኛ ከሆኑ ለዚህ በሺታ ምርመራ የሚሆን ናሙና እንዲሰጡ በትህትና እንጠይቃለን። ከዚህ በተጨማሪ የተወሰደዉ ናሙና ለምርምር ስራ ስለምንጠቀም አሁም ፍቃደኛ መሆንዎን እንዲገልጡልን እንጠይቃለን።ከዚህ ጥናት የበሺታ አምጪዉ ጀርም ከተገኘ ትክከለኛዉን መድሐኒት በነጻ ያገኛሉ። ከዚህ በተጨማሪ ከቲቢ ጋር የተያያዙ ጥያቄዎችን እንጠይቆታለን። በመጠይቁ ወቅት የሚሰጡት መልሶች እና አስተያየቶች በሙሉ በምስጢር የተጠበቁ ይሆናሉ። በዚህ የጥናት ምርምር ላለመካፈልና በመሃል በማንኛዉም ጊዜ ለማቆም መብትዎ የተጠበቀ ነዉ። ስለዚህ እስካሁን የተባለዉን ግንዛቤ ዉስጥ አስገብተዉ መልካም ፍቃደኝነትዎን በመፈረም እንዲገልጹልኝ እጠይቆታለሁ።በጥናትና ምርምር በመሳተፎ በጣም እናመስግንዋታለን።

በዚሁ የጥናት ምርምር ለመሳተፍ ፍቃደኛ ነዎት?   1. አዎን   2. አይደለሁም

የተጠያቂዉ ስም--ፊርማ----------------ቀን----------------

የአጥኝው ስም--ፊርማ--------------------ቀን----------

ስሚር ኔጋቲብ የሚባለዉን የሳንባ ቲቢን ለመመርመር አማራጭ የምርመራ ዘዴዎች ያላቸዉን ፋይዳ ለማወቅ የተዘጋጀ መጠይቅ

ትዕዛዝ:-የተጠያቂዉን መልስ ከተዘረዘሩት አማራጮች ያክበበት

ተ.ቁ	ጥያቄ	መልስ
I	የህመምተኛዉ መለያ	የህመምተኛዉ ካርድ ቁጥር _____ ላአብራቶሪ መለያ: _____
1	ዕድሜ	
2	ያታ	1.ወንድ 2. ሴት
3	አድራሻ	1. ገጠር 2. ከተማ 3. ስልክ ቁጥር ( )
4	ሥራ	1. የግል ሥራ 5.ነጋዴ 2. የመንግስት ሠራተኛ 6. የቀን ሠራተኛ 3. ግብርና 7. የቤት እመቤት 4. ተማሪ 8. ሌላ_____
5	የት/ት ደረጃ	1. ለት/ት ያልደረስ/ች 4. ማንበብና መጻፍ የሚችል

89

		2. ክፍል 1-8 ያጠናቀቀ/ች 5. ኮሌጅ/ዩኒቨርስቲ ያጠናቀቀ/ች
		3. ክፍል 9-12 ያጠናቀቀ/ች 6. ያልተማረ/ች
6	የጋብቻ ሁኔታ	5. ያገባ/ች 3. የተፋታ/ች
		6. ያላገባ/ች 4. የሞተባት/የሞተችበት
7	ሳል አለብዎት?	7. አዎ 2. የለም
8	ለጥያቄ ቁጥር-7መልስህ/ሽ አዎ ከሆነ፣ ምን ያህል ጊዜ ቆይቶል፤	8. < 2 ሳምንት 2. 2-4 ሳምንት 3. >4 ሳምንት
9	የደረት ህመም	1. አዎ 2. የለም
10	ስትተነፍስ/ሽ፣ ትንፋሽ ያጥርሃል/ሻል?	1. አዎ 2. የለም
11	ማታ ማታ ያልብዎታል?	9. አዎ 2. የለም
12	ክብደትዎ ቀንሶዋል?	10. አዎ 2. የለም
13	የምግብ ፍላጎትህ/ሽ ቀንሶዋል?	11. አዎ 2. የለም
14	ትኩሳት አለብዎት?	12. አዎ 2. የለም
15	የመድከም ስሜት አለብህ/ሽ?	13. አዎ 2. የለም
16	አክታህ/ሽ ደም የተቀላቀለ ነዉ.	14. አዎ 2. የለም
17	ከቲቢ ህመምተኛጋር አብረዉ ኖረዋል ?	15. አዎ 2. የለም
18	ለህመሙ Antibiotic ወስደዋል?	16. አዎ 2. የለም
19	አሁን የቲቢ መድሃኒት እየወሰዱ ነዉ?	17. አዎ 2. የለም
20	የቲቢ መድሃኒት ወስደዉ ያዉቃሉ?	18. አዎ 2. የለም
21	ለጥያቄ ቁጥር 20 መልስዎ አዎ ከሆነ ህክምናዉን አጠናቀዋል	19. አዎ 2. የለም
22	HIV ተመርምረዉ ያዉቃሉ?	20. አዎ 2. የለም
23	HIV ከተመረመሩ፣ ዉጤትዋስ?	21. የለብኝም 2. አለብኝ
24	የአክታ ናሙና አይነት	22. የረፍድ አክታ 2.የማለዳ አክታ

25	አጠቃላይ የናሙናዉ ሁኔታ	ፐሩለንት
		ሙኮ-ፐሩለንት
		ደም የተቀላቀለበት
		ምራቅ
26	ኤፍቢ ዉጤት	1. ፖዘቲብ (Positive) 2. ነጋቲብ (Negative)

Annex V: Declaration Sheet

I, the undersigned, MSc Medical Microbiology student declare that this thesis is my original work in partial fulfilment 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: Dossegnaw Aragaw   Signature _____Date_____

This thesis has been submitted for examination with my approval as an adviser.

1. Dr.Gemeda Abebe

   Signature _____   Date_____

2. Mr. Mulualem Tadesse

   Signature_____   Date_____

This thesis has been submitted with my approval as an examiner.

Name of examiner: Dr. Alemseged Abdissa (PhD)

   Signature: _____   Date: _____