ADDIS ABABA UNIVERSITY SCHOOL OF GRADUATE STUDIES

EXTERNAL QUALITY ASSESSMENT OF AFB SMEAR MICROSCOPY IN TUBERCULOSIS

BY

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LIST OF ABBREVIATIONS

AFB	Acid fast bacilli
AIDS	Acquired Immunodeficiency Syndrome
BCG	Bacillus Calmette-Guérin
DOTS	Directly Observed Treatment Short course
EHNRI	Ethiopian Health and Nutrition Research Institute
EQA	External quality assessement
HIV	Human Immunodeficiency Virus
IUATLD	International union against tuberculosis and lung disease
LT	Laboratory technician
MC	Methylcellulose
MOH	Ministry of Health
NALC	N-acetyl-L-cystein
NTP	National tuberculosis programme
NTLCP	National tuberculosis and leprosy control programme
OSE	On site evaluation
PAM	Polyacrylamide
PBS	Phosphate buffered saline
PPD	Purified protein derivative
PT	Panel testing
PTB	Pulmonary tuberculosis
QA	Quality assurance
QC	Quality control
QI	Quality improvement
SNPTB	Smear negative pulmonary tuberculosis
TB	Tuberculosis
TST	Tuberculin skin test
WHO	World Health Organization
ZN	Ziehl Neelsen

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Abstract

Tuberculosis is the world's most serious infectious disease, with over 2 million deaths each year. About a third of the world's populations are latently infected with *Mycobacterium tuberculosis.* More than 95% of tuberculosis cases and deaths occur in resource-poor countries of the less-developed world. The diagnosis of tuberculosis mostly relies on tuberculin skin test, chest radiography, culture, and smear microscopy. Sputum smear microscopy is the most widely used technique for the diagnosis of pulmonary TB (PTB) especially in high prevalence countries. For effective TB detection, it is essential to assure the quality of smear microscopy. Quality Assurance (QA) is a system designed to continuously improve the reliability and efficiency of laboratory services. Panel testing, one of the QA systems that can provide data on laboratory capabilities and assess the current status of laboratory performance and detect problems associated with diagnostic performance. This study aims to prepare panel slides using three different methods, evaluate the efficiency of AFB smear microscopy in different laboratories in Ethiopia and the proficiency of peripheral laboratory technicians following training. Until now panel slides have been prepared by two methods. The methods use AFB appositive and negative sputum samples treated with sodium hydroxide (NaOH) and N-acetyl- L-cysteine (NALC). The third procedure of preparation of sputum smear slides for panel testing using new artificial sputum preparation method was developed and examined using a cultured monocyte cell line, cultured avirulent mycobacteria and methyl cellulose(MC) or polyacrylamide gel (PAM) for viscosity and background. Since it is expensive and tedious to grow monocytes in the laboratory we used pus drawn from acute abscesses in patients admitted to St. Paul hospital. Panel slides were prepared using the above three methods and compared. Slides prepared using NaOH were consistent with positivity grades but were not similar with real direct sputum smears macroscopically or microscopically. But slides made using PAM and MC were consistent with panel positivity grades and were similar to real direct sputum smears. Slides made with the three methods were found as they can be used for EQA and training in tuberculosis laboratories and microscopy centers. The slides were sent to 56 laboratories in the country to assess the performance of laboratory technicians. The result shows that the personnel were proficient enough to detect positive smears. A total of 29 technicians were trained and tested. Their results show that there was definite improvement after training. The results before training show that there is a high possibility of false negativity as a result of low performance of laboratory technicians. So panel slides have significant importance in assessing the performance of laboratory technicians and evaluating their proficiency before and after training. Moreover since the efficiency of lab technicians has significant effect on smear microscopy result, continuous external quality assessment has to be conducted using methods like panel slides.

Key words: *External quality assessment, panel test, validity, Mycobacterium tuberculosis*

1. Introduction

1.1. Tuberculosis

1.1.1. The disease

Tuberculosis (TB) is a bacterial disease caused by *Mycobacterium tuberculosis*. TB usually affects the lungs it is called pulmonary TB, but, sometimes can affect other organs it is called extrapulmonary TB; the later occurs especially among children and people with HIV/AIDS (Dye *et al.,* 1999).

Almost one third of the world's population is infected with TB, but a healthy immune system usually prevents active disease. TB kills as many as half of people with HIV worldwide (WHO, 1998).

The name tuberculosis comes from tubercles. These are small, hard lumps that form when the immune system builds a wall around the TB bacteria in the lungs. There are two kinds of active TB namely primary TB and reactivated TB. Primary TB occurs soon after a person is first exposed to *M. tuberculosis*. Reactivated TB occurs in people who were previously exposed to TB. If their immune system is weakened, TB can break out of the tubercles and cause active disease. Most of the cases of TB occurring in people with HIV are due to reactivation of a previous TB infection (Stewart *et al.,* 2003).

1.1.2. Mycobacterium tuberculosis

The bacterium *Mycobacterium tuberculosis* belongs to the class actinomycetes, order actinomycetales, family mycobacteriaceae and genus *Mycobacterium* (Shinnick and Good, 1994). It is an aerobic, non-spore forming, non-motile organism with high cell wall content of high molecular weight lipids. Due to having a slow generation time (15 to 20hours) growth of *M. tuberculosis* is very slow. Therefore visible growth takes from 3 to 6 weeks on solid media. The organism appears as slightly bent, beaded rod 2 to 4 μm long and 0.2 to 0.5 μm wide. It tends to grow in parallel groups, producing the colonial characteristic of serpentine cording or two organisms adhere at one end to form a V shape

(Haas, 2000).The optimal growth temperature for *Mycobacterium tuberculosis* is 37 ^{o} C (Prescott *et al.,* 1999) and it grows readily on substrates using glycerol as a carbon source and ammonia or amino acids as nitrogen sources (Murray *et al.,* 2003).

The cell wall of *M. tuberculosis* has unusually high lipid content and has many unique components such as cord factor which causes the bacilli to clump in cell culture and again impose a problem in experimental infections (Bloom and Fine, 1994). The lipids are long chain fatty acids and present difficulties in staining. Therefore, heat or increased concentration of stain is used to achieve staining (Laidlaw, 1989). However, once stained it is extremely difficult to decolorize the cells even with mineral acids or acid-alcohol; thus the name acid fast bacilli (AFB) was given to the bacterium. This unique cell wall of mycobacteria is also responsible for its resistance to the lethal effects of drugs (Evans, 1998).

The bacterium is resistant to drying and survives long periods in dried sputum; it keeps its viability for weeks at +4ºC and for years at –70ºC. It is as susceptible as other bacteria to heat, X- and UV-rays and alcohol (NTP, 2001).

1.1.3. Transmission, infection and disease development

Tuberculosis transmission from person to person is primarily airborne. During coughing, sneezing or speaking a person spreads aerosol containing *Mycobacterium tuberculosis,* which after the evaporation of water remains in droplet nuclei in the air for prolonged periods of time. Transmission occurs when an exposed individual inhales these droplet nuclei. Those bacteria reaching the alveoli are ingested by local macrophages. If the macrophages are not able to destroy the bacteria, cell mediated immunity reaction is activated, and delayed-type hypersensitivity generated (Lucas, 2003).

Inside the lungs, in addition to local spreading, bacteria are transmitted from the initial primary focus through lymphatics to hilar and mediastinal lymph nodes and by the blood stream to distant sites. If the spread is uncontrolled, the disease develops in to miliary tuberculosis (Davies, 2003). However, if the host's immune response overcomes the

bacterial invasion, the bacteria are contained by the macrophages and isolated by caseous granuloma formation and consequently active TB is prevented. This condition is classified as a latent tuberculosis infection (Lucas, 2003).

M. tuberculosis is capable of adapting to low oxygen content and uses lipids as energy and metabolic source and subsequently remain dormant in tissues for years and decades (Hernández-Padlo *et al*., 2000). Most infected people contain the infection by efficient immune response. However, if the cell-mediated immune system weakens due to various reasons, such as HIV infection, malnutrition, ageing and immunosuppressive treatments, TB will reactivate. AIDS is the strongest known factor in enhancing activation of TB infection (Davies, 2003).

When TB reactivates, the caseous material in granulomas, liquefies and may be expelled into the bronchi, resulting in the formation of cavities. In the cavities bacteria multiply effectively in aerobic conditions. Cavities may harbour up to 10^8 bacteria which can spread to other bronchial segments and be excreted in the sputum, leading to the infectivity of the patient (Zellweger, 1997). These patients on smear examination turn out to be sputum smear positives.

1.2. Epidemiology

1.2.1. Global

Tuberculosis, along with AIDS and malaria, is one of the leading causes of death (Corbett *et al.,* 2003; WHO, 2006).Worldwide, there were approximately 8.8 million new TB cases in 2005 of which 7.4 million were in Asia and sub-Saharan Africa. A total of 1.6 million TB deaths including 195 000 patients infected with the human immunodeficiency virus (HIV) was reported globally (WHO, 2007). Africa has the highest estimated TB incidence rate (356/100 000) due to an HIV-driven epidemic, but the majority of TB patients live in most populous countries of Asia: Bangladesh, China, India, Indonesia and Pakistan (Dye, 2006).

TB epidemiology has been changed in the past two decades due to the influence of the AIDS epidemic. HIV infection has been estimated to account for an excess of 34% of new cases of TB (Cantwell and Binkin, 1997). Among those infected with HIV, one in ten per year will develop the disease (Pozniak, 2000). It was estimated that 12 million patients were coinfected with HIV and *Mycobacterium tuberculosis* as of 2000, with the majority living in sub-Saharan Africa and Southeast Asia (Palomino, 2005).

In most countries, more cases of TB are reported among men than women. This difference is partly because women have less access to diagnostic facilities in some settings (Hudelson, 1996). In populations with higher rates of HIV infection, women 15–24 years old constitute a higher proportion of TB patients (Corbett *et al.,* 2003). TB cases occur predominantly (approximately 6 million of the 8 million) in the economically most productive (15- to 49-year-old) age group (Frieden *et al., 2003*).

More than 90 million TB patients were reported to WHO between 1980 and 2005; 26.5 million patients were notified by DOTS programmes between 1995 and 2005, and 10.8 million new smear positive cases were registered for treatment by DOTS programmes between 1994 and 2004 (WHO, 2007).

1.2.2. TB in Ethiopia

Tuberculosis has been identified as a major public health problem in Ethiopia since the 1950s. Though the efforts to control TB started in the early 1960s with the establishment of TB Centres and sanatoria in two major urban areas in the country, Addis Ababa and Harrar, these centres had practically no impact in reducing the role of tuberculosis. After the introduction of the concept of national TB control programs by the World Health organization (WHO), the Ethiopian Ministry of Health (MOH) adopted this concept and subsequently opened the National TB control Programme in 1976. The country has been implementing the WHO recommended DOTS (Directly Observed Treatment Short-course) strategy since 1992 (FMOH, 2005). Currently DOTS is delivered to all districts in Ethiopia, and is being implemented in 119 hospitals, 519 health centres and 114 health

stations across the country. By the year 2006 the country was covered under DOTS (WHO 2009).

Ethiopia ranks seventh in the list of 22 high burden countries severely affected by tuberculosis (WHO, 2008). According to the World Health Organization (WHO) Global TB Report 2009, the country had more than 481,000 TB cases in 2007, with an estimated incidence rate of 387 cases per 100,000 people. According to the Ministry of Health hospital statistics data, tuberculosis is one of the leading causes of morbidity, the cause of hospital admission and death in Ethiopia (WHO, 2009).

1.3. Diagnosis

The diagnosis of TB relies on clinical manifestation(signs and symptoms), radiology (chest X-rays), a tuberculin skin test (PPD), blood tests, as well as microscopic examination and microbiological culture of bodily fluids (David,2007).

1.3.1. Clinical manifestation

Pulmonary tuberculosis patients usually have unexplained weight loss and productive cough for more than three weeks. Symptoms like haemoptysis, chest pain, fever, night sweat, and anorexia have also been shown to be common among TB patients (WHO, 1996).

Tuberculosis also affects other parts of the human body, including kidneys, spine or brain. This is called extrapulmonary TB. In extrapulmonary TB, symptoms vary according to the organs involved. For example, tuberculosis of the spine may cause backache, and tuberculosis in kidneys might cause blood in urine, which is called haematuria (WHO, 1996).

1.3.2. Tuberculin skin test

The Mantoux tuberculin skin test (TST) or the special TB blood test can be used to test for *M. tuberculosis* infection. Additional tests are required to confirm TB disease. The Mantoux tuberculin skin test is performed by injecting a small amount of fluid called tuberculin, which is a purified protein derivative (PPD), into the skin in the lower part of the arm. The test is read within 48 to 72 hours by a trained health care worker, who looks for a reaction (indurations) on the arm. This test is positive in TB infected people because of cell mediated hypersensitivity reaction (Murray *et al*., 1990).

1.3.3. Chest radiograph

A posterior-anterior chest radiograph is used to detect chest abnormalities. Lesions may appear anywhere in the lungs and may differ in size, shape, density, and cavitation. These abnormalities may suggest TB, but cannot be used to definitively diagnose TB. However, a chest radiograph may be used to rule out the possibility of pulmonary TB in a person who has had a positive reaction to a TST or special TB blood test (QuantiFERON-TB Gold test (QFT-G) and T-SPOT.TB test) and no symptoms of disease (Rao *et al.,* 1971).

1.3.4. Diagnostic Microbiology

Accurate diagnosis of tuberculosis is based on the identification of *M. tuberculosis* in clinical samples such as sputum. Culture of *M. tuberculosis* is the golden standard, but it is time consuming and expensive for most low-income countries. In low-income countries, microscopy of sputum for acid-fast bacillus is often the sole laboratory test available for diagnosis of pulmonary tuberculosis.The presence of acid-fast-bacilli (AFB) on a sputum smear or other specimen indicates TB disease. Acid-fast microscopy is easy and quick. In settings like Ethiopia, where disease prevalence is high, all AFB in the smear can be considered as *M. tuberculosis* (Hargreaveaves *et al.,* 2001).

1.4. Direct Smear Microscopy

A person with suspected pulmonary TB is asked to give a sputum sample (spitting phlegm into a small container). This is examined to see if the bacterium causing tuberculosis is present (Hawken *et al*., 2001). Detection of acid-fast bacilli (AFB) in stained smears examined microscopically may provide the first bacteriologic clue of TB. Direct smear microscopy is the most rapid, inexpensive and specific method for the detection of infectious cases of pulmonary tuberculosis. Very often, sputum microscopy alone is considered sufficient for diagnosis, when combined with clinical findings (Rao *et al.*, 1971). This test is based on the principle of Ziehl Neelsen diagnostic technique of direct smear microscopy of sputum. The unique properties of bacterial cell wall of *M. tuberculosis* allows it to retain the primary stain even after exposure to strong acid solutions, thus they are called acid-fast. In Ziehl Neelsen staining procedure, using carbol fuchsin and methylene blue, the acid-fast organisms appear red.

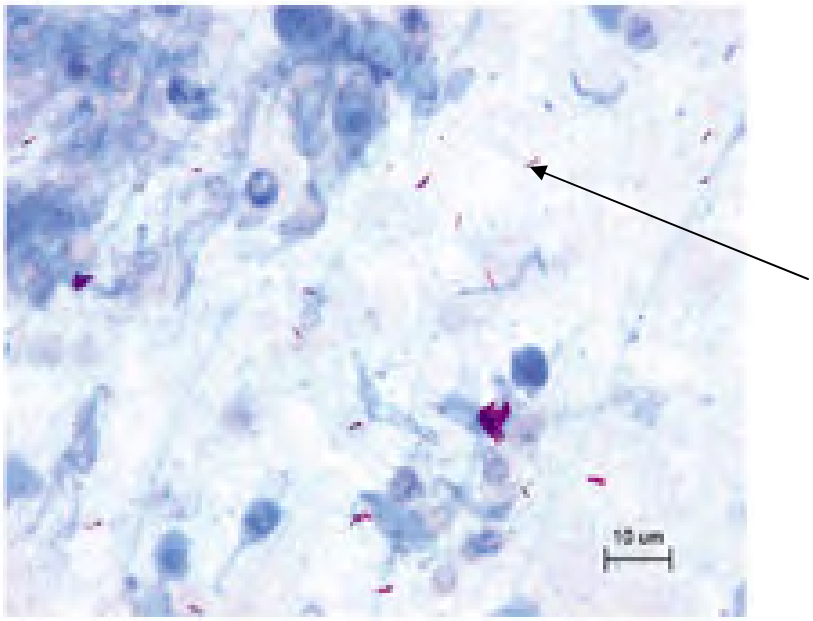


Figure1. *Mycobacterium tuberculosis* in sputum (Yamada *et al*., 2006)

Since it is performed quickly, has a high specificity and cheap, direct smear microscopy is the primary method for diagnosing TB in the high prevalence countries (Karen *et al*.,

2006). Smear microscopy for acid-fast bacilli (AFB) is sensitive technique for case detection and diagnosis of patients with infectious TB (Kusznierz *et al.,* 2004). Therefore it is the first priority for national tuberculosis programmes (NTPs) in high-prevalence countries. No other established technique offers the same advantages of accuracy, speed and accessibility (IUATLD, 2005).

Smear microscopy is more specific than chest radiography and more rapid than culture. Field workers can carry it out in the peripheral health unit, using a multi-purpose instrument, close to the dwelling of the patient but sufficiently centralized to ensure monitoring of performance (IUATLD, 2005).

However, direct smear microscopy is characterized by poor sensitivity. About 40–60% of patients with pulmonary disease and 75% of patients with extra pulmonary disease remain undiagnosed by this method. A minimum number of 10^4/mL bacilli are required for microscopy (Davies and Pai, 2008). Its main limitations are its relatively low sensitivity, especially in individuals co-infected with HIV, and variable quality of the test in programme conditions necessitates an effective external quality assessment (EQA) system following international guidelines (Karen *et al*., 2006). Furthermore it needs maintenance of equipment, consistent supply of reagents, and proper training in interpretation of the slides. If the sensitivity of smear microscopy could be improved, it would be a valuable instrument for tuberculosis control and would improve the diagnosis of tuberculosis in both adults and children (Hayleyesus *et al*., 2007).

An increase in the annual number of notifications of all forms of tuberculosis (TB) has been seen in many low-income countries with high TB and HIV prevalence, and is attributed primarily to the predisposing effect of HIV on TB. Associated with this increase in all forms of TB, the rate of increase in smear-negative TB has been greater than that of smear-positive TB in several low income countries (Hawken *et al*., 2001).

The high rate of smear negative tuberculosis seen in high HIV prevalence setting may in part be due to under reading of sputum smears as a result of overwhelming burden of tuberculosis that leads to rapid and inaccurate sputum examination (Hawken *et al.*, 2001).

1.4.1. Smear negative pulmonary tuberculosis (SNPTB)

Smear negative PTB case is defined as a patient with three initial examinations negative for AFB by direct microscopy, and no response to a course of broad-spectrum antibiotics. Having three additional smear examinations that shows negative result and radiological abnormalities consistent with pulmonary tuberculosis or positive culture confirms the smear negative pulmonary tuberculosis (FMOH, 2005).

Almost 25% to 60% of patients with PTB have SNTB disease. The diagnosis of TB may be missed initially in 50% of these patients. This error may incur delays in treatment by 12.5 weeks until the return of positive culture results. The utility of rapid diagnostic tests in the early detection of smear-negative TB is, therefore, an important clinical consideration. It is also of considerable public health interest (Lim *et al.,* 2000).
As shown in table 1, a high proportion of TB patients visiting different health centres in Ethiopia are found to be smear negative (FMOH, 2005).

Table1. TB case notification rate in Ethiopia 1992-96

Year (EC)	Total New cases	Smear positive	%	Smear negative	%	EPTB	%	TB case notification rate Per 100,000 population	
								Smear positive	All forms
1992	83,334	26,459	32	30,333	36	26,542	31	42	131
1993	90,729	32,423	36	28,994	32	29,312	32	50	139
1994	105,250	35,915	34	32,197	31	37,138	35	53	157
1995	108,488	37,014	34	32,656	30	38,818	36	54	157
1996	121,026	41,430	34	37,119	31	42,477	35	59	173

1.4.1.1 Causes of smear negative pulmonary tuberculosis

In many sub-Saharan countries, the number of patients registered with SNPTB has increased. This may be due to several factors: co-incident HIV infection, poor diagnostic practices, false negative sputum smears, mistaking other respiratory infections or diseases for tuberculosis and paediatric (childhood) tuberculosis (Munyati *et al.,* 2005).

1.4.1.1.1. HIV infection

The altered pathology in HIV-associated TB contributes to the increase of the proportion of smear-negative TB, for two reasons. The mean concentration of bacilli in the sputum of HIV infected patients is reported to be lower than in HIV-negative patients, with a consequent decrease in the sensitivity of the sputum smear examination. This decreased

sensitivity of the sputum smear in HIV-associated TB may produce an increase in the number of smear-negative TB cases (Hawken *et al*., 2001). The TB and HIV epidemic in sub- Saharan Africa has been associated with an increase in the proportion of SNPTB cases registered under programme conditions, usually in areas where such cases are not confirmed by culture. As rates of HIV rise, an increase in the proportion of PTB patients with negative sputum smears may be anticipated, due to the effects of increasing immunosuppression reducing pulmonary cavity formation and sputum bacillary load (Hargreaves *et al.,* 2001). The HIV epidemic has led to huge rises in incidence of tuberculosis in the worst affected countries, with disproportionate increases in SNPTB in children and adults. HIV changes the presentation of SNPT from a slowly progressive disease with low bacterial load and reasonable prognosis, to one with reduced pulmonary cavity formation and sputum bacillary load, more frequent involvement of the lower lobes, and an exceptionally high mortality rate (Hayleyesus *et al.,* 2007).

Many patients with PTB who are co-infected with HIV with late stage HIV disease (CD4+ count less than or equal to 200 per mm^3) and those who are severely immunosuppressed are more likely to be sputum smear-negative. However, in spite of best efforts at sputum collection, processing, and examination, some patients with active PTB do not produce adequate sputum, while others who produce adequate sputum also remain smear-negative for reasons that are as yet unknown.

1.4.1.1.2. Childhood tuberculosis

It is estimated that each year, more than 8.4 million people develop TB disease and two million die. Of these, it is estimated that nearly one million (11%) are children ≤ 15 years of age.

TB in children remains important, not only because of its associated morbidity and mortality, but also because infected children constitute a reservoir from which future tuberculosis cases can emerge. Therefore, efforts should be aimed at identifying and adequately treating children with active disease and providing preventive treatment to

infected patients without disease. Children who have been in close contact with a sputum smear negative case must be screened for TB (David *et al.,* 1999).

The diagnosis of tuberculosis in children can be difficult and generally is time consuming. Smears are almost never positive (David *et al*., 1999) because their pulmonary lesions are small, cough is not productive, and there is little or no expulsion of bacilli (Hayleyesus *et al.,* 2007).

1.4.1.1.3. False negative sputum smears

False negative sputum smears are positive sputum specimens misread as negative. There are several technical reasons that may give rise to a false negative sputum examination. These include inappropriate sputum collection procedures, inadequate storage of sputum specimens and stained smears, failure to select suitable sputum particles for smear preparation, inadequate preparation of smears or staining of slides and inadequate examination of the smear (Hawken *et al*., 2001).

1.4.1.2. Problems associated with smear negative pulmonary tuberculosis

The problem of SNPTB is not simple. In acute-care settings, as many as 8 to 10 patients are suspected to have tuberculosis for every one confirmed case (Richard, 2001). The detection and management of PTB is a principal aim of tuberculosis control programs. However, SNPTB is an increasing clinical and epidemiological problem, particularly in areas that are affected by the dual TB/HIV (Fernanda *et al.,* 2006).

Although patients with sputum SNTB are less infectious than patients with smear-positive TB, they also contribute to TB transmission. In The Netherlands, patients with smear-negative, culture-positive TB are responsible for 13% of TB transmission (Tostman *et al*., 2008). A recent DNA fingerprinting study from San Francisco attributed 17% of TB transmission in this low prevalence setting to patients with SNPT (Fernanda *et al.,* 2006).

SNPTB patients had a good prognosis before the impact of the HIV epidemic in sub-Saharan Africa. In the era of HIV however, smear-negative PTB patients have worse outcomes than smear-positive PTB patients, despite standard TB treatment. One reason could be that smear-negative PTB may be over diagnosed due to other HIV-related opportunistic infections that mimic TB (Hargreaves *et al*., 2001).

The HIV epidemic has led to large increases in the frequency of smear-negative pulmonary tuberculosis, which has poor treatment outcomes and excessive early mortality compared with smear-positive disease (Hayleyesus *et al*., 2007). Smear-negative PTB patients presenting with signs of concurrent HIV infection are at particularly high risk of death. The high mortality of smear-negative PTB patients suggests that smear-negative PTB cases should be given higher priority in areas of high HIV seroprevalence (Hargreaves *et al*., 2001).

Diagnosis of SNPT is a difficult task, and in developing countries, the majority of these cases has been treated only on the basis of clinical and chest radiographic findings. Without a standardized clinical work up, the misdiagnosis rates have been estimated as high as 35% to 52%. Sputum cultures increase the sensitivity of diagnosis substantially, but at increased expenses and complexity. New diagnostic approaches that could identify patients with SNPT, such as nucleic acid amplification assays (PCR), are expensive and have not been validated in developing countries under field conditions (Fernanda *et al*., 2006).

Even in the absence of HIV, the diagnosis of smear negative PTB can challenge the most experienced physicians. There is concern that smear-negative PTB may be over-diagnosed and treated in areas with a large burden of disease and limited diagnostic facilities. In HIV positive patients, the risk of rapid progression of TB if the diagnosis is missed, or if treatment is delayed, must be balanced against the risks and side effects of unnecessary treatment (Hargreaves *et al.,* 2001).

1.4.2. Improving the Sensitivity of Microscopy

There is a need to identify methods to improve the sensitivity of microscopy. Physical and chemical sputum processing methods, including centrifugation, sedimentation, and bleach, have been studied and found to show promise (Karen *et al.,* 2006). Microscopy to detect acid-fast bacilli can be improved by sputum liquefaction and concentration by centrifugation and gravity sedimentation. Liquefaction of sputum with sodium hypochlorite and concentration by either centrifugation or sedimentation is the most widely studied procedure. Available solvents include sodium hypochlorite (household bleach), sodium hydroxide, N-acetyl-L-cysteine-sodium hydroxide solution, and ammonium sulphate and sodium hydroxide solution. The number of fields reviewed during microscopic examination may affect the sensitivity of smear microscopy (Adithya *et al.,* 2008).

1.4.3. Quality Assurance System

For effective TB detection, it is essential to assure the quality of smear microscopy (Yamada *et al*., 2006). A quality assurance system for AFB smear microscopy is necessary to achieve a high degree of confidence. The IUATLD and the WHO recommend quality assessment of smear microscopy as an essential aspect of the activities of the National TB Control Program (NTP) (Kusznierz *et al.*, 2004).

Quality Assurance (QA) is a system designed to continuously improve the reliability and efficiency of laboratory services. As defined by both the WHO and the IUATLD, a quality assurance program for AFB smear microscopy has several components. The first component Quality Control (QC) is a systematic internal monitoring of working practices, technical procedures, equipment, and materials, including quality of stains (IUATLD, 2007). The second component of quality assurance system is Quality Improvement (QI). This is a process by which the components of smear microscopy diagnostic services are analyzed with the aim of looking for ways to permanently remove obstacles to success. Data collection, data analysis, and creative problem solving are the key components of this process. It involves continued monitoring, identifying defects,

followed by remedial action including retraining when needed, to prevent recurrence of problems. QI often relies on effective on-site evaluation visits (APHL, 2002).The third component of quality assurance system is External Quality Assessment (EQA) a process to assess laboratory performance.

1.4.4. External Quality Assessment (EQA) voice voise latter

EQA includes on-site evaluation of the laboratory to review QC and should include on-site rereading of smears. EQA also allows participant laboratories to assess their capabilities by comparing their results with those obtained in other laboratories in the network (intermediate and central laboratory) through panel testing and rechecking (APHL, 2002). TB control requires a functional laboratory set-up with quality diagnostic services and a trained diagnostician and a microscopist (FMOH, 2002). However, the performance of such laboratories depends on continuous monitoring and quality improvement mechanisms put in place (WHO, 1994). The principal objective of external quality assessment (EQA) is to standardize sputum microscopy for the detection of infectious TB cases across the peripheral diagnostic centres (FMOH, 1999).

The choices for how to implement EQA in each country will depend on both the available resources and the ability to obtain additional resources to support the EQA activities. Each method has distinct advantages and disadvantages, as well as varying levels of resource requirements.

On site evaluation (OSE) of the peripheral laboratories is an essential component of a meaningful EQA programme. A field visit is the best method to obtain a realistic picture of the conditions and practices in the laboratory (IUATLD, 2007). On site evaluation has some advantages like permitting direct personal contact, motivating the staff, observation of actual work, it identifies causes of error and it permits verification of equipment quality and function. Its disadvantages are its selectivity, usually not country wide if left solely for reference laboratory, it is labour intensive and costly.

Blinded rechecking (BRC) refers to the process by which a random selection of slides collected from the routine workload at a peripheral laboratory (the Test Lab) is re-examined at an intermediate or reference laboratory (the Controlling lab). The purpose of the exercise is to allow a statistically valid assessment of the proficiency of the peripheral laboratory. Each round of slide rechecking must be followed by feedback in the form of a written report, showing details of incorrect scorings and offering suggestions for quality improvement (corrective actions).

Low work load for peripheral laboratory, motivating improved daily performance and reflecting reality of routine performance are some of the advantages of blinded rechecking. However it has disadvantages like promoting heavy work load for higher level centers, its unavoidable inaccuracies, biased if not blinded and the staff must be made available.

Panel testing (PT) is an EQA method that is used to determine whether the laboratory technicians are adequately performing AFB smear microscopy (APHL, 2002). It also facilitates evaluating individual performance in staining and reading. In general, PT is useful to Supplement rechecking program, provide information on the capabilities of the peripheral laboratories prior to implementing a rechecking program, assess status level of performance or to quickly detect problems associated with very poor performance, evaluate proficiency of laboratory technicians following training and monitor performance of individuals when adequate resources are not available to implement a rechecking program (IUATLD, 2007).

Test panels of both unstained and stained slides are recommended for panel testing. The use of unstained slides has been credited with the advantage of verifying several aspects of the laboratory technicians' (LT) technical performance, including preparation of staining reagents, staining procedure, and the reading and reporting of results. The stained smears, if used alone, will only assess the LT's reading capability, and will not provide information on his/ her ability to stain smears nor on the quality of stains used (Martin *et al.,* 1978). The report on both the result as well as the quality of the smear and

stain should help the reference laboratory determine the source of performance problems (IUATLD, 2007).

The preparation of panel slides is based on the number of acid-fast bacilli (AFB) in the total area of a prepared standard smear (Toman, 2004). This facilitates simulation of graded specimens. A sample of such a specimen needs to be validated before it is sent out for testing. It is necessary to preserve the white blood corpuscles (WBC) and epithelial cells in the simulated specimen (Martin *et al*., 1978) for it is particularly important for initial focusing of low-grade specimens.

Two procedures have been recommended by the World Health Organization (WHO) for preparation of panel testing slides one using digestion with Sodium hydroxide (NaOH) and the other using N-acetyl L-cystine (NALC) (APHL, 2002). These methods use AFB positive and negative sputum specimens collected from patients. NaOH and NALC are used as mucolytic agents to digest and homogenize the sputum.

Although the above-mentioned procedures are simple and rapid, they have several disadvantages (Yamada *et al*., 2006). First, large amounts of AFB-negative sputa are needed for dilution of AFB-positive sputa, but good quality AFB-negative sputum is often difficult to obtain. Secondly, liquefaction of high-grade positive AFB sputum and its subsequent manipulation presents a biohazard and hence the procedure needs to be done using a bio-safety cabinet. Third, slides prepared with AFB positive sputum are difficult to standardize relative to graded controls. Finally, the appearance of smear slides prepared from NaOH and NALC-treated sputum are not macroscopically or microscopically similar to real smears (Yamada *et al.,* 2006).

The third procedure of manufacture of test slides for panel testing using new artificial sputum preparation method was developed and examined using a cultured monocyte cell line, cultured avirulent mycobacteria and methyl cellulose or polyacrylamide gel for viscosity and background (Yamada *et al.,* 2006). The study reported that the new artificial sputum was similar to real sputum in viscosity and macroscopic and microscopic appearance; it was also consistent in panel positivity grades. Therefore it

could contribute to the EQA and training in tuberculosis laboratories or microscopy centers (Yamada *et al*., 2006).

Since it is tedious and expensive to grow monocytes, it was decided to use pus cells from patients and utilize them along with BCG and methyl cellulose or polyacrylamide gel to prepare panel slides.

Taking all these conditions into consideration, panel slides were prepared using artificial materials and used to assess the proficiency of tuberculosis laboratory technicians before and after training. Address the issue of smear negative tuberculosis and quality of AFB smear microscopy in different laboratories of Ethiopia.

1.5. Objectives

1.5.1. General objective

To prepare panel slides and validate using three different methods, evaluate the efficiency of AFB smear microscopy in different laboratories in Ethiopia and the proficiency of peripheral laboratory technicians following training.

1.5.2 Specific objectives

- To prepare panel slides using patients' sputa and NaOH and pus cells, avirulent mycobacteria *M. bovis BCG* and methylcellulose or polyacrylamide gel.

- to compare the macroscopic appearance (size, thickness, evenness, staining), microscopic appearance (cells, mucus-like component), reproducibility, quality control, and suitability of unstained as well as stained smears as slide panels prepared from later two methods with WHO recommended method.

- To assess the current level of performance of laboratory personnel in Microscopy Centres in Ethiopia and to evaluate proficiency of laboratory technicians in peripheral laboratories before and after training.

2. Materials and Methods

2.1. Study Center

The study was conducted at National Reference Tuberculosis Laboratory, Ethiopian Health and Nutrition Research Institute, Addis Ababa from 1st November 2008 to 31st May 2009.

2.2 Sputum specimen

AFB negative and positive sputum specimens were collected from St. Paul specialized TB hospital and was processed as per the procedure set by IUATLD, 2002. Miss

2.3. Pus cell

Pus containing WBC was collected from people undergoing surgery in St. Paul hospital and treated with 40% formalin. After vortexing for thirty seconds, two smears were made using a 10 μL loop to confirm the amount of pus cells present. The slides were dried and heat fixed, stained using the Ziehl-Neelsen method and observed at 100 X and 1000 X. then 1 mL of 4% NaOH was added to each mL of pus and Vortexed vigorously for 1 min. About 20 mL of a mixture of pus and NaOH was put in a 50 mL conical tube and distilled water was added up to 40 mL and mixed by inverting several times. The mixture was centrifuged at 3000x g for 20 minutes and the supernatant was decanted into a disinfectant. The sediment (pus cells) was stored in 40 % formalin.

2.4. *Mycobacterium bovis* strain

Avirulent *M. bovis* strain from BCG vaccine was allowed to grow in Middlebrook 7H9 broth for 2 weeks, and suspension matching McFarland No. 1 turbidity standard suspension was prepared. The broth was vortexed with glass beads vigorously for 3 minutes to disperse cell clumps and was put to settle for 15 minutes. The supernatant was centrifuged at 1000 x g for 5 minutes. The supernatant was transferred to a new tube. The turbidity of the dispersed bacteria was measured and suspension matching McFarland No. 0.5 turbidity standard was prepared. The number of AFB was enumerated after ZN staining. Enumeration of ZN stained AFB was done by reading in 100 oil immersion

fields. The suspension was diluted using PBS to obtain approximately 20 AFB per oil immersion field (3+ grade suspension) and the number of AFBs was enumerated after reading 100 oil immersion fields in 6 slides. The enumerated 3+ grade suspension was diluted four-fold with PBS to obtain approximately 5 AFB/ oil immersion field (2+ grade suspension) and the number of AFBs was enumerated after reading 100 oil immersion fields in 6 slides. The enumerated 2+ grade suspension was further 10-fold diluted with PBS to obtain approximately 50 AFB/ 100 oil immersion fields (1+ grade suspension) and the number of AFBs was enumerated after reading 100 oil immersion fields in 6 slides. The enumerated 1+ grade suspension was further 10-fold diluted with PB to obtain approximately 5 AFB/ 100 oil immersion fields (Scanty-grade suspension) and the number of AFBs was enumerated after reading 100 oil immersion fields in 6 slides. A 500µl sample of each AFB grade suspension was mixed to 10µl sediment of pus cell.

2.5. Preparation of Panel Slides for AFB Smear Microscopy

2.5.1. Method-1

In method 1 panel slides were prepared for AFB smear microscopy using raw sputum. Negative sputum suspension was prepared first. In a class II biosafety cabinet, 1 drop of 40% formalin per 1 mL of negative sputum was added and the sputum was vortexed vigorously for 30 seconds, tube was inverted, and vortexed again for 30 seconds. The sputum was incubated for 1 hour at room temperature. The pooled negative sputum was vortexed for 2 minutes and 2 direct smears were made using a 10 µL loop. The slides were dried and heat fixed. The smears were stained using the Ziehl-Neelsen method and observed at 100 X to confirm that the starting material was negative for AFB and had pus cells of >10 per high power field. About 3 mL of the negative sputum was placed in to a 50 mL conical tube and 1 mL of 4% NaOH was added and inverted 3-5times to mix. to this mixture 20 mL of distilled water was added, and mixed well by inverting 3-5 times. Whenever mucolysis was incomplete a pinch of NALC was added and shacked gently till mucolysis was complete. The solution was incubated in a 55-60^{o}C water bath for 10 minutes, mixing by inversion several times during the incubation period. The negative sputum suspension was used for preparing dilutions of the positive stock suspension (APHL, 2002).

Positive stock suspension was prepared secondly. A drop of 40% formalin per 1 mL of sputum was added to the tube of positive pooled sputum specimen and vortexed vigorously for 30 seconds. Then the tube was inverted and vortexed again for 30 seconds. The sputum was incubated for 1 hour at room temperature and after vortexing for two minutes; two direct smears were made using a 10μL loop to confirm the AFB load of the starting material. Slides were dried, heat fixed and stained using the Ziehl-Neelsen method and observed at 1000 X (oil immersion). Then the sputum was vortxed for 1 minute and 3 mL was placed in to a 50 mL plastic conical (Falcon) tube. To each tube, 1 mL of 4% NaOH was added and vortexed vigorously for 1 minute. Then 20 mL of distilled water was added and mixed by gently inverting several times and incubated in a 55-60^{o}C water bath for 30 minutes, mixing by inversion several times during the incubation period. Distilled water was added up to 40 mL and mixed by inverting several times and centrifuged at 3000 x g for 20 minutes. The supernatant was decanted into tuberculocidal disinfectant (sodium hypochlorate) and the sediment was resuspended in 1mL PBS and vortexed for 1 minute to achieve a homogeneous suspension. Six slides of the resuspended sediment were prepared using a 10 μL loop. The slides were allowed to air dry and fixed by heating (APHL, 2002).

Slides were stained using the Ziehl-Neelsen staining method. Three different laboratory technicians read each of the six slides to determine the average number of AFB per field. This average is represented by A in the formula below (see Table 2).

Table 2. Target Dilutions and Concentrations for Smear Panel Preparation

Target Dilutions and Concentrations for Smear Panel Preparation				
Reporting Scale	**Expected concentration of AFB to observe**	**Target concentration of AFB in each dilution**	**Formula**	**Preparation of positive suspension**
3+	> 10 AFB/field	50 AFB/field or 5000 AFB/100 fields	50 AFB/field x 1 mL ÷ A = B ml of A	B ml of A + (1-x ml of negative stock) = '3+' stock
2+	1-9 AFB/field	5 AFB/field or 500 AFB/100 fields	5 AFB/field x 1 mL ÷ 50 AFB/field = 0.1 mL of '3+' stock	0.2 of B + 0.8 ml of negative stock = '2+' stock
1+	10-99 AFB/100 fields	50 AFB/ 1000 fields	50 AFB/100 fields x 1 mL ÷ 10 AFB/field = 0.05 mL of '2+' stock	0.05 ml of C + 0.95 ml of negative stock = '1+' stock
Actual/Scanty	1-9 AFB/100 fields	5 AFB/ 100 fields	5 AFB/ 100 field x 1 mL ÷ 20 AFB/100 fileds = 0.1 mL of '1+' stock	0.1 ml of D + 0.9 ml of negative stock = 'Scanty' stock

2.5.2. Method-2

Method two was prepared using pus cells, cultured BCG and polyacrylamide. A polyacrylamide stock was prepared by mixing 22.2 g acrylamide, 0.6 g N, N'-methylenebisacrylamide and distilled water to 100 ml. The stock was stored at $4^{o}C$, protected from light. PAM was made from 1.75 ml polyacrylamide stock, 6.25 ml distilled water, 2 ml TBE buffer (pH 8.0), and 100 micro liter of 10% ammonium persulfate. After mixing well, 8µl of TEMED, a polymerisation accelerator, was added and mixed; 0.5ml of this mixture was immediately added to a cell-mycobacteria sample and mixed thoroughly. The remainder of the PAM mixture containing TEMED was kept for several minutes to confirm proper polymerisation. After final mixing, the mixture containing pus cells, mycobacteria and PAM was left for 2 days at room temperature on the laboratory table in ordinary room lighting until it became viscous. Panel test smears were prepared with a microbiological loop (Yamada *et al.,* 2006).

2.5.3. Method-3

Method 3 was prepared using pus cells, cultured BCG and methylcellulose (MC). MC was dissolved using distilled water to prepare 2% solution; 0.5 ml of this solution was immediately added to a cell-mycobacteria mixture and mixed thoroughly. The final concentration of MC then became 1% and the final product was incubated at room temperature for about 1 hour. Panel test smears were prepared using a microbiological loop.

2.6. Validation of panel test slides

From each method and each grade of positivity 6 slides were chosen randomly. Heat fixed and stained with ZN method and 3 laboratory technicians read each smear at 1000X and recorded the AFB/field for each smear. Average and standard deviation (SD) for each grade was calculated using the results from 6 smears.

Mean: Mean is computed from slide test results 1-6.

Standard deviation: The standard deviation is computed from slide test results 1-6. The following formula was used to compute Standard deviation.

$$\frac{\sqrt{n\sum x2-(\sum x)^2}}{n\,(n-1)}$$ Where x= average and n=6

Consistency: The consistency column result is computed using the following formula:

Mean [M] minus 2 standard deviations [SD] (M-2SD) and Mean [M] plus 2 standard deviations [SD] (M+2SD). Table 3 gives the acceptable values for each grade of smear for declaring the batch as 'acceptable' for panel testing.

Table 3. Acceptable values for each grade of smear for declaring for Panel Testing

Grade	X-2SD	X+2SD	Consistency
3+	≥11/ field		True (sufficient)
2+	≥1/ field	≤10/ field	True (sufficient)
1+	≥10/ 100 fields	≤99/ 100 fields	True (sufficient)
Scanty	≥1/ 100 fields	≤9/ 100 fields	True (sufficient)
Negative		0 / 100 fields	True (sufficient)

When the value of 'M-2SD' or 'M+2SD' was outside the values suggested above, then the consistency was considered as false (insufficient).

When the consistency is false-then there is too much variation in the number of AFB per slide and this sample is not of sufficient consistent to use in a PT test for a reliable evaluation of performance. The above mentioned formula provides an objective

evaluation of consistency. Both average and SD were calculated using an Excel sheet (appendix 1).

The prepared panel slides were stored in slide boxes in a dry place.

2.7. Assessment of Smear microscopy in Ethiopia

Stained and unstained slides were distributed to 56 health centers and hospitals in the nine regions of Ethiopia to assess the reading, interpretation, grading and staining ability of the laboratory technicians. Along with the slides, panel slide reading sheet and panel testing recording and feed back form (appendix 2 and 3) were provided. The panel test included slides of different grades. The laboratory technicians read the slides stained and/ unstained and fill the forms. The prepared panel slides were also used to evaluate the proficiency of laboratory technicians before and after training. The training was conducted at Dessie regional laboratory and 29 technicians from different peripheral laboratories were participated.

3. Results

This study was conducted at the National Reference Tuberculosis Laboratory, EHNRI, and A.A. from Nov. 2008 to May 2009. Panel slides for AFB smear microscopy were prepared using the standard NaOH treated sputum samples (Method-1) and artificial sputum prepared using two other new methods. These two innovative methods for preparation of panel slides were applied using cultured Bacillus Calmette-Guérin (BCG) strain of *Mycobacterium bovis*, pus cells and one with polyacrylamide (Method-2) and other, methyl cellulose (Method-3).The efficiency of laboratory technicians in AFB smear microscopy in different hospitals and health centres of different regions of Ethiopia were assessed and the performance of peripheral laboratory technicians before and after training were evaluated.

3.1. Preparation and examination of panel slides

Panel slides prepared by the three different methods were of different grades of positivity including negative slide as shown in table 4.

Table 4. Different methods of preparation of panel slides

Method	Description	Total slides prepared
Method-1	NaOH treated sputum	2000
Method-2	BCG+Pus ells+ Polyacrylamide	1500
Method-3	BCG+Pus cells +Methylcellulose	1500
Total		5000

Table 5 shows the slides prepared with different grades of positivity and the negative slides.

Table 5. Different grades of slides prepared using three methods

Method	Negative	Scanty	1+	2+	3+	Total
Method-1	600	500	300	200	400	2000
Method-2	200	450	600	150	100	1500
Method-3	300	200	300	500	200	1500
Total	1100	1150	1200	850	700	5000

Negative= no AFB, 1+=10-99/100field

Scanty= 1-9 AFB/100field 2+= 1-9/field

3+= >10/field

From each method, slides of different grades were prepared. A total of 1100 negative, 1150 scanty, 1200 1+, 850 2+ and 700 3+ slides were prepared.

After preparation, a total of 10 slides from each grade belonging to different methods were examined by two laboratory experts for unstained macroscopic appearance. The smears were then stained using Ziehl-Neelsen method and re-examined by same experts for macroscopic appearance after staining (Table-6 and 7).

Table 6. Macroscopic appearance of unstained smears

	Criteria			
	Size (3x2cm)	**Oval Shape**	**Reading of news prints through the smears**	**Evenness of smear**
Expert-1				
Method-1	10/10 (100%)	10/10 (100%)	10/10 (100%)	10/10 (100%)
Method-2	10/10 (100%)	10/10 (100%)	10/10 (100%)	10/10 (100%)
Method-3	9/10 (90%)	10/10 (100%)	10/10 (100%)	7/10 (70%)
Expert-2				
Method-1	10/10 (100%)	10/10 (100%)	10/10 (100%)	10/10 (100%)
Method-2	10/10 (100%)	10/10 (100%)	10/10 (100%)	10/10 (100%)
Method-3	9/10 (90%)	10/10 (100%)	10/10 (100%)	8/10 (80%)

Macroscopic appearance of unstained smears showed to be of oval shape and uniform size of 3x2cm. However, with method-3 the size was smaller (about 2.5cm) for at least one smear. News paper prints could be read properly through all the smears made using methods 1 and 2 but smears of method two are more opaque to read through them. The smears were evenly made using methods 1 and 2; however, with method-3 the material was more concentrated in the centre. (Figure 2)

A B C D

Figure 2: Macroscopic appearance of unstained smears

A) Direct smear from sputum, B) PAM method, C) MC method, D) NaOH method

PAM= polyacrylamide; MC=methylcellulose; NaOH=sodium hydroxide

Table 7. Macroscopic appearance of stained smears

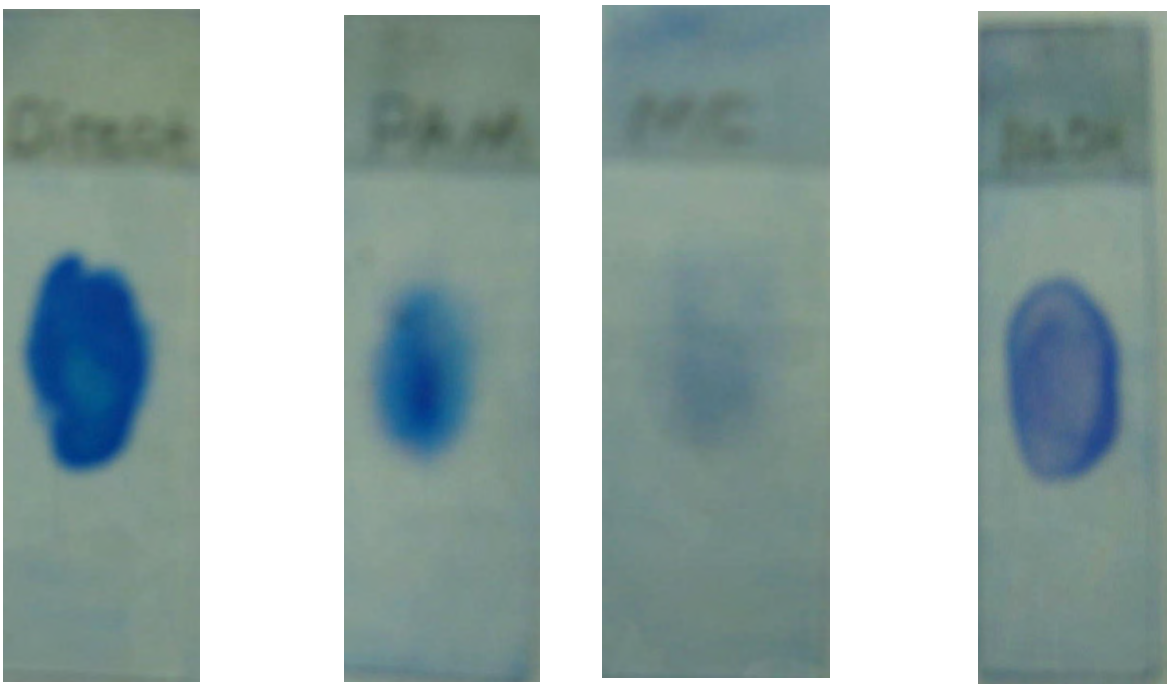
	Criteria			
	Evenness of staining	**Uniform Blue appearance**	**Red colored patches in the smear**	**Any other observation**
Expert-1				
Method-1	10/10 (100%)	10/10 (100%)	0/10 (0%)	Not changed
Method-2	9/10 (90%)	10/10 (100%)	0/10 (0%)	Appears like stained sputum
Method-3	10/10 (100%)	10/10 (100%)	0/10 (0%)	Appears thinner after staining
Expert-2				
Method-1	10/10 (100%)	10/10 (100%)	0/10 (0%)	
Method-2	9/10 (90%)	10/10 (100%)	0/10 (0%)	
Method-3	10/10 (100%)	10/10 (100%)	0/10 (0%)	Appears thinner after staining

Macroscopic appearance of the stained smears showed that all the smears made by different methods were uniformly blue in colour due to methylene blue without any red patches of carbol fuchsin, indicating that the artificial substances, polyacrylamide and methyl cellulose did not interfere with the action of our 3% acid alcohol used as decolorizing agent. The smears were evenly stained.

An important observation was that the smears made using method-2 appeared more like sputum smear; however, the smears from method-3 appeared thinner after staining.

The ZN stained smears were then examined using ZEISS bright field microscope under x100 and x1000 magnifications by the same experts. The observations are given in Tables 8 and 9. Figure 3 shows macroscopic appearance of ZN stained panel slides

prepared using three methods and figure 4 shows the microscopic appearance of ZN stained panel slides prepared using the three methods.



A B C D

Figure 3: Macroscopic appearance of stained smears

.

A) Direct smear from sputum specimen, B) PAM method, C) MC method, D) NaOH method

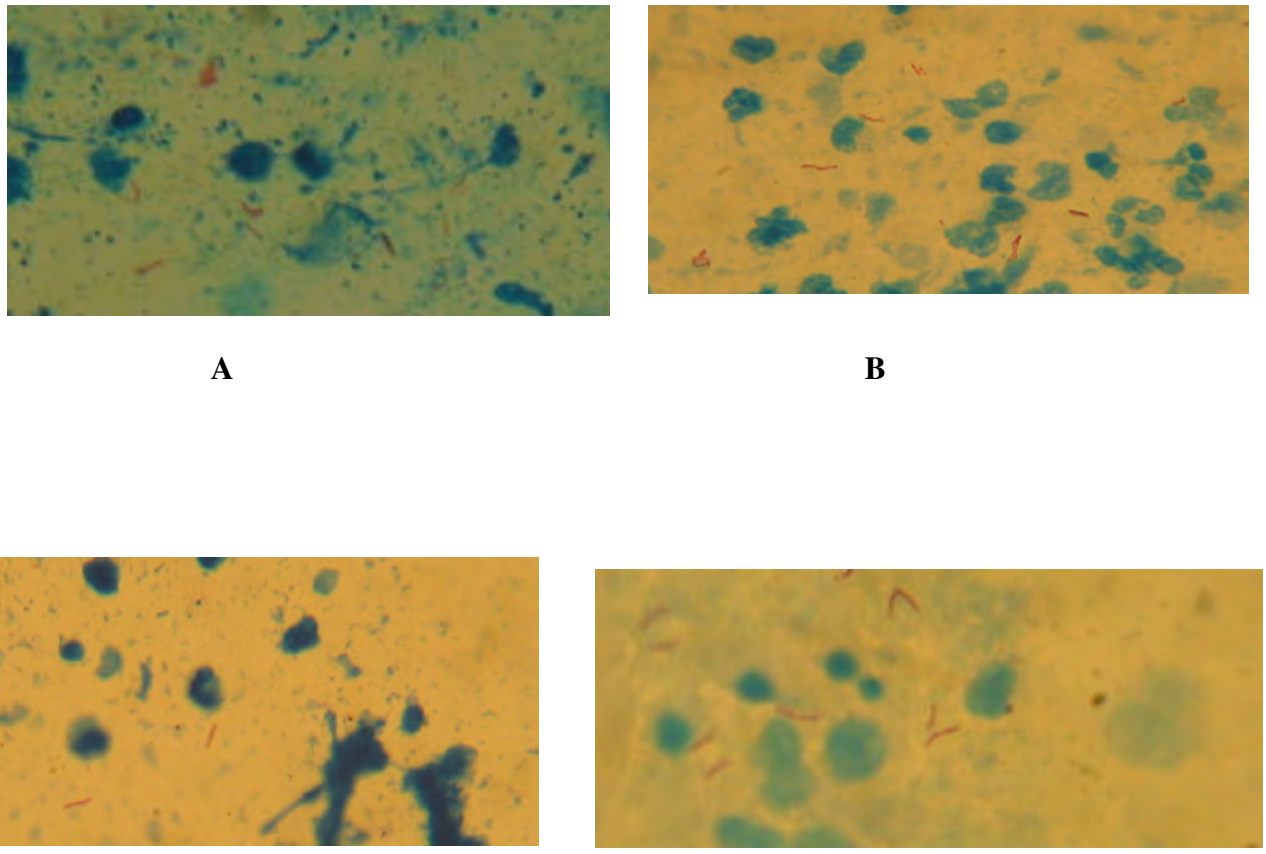
PAM= polyacrylamide; MC=methylcellulose; NaOH=sodium hydroxide

It was observed that the staining was uniform and required numbers of cells were present in all the smears. In smears made by methods 2 and 3, the mucus and fibrin like material was also observed, while it was not seen in smears made using method 1. The red stained patches were not seen under microscope.

Table 8. Microscopic appearance of ZN stained smears under x100 magnification

	Criteria			
	Evenness of staining	**Cells >20/low power field**	**Mucus and fibrin like material**	**Red colored patches**
Expert 1				
Method-1	10/10 (100%)	10/10 (100%)	0/10 (0%)	0/10 (0%)
Method-2	10/10 (100%)	10/10 (100%)	10/10 (100%)	0/10 (0%)
Method-3	10/10 (100%)	10/10 (100%)	10/10 (100%)	0/10 (0%)
Expert 2				
Method-1	10/10 (100%)	10/10 (100%)	0/10 (0%)	0/10 (0%)
Method-2	10/10 (100%)	10/10 (100%)	10/10 (100%)	0/10 (0%)
Method-3	10/10 (100%)	10/10 (100%)	10/10 (100%)	0/10 (0%)

All the smears prepared using the three methods showed evenness in staining. Sufficient amount of pus cells were seen under low power field which are used as a back ground especially for slides of low positive grades and negative slides. Mucus and fibrin like materials were not seen in slides prepared with method-1. Slides of method-2 and 3 had mucus and fibrin like materials which makes them similar to real sputum smears. Red colored patches were not seen in slides prepared by the three methods.



A **B**

C **D**

Figure 4. Microscopic appearance of smears**.**

A) Directly prepared from sputum specimen, B) PAM method, C) MC method, D) NaOH method
PAM=polyacrylamide, MC=methylcellulose, NaOH=sodium hydroxide

Table 9. Microscopic appearance of ZN stained smears under x1000 magnification

	Criteria			
	Polymorphonuclear leucocytes	**Macrophages**	**Epithelial cells**	**Acid fast bacilli**
Expert 1				
Method-1	1/10 (10%)	10/10 (100%)	2/10 (20%)	10/10 (100%)
Method-2	10/10 (100%)	0/10 (0%)	0/10 (0%)	10/10 (100%)
Method-3	10/10 (100%)	0/10 (0%)	0/10 (0%)	10/10 (100%)
Expert 2				
Method-1	0/10 (0%)	10/10 (100%)	2/10 (20%)	10/10 (100%)
Method-2	10/10 (100%)	0/10 (0%)	0/10 (0%)	10/10 (100%)
Method-3	10/10 (100%)	0/10 (0%)	0/10 (0%)	10/10 (100%)

Under x1000 magnification, the smears made using NaOH treated sputum showed presence of macrophages and acid fast bacilli; in two smears epithelial cells were also observed. However, the smears made using methods 2 and 3, showed presence of polymorhonuclear leucocytes and acid fast bacilli; macrophages and epithelial cells were not seen. This was because the smears were prepared using pus collected from patients suffering from acute abscesses.

3. 2. Validation of panel Slides.

A set of six smears each from negatives and different grades of positivity manufactured by the above methods were then examined by three different technologists from National Reference Laboratory. The results were used to validate the smears as recommended by WHO (APHL, 2002). Whenever the slides were not of sufficient consistency and failed the validation, the whole batch was discarded.

The results for validation of slides are depicted from Table 10 to Table 13.
It was observed that all the slides of different grades passed the validation. Also, all the manufactured negative slides were actually negative on examination (results not depicted in the tables)

Table 10. Validation results for 3+ smears manufactured using the three methods.

	Number of AFBs per 100 oil immersion fields						**Mean (M)**	**SD**	**M-2SD**		**M+2SD**		**Validity**
Method	Slide 1	Slide 2	Slide 3	Slide 4	Slide 5	Slide 6			Expected	Actual	Expected	Actual	
1	2790	2039	2188	2224	2530	2452	2371	273	>1000	1824	>1000	2917	TRUE
2	1203	1246	1222	1256	1195	1270	1232.0	30.1	>1000	1171.8	>1000	1292.2	TRUE
3	1976	1870	1854	1866	1872	1880	1886.3	44.7	>1000	1796.8	>1000	1975.8	TRUE

Table 11. Validation results for 2+ smears manufactured using the three methods.

	Number of AFBs per 100 oil immersion fields						**Mean (M)**	**SD**	**M-2SD**		**M+2SD**		**Validity**
Method	Slide 1	Slide 2	Slide 3	Slide 4	Slide 5	Slide 6			Expected	Actual	Expected	Actual	
1	345	320	287	432	489	586	409.8	114.2	>100	181.5	<=1000	638.1	TRUE
2	632	654	672	650	631	645	647.3	15.3	>100	616.8	<=1000	677.9	TRUE
3	435	467	423	417	421	452	435.8	19.9	>100	396.1	<=1000	475.6	TRUE

Table 12. Validation results for 1+ smears manufactured using the three methods.

	Number of AFBs per 100 oil immersion fields						**Mean (M)**	**SD**	**M-2SD**		**M+2SD**		**Validity**
Method	Slide 1	Slide 2	Slide 3	Slide 4	Slide 5	Slide 6			Expected	Actual	Expected	Actual	
1	67	48	64	67	56	51	59	8	>=10	42	<100	75	TRUE
2	24	23	28	22	31	21	24.8	3.9	>=10	17.1	<100	32.6	TRUE
3	34	30	32	37	31	33	32.8	2.5	>=10	27.9	<100	37.8	TRUE

Table 13. Validation results for scanty positive smears manufactured using the three methods.

	Number of AFBs per 100 oil immersion fields						**Mean (M)**	**SD**	**M-2SD**		**M+2SD**		**Validity**
Method	Slide 1	Slide 2	Slide 3	Slide 4	Slide 5	Slide 6			Expected	Actual	Expected	Actual	
1	1	3	2	2	2	1	2	1	>0	0	<10	3	TRUE
2	3	2	3	5	2	6	3.5	1.6	>0	0.2	<10	6.8	TRUE
3	3	3	2	1	4	3	2.7	1.0	>0	0.6	<10	4.7	TRUE

Table 14, shows a comparison of macroscopic and microscopic observations of slides prepared using three different methods and their utilization as panel test slides for routine use.

Table 14. Comparison of three methods for manufacture of panel smears

Parameter	Method 1	Method 2	Method 3
Unstained smear Macroscopic examination			
Appropriate Size	100%	100%	90%
Oval Shape	100%	100%	100%
Reading of news prints	100%	100%	100%
Evenness	100%	100%	75%
ZN Stained smear macroscopic examination			
Evenness of staining	100%	90%	100%
Uniform blue appearance	100%	100%	100%
Red coloured patches	0%	0%	0%
ZN stained smear microscopic examination x100			
Evenness of staining	100%	100%	100%
Cells > 20	100%	100%	100%
Mucus and fibrin	0%	100%	100%
Red coloured patches	0%	0%	0%
ZN stained smear microscopic examination x1000			
Polymorphonuclear leucocytes	10%	100%	100%
Macrophages	100%	0%	0%
Epithelial cells	20%	0%	0%
AFB	100%	100%	100%
Validation	100%	100%	100%

3.3. Assessment of proficiency of laboratory technicians

Panel smears prepared using method 1 were used for panel testing of lab personnel working in health centers and hospitals found in different regions of Ethiopia. The panel slides were sent to 56 microscopy centres and consisted of 6 smears – 3 stained and 3 unstained. Along with the slides panel slide reading form (appendix 2) and Panel Testing Recording and Feedback Form (appendix 3) were sent. Among these 6 smears, 2 were negatives and one each was from different grades of positivity. Four laboratories did not send back the results of their reading.

The results were available for analysis from a total of 52 laboratory personnel (appendix 5).

Table 15. Results of panels sent to peripheral laboratories

Total Personnel tested	**Personnel showing no error**	**Personnel showing errors**	**Type of errors**	**Comments**
52	40	12	QE – 5 LFP – 3 LFN – 1 HFP - 1 No result of unstained smears -3	One personnel had 2 QE. The results from three Labs were incomplete.

QE – Quantification Error; LFP – Low false positive; LFN – Low false negative; HFP – High false positive

Three Labs didn't send the results of unstained smears this is may be due to the shortage of staining materials.

3.4. Evaluating the proficiency of Lab technicians following training

The panels were also used for a training in which 29 lab personnel were tested before and after training. The training was given for peripheral lab technicians (appendix 6).
Table 16 depicts the results from that training.

Table16. Pre and post training panel testing results using smears manufactured by method 1

	Individual results (No 29)		**Errors**	
	No Error	With Error	Minor Errors	Major Errors
Before training	4	25	37	17
After training	6	23	30	4

Before training 4 individuals (14%) show no error but after training it increased to 6 individuals (21%). There were 37 minor errors and 17 major errors before training. But after training the technicians show great improvement. There were 30 (81%) minor errors and 4 (14%) major errors.
Chi square test was done to compare performance of lab technicians before and after training. Ho: which was stated as; "At 1% level of significance, there is no significant improvement of performance of Lab technicians after training." was rejected. The statistical analysis shows that there is significant improvement of Lab technicians in performance after training.

4. Discussion

In the present study, a total of 5000 slides of different grades were prepared by three methods and examined both macroscopically as well microscopically, validated and used under field conditions.

The smears made from NaOH treated sputum (Method 1) were considered as Gold Standard as it is WHO recommended method (APHL, 2002). Both newer methods (Methods 2 and 3) were compared with the Method 1.

Method 1 was rapid and simple as the sputum samples could be immediately processed and the panel slides could be made on the same day. However, the quality of sputum sample got deteriorated as the mucus and fibrin part got damaged during processing and the slides were easy to examine than actual specimens. The smears made by this method are not macroscopically or microscopically similar to direct sputum smears. This method has a disadvantage of dealing with live virulent mycobacteria from patients and in the present setting there is always a possibility of MDR TB as Ethiopia ranks 15th among the 27 countries with high MDR TB rate (WHO, 2009). This necessitates working in a Biosafety Level III laboratory and using Biosafety Cabinet of Class IIB. Also, in this method it was difficult to get good quality negative sputum samples. The negative sputum specimen needs to have sufficient amount of pus cells as they are used as a back ground especially for negative and low positive grade slides.

Considering the Methods 2 and 3, we used culture of Bacille Calmette Guérin (BCG). Though it took longer time, 2 weeks in liquid culture, it was safe to handle these bacteria, as it is avirulent strain. As a result it is not mandatory to work in Biosafety Level III laboratory and using Biosafety Cabinet of Class IIB. Since the cell culture for monocytes, as used by Yamada *et al.,* (2006), a tedious, expensive and time consuming procedure we opted for pus drawn from acute abscesses in patients admitted to St. Paul hospital. However, while collection and processing the sample all the universal precautions were followed. Also, after addition of formalin it was rendered non-infectious and the cells could stay intact for at least 6 months. This makes our method inexpensive easy and

rapid. As proposed by Yamada *et al*., (2006), the monocytes were obtained from culture collections (American type culture collection) which are expensive especially in our conditions. The culture of monocytes is also expensive as it requires sophisticated equipments and expensive culture media. The procedure is tedious and time consuming because it needs to follow the procedures properly and it took about three weeks to harvest sufficient amount of cells (Yamada *et al.,* 2006). But in our method pus cells collected from patients is obtained from pus which is normally a waste after surgery so it is obtained for free. We can use the pus cells immediately after treating it with NaOH and formalin so it is not time consuming.

One problem of these methods was dispersing the clump of bacteria. The clumps can be dispersed by passing through acrodisk filter (pore size 5 micron) (Yamada *et al., 2006).* Since these filters were not available in our laboratory we tried and succeeded to disperse the clumps by vortexing the broth with beads and centrifugation with low speed. In the case of the acrodisk filter the price of the filter is expensive and also it is not easily available. Further more; most of the bacteria can be trapped by the filter. This method of dispersing bacteria was more advantageous as we can get large number of bacteria. It is also inexpensive and easy as we can use readily available materials to disperse the bacteria.

Working with methyl cellulose was easy, as only 2% solution was to be mixed with bacteria and cells. The disadvantage of this method was that the 2% solution of methyl cellulose was a gel and it was very difficult to adjust the number of bacteria in different graded samples as bacteria could remain concentrated at a particular place in that gel (Yamada *et al.,* 2006). In this study we vortexed the cell-methyl cellulose mixture with glass beads to homogenize and it was easy to adjust the number of bacteria.

The polyacrylamide method was laborious as first we had to prepare a polyacrylamide stock from acrylamide using a number of chemicals and then this mixture was to be added with cells, bacteria and few more chemicals as mentioned in the Material and Methods section. Then this solution was to be left at room temperature for 2 days for polymerization to occur before preparing the slides. Also, there was a risk as acrylamide

is toxic before polymerization. However, it has advantages like; the protective measures are only lab coats and gloves. Also once the polymerization occurred; the stock could be used for long periods of time and very small amount of the stock is used to prepare large number of slides (Yamada *et al*., 2006).

Considering the macroscopic appearance of the prepared smears, it was noted that the shape was oval for all the three methods and thickness was good enough to allow reading of news prints through it. However, since methyl cellulose is a viscous substance as discussed earlier, it was slightly difficult to make smears of uniform size and evenness using this method. Also, since beads were used to homogenize the mixture only 10% of the examined slides had size problem which was also not a major problem as the size was just half a cm less than the expected. Problem with evenness was more (75%) due to the same reason of viscosity.

After Ziehl Neelsen staining, the smears were examined macroscopically for evenness. The uneven smears from method 3 were now found evenly stained showing that though they appeared thick under unstained condition they became uniform and rather thinner after staining. It was also seen that the smears were uniformly blue without any red patches, indicating that the polyacrylamide and methyl cellulose did not interfere with 3% acid alcohol being inert substances. Also indicating that all the smears had good staining property.

Under x100 magnification of the microscope the staining was again found to be even with required number of cells. Since NaOH damages the mucus and fibrin present in sputum sample, these components were absent in the smears. This is an inherent problem with NaOH treated sputum and thus to make panel slides more resembling with sputum samples. WHO has also recommended the NALC method wherein the mucus substance may remain intact (APHL, 2002).

The kind of cells in the three methods varied depending upon what material was used to prepare the slides. The NaOH treated sputum, since it was from TB patients and TB suspects had macrophages, polymorphonuclear leucocytes (PMNL) and epithelial cells

along with acid fast bacilli; however, as indicated before, these slides lacked mucus and fibrin. On the other hand, since the other two methods used pus samples from acute abscesses, only PMNL was found along with AFB and also mucus and fibrin. Considering this, since it is not expected from a person in microscopy center to differentiate between macrophages and PMNL, it is acceptable to use the PMNL as pus cells along with mucus and fibrin from pus. The morphology of AFB was uniform in all the three different methods.

All the grades of positivity from different methods were validated by a panel of three technologists from the National reference Laboratory. As per WHO, if the panel are validated they can be used for panel testing as part of external quality assessment (EQA) in the field (IUATLD, 2007).

TB control requires a functional laboratory set-up with quality diagnostic services and a trained diagnostician and microscopist. However, the performance of such laboratories depends on continuous monitoring and quality improvement mechanisms put in place. The principal objective of external quality assessment (EQA) is to standardize sputum microscopy for the detection of infectious TB cases across the peripheral diagnostic centers and to validate the reported acid-fast bacilli (AFB) microscopy results from these centers (Estifanos *et al*., 2005).

As per WHO, the EQA has to be performed by three different methods viz. on site evaluation (OSE), panel testing (PT) and random blinded rechecking (RBRC) (IUATLD, 2007). However, considering the financial and human resources, countries can decide which method to use (FMOH, 1999). In Ethiopia, the National TB Programme addressed this issue of EQA by making Ethiopian Health and Nutrition Research Institute responsible for EQA (EHNRI/TLCP, 2008). However, the bigger challenge was to have the panel slides in large numbers and since good quality sputum was many times not available in the National Reference Laboratory it was decided to have some alternate to the natural sputum. Thus the slides were prepared by different methods in this study to meet the need of the country. The two new methods (methods 2 and 3) enabled us to prepare large number of slides in very short period of time.

The need for EQA in Ethiopia had become critical because of a high incidence of smear negative TB. Over the years the observation was that the cases with smear positive TB were becoming lesser and the percentage remained between 32-36%. This could be explained considering the increasing number of HIV/AIDS patients, who are usually smear negative at the later stage of HIV disease (Harries *et al.,* 2001).

Another reason that can be attributed is childhood TB, wherein also the child is unable to produce good quality sputum to make a definitive diagnosis (David *et al.,* 1999).

In addition to the above reasons, there is another reason which is low performance of lab personnel and chances of false negative smears observed in the field. Panel testing gives us a chance to test the personnel in the field and know their performance.

The use of a test panel of unstained slides has the advantage of testing several aspects of the designated regional level LT's technical performance, including preparation of staining reagents, staining procedure, reading and reporting of results. The use of stained smears alone, will only assess reading capability, and will not provide information on the LT's capability to stain smears. The results of sputum AFB microscopy are known to be influenced by various factors including the proficiency to read smears by microscopists (EHNRI/TLCP, 2008).

Considering this, sets of stained and unstained panel slides were sent to 56 microscopy centers and 52 lab personnel undertook this testing. The analysis of the results showed that 40 among the 52 personnel (77%) performed well without any major or minor error. Only one person had a major error of high false positivity, three had low false positivity and 5 had quantification errors. Only one person had low false negative error. Considering that both stained and unstained slides were provided for PT, the results showed that the stains were working properly, at least in 49 tested centres and the personnel were proficient enough to detect positive smears. Three personnel didn't provide a report of the unstained slides. It is expected from every laboratory to have the necessary staining reagents. So we recommended that they should have the reagents for optimum performance of their laboratories. These results may not show their actual

performance in routine work as the personnel know that they are being tested, might pay more attention to such slides and the time was not fixed as supervisors were not on site. (IUATLD, 2007). For reliable panel testing it is recommended if experts are on site and supervise.

Several documents recommend the need for training laboratory technicians to improve the quality of sputum AFB microscopy. The results of sputum AFB microscopy are known to be influenced by various factors including the proficiency to read smears by microscopists (Selvakumar *et al.,* 2004).

A study conducted in India tried to assess the proficiency of LTs under training to read sputum AFB smears using prepared panel slides. The result shows low false-negatives decreased from 58% to 22% after training (Selvakumar *et al.,* 2004).

Our study also tests the proficiency of technicians before and after training. This was done using the panel slides manufactured using NaOH treated sputum prepared in this study. A total of 29 technicians were trained and tested. It was found that there was a huge reduction in major errors which fell from 17 to just 4 after training. The results show that there was definite improvement. After training two additional personnel did not show any error. The minor errors were reduced from 37 to 30 and major errors were reduced from 17 to 4. The improvement of the performance of LTs after training is significant statistically.

The results in the pre training test show that there is high possibility of false negativity. Since the test was done in the presence of experts the time and the number of fields to be observed was fixed. So low performance of the lab technicians contribute to smear negativity in this study.

It is very essential that every laboratory technician should be trained in sputum AFB microscopy (Selvakumar *et al.,* 2004). Training has improved the performance of LTs. And this shows that the problem of low case finding in our country can be improved through training of LTs. This study showed that panel slides are essential tools for the training of lab personnel in peripheral laboratories. Thus panel slides prepared in this study were also used for routine use for the national issue.

5. summary, conclusion and Recommendations

5.1. Summary

This study, the use of panel slides for external quality assessment of AFB smear microscopy in tuberculosis was undertaken in National Reference Laboratory from Nov 08 to May 09. Following are the main features of this study-

- A total of 2000 slides were prepared from naturally produced and NaOH treated sputum (Method 1). These slides after validation were used to panel test 53 technologists in the microscopy centers and also to perform pre and post training testing of 29 technicians.
- A total of 1500 slides each were prepared using cultured BCG strain of *Mycobactetrium bovis*, pus cells from patients with acute abscesses and one of the following chemicals – polyacrylamide gel (Method 2) and methyl cellulose (Method 3).
- The slides prepared by the three methods belonged to different grade of positivity and also the negative slides.
- All the slides prepared by three methods were uniform in size, shape, thickness and evenness on macroscopic examination of unstained smears.
- On macroscopic examination of Ziehl Neelsen stained smears, representative samples of all the slides were found to have even staining with a uniform blue appearance.
- The slides prepared by Method 2 (polyacrylamide gel) resembled that of sputum slides.
- The slides prepared by Method 3 (methyl cellulose) were slightly opaque when unstained, but became very thin after staining.
- All the expected cells i.e. macrophages, polymorphonuclear leucocytes (PMNL) and epithelial cells and AFB were present in slides prepared from method 1 (NaOH treated sputum); however, in the other two methods only PMNL and AFB were there.

- Mucus and fibrin which made appearance like natural sputum were absent in Methods 1 while they were present in slides made by Method 2 and 3.
- Slides made by all the methods in different grades were validated by three experts from National Reference Laboratory.
- The panel slides prepared were used to assess the current performance of laboratory technicians and to evaluate the proficiency of peripheral lab technicians before and after training.

5.2. Conclusion and Recommendations

- Using both methods of polyacrylamide gel and methyl cellulose, were acceptable in comparison to the Gold standard (NaOH treated sputum) and can be used for preparation of panel slides.
- Panel slides made by both methods are recommended for EQA and as a tool of training for laboratory technicians.
- The polyacrylamide method is more recommended for EQA and training because it is microscopically and macroscopically similar to direct sputum smears. However panel slides made from methyl cellulose are macroscopically different from real sputum smears before and after staining but they are similar with respect to their microscopic appearance.
- It is recommended that panel testing will be more effective if it is performed in the presence of supervisors.
- The use of panel slides to assess the efficiency of laboratory technicians and to evaluate them during training is significant

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7. Appendices

Appendix 1: validation of panel slides

Validation of Panel slides

Date	Batch No	Grade	Number of slides	Number of AFBs per 100 Oil Immersion Fields								M-2SD		M+2SD		Validity
				Slide 1	Slide 2	Slide 3	Slide 4	Slide 5	Slide 6	Mean (M)	SD	Expected	Actual	Expected	Actual	
02/06/2008	0012	3+	89	1112	2123	2121	2211	2345	2219	2022	453	>1000	1115	>1000	2928	TRUE
02/06/2008	0013	2+	94	450	534	612	480	540	550	528	57	>100	414	<=1000	641	TRUE
02/06/2008	0014	1+	99	45	80	30	60	40	50	51	17	>=10	16	<100	86	TRUE
02/06/2008	0015	SCANTY	96	6	8	6	3	5	6	6	2	>0	2	<10	9	TRUE
02/06/2008	0016	1+	92	15	16	17	18	16	81	27	26	>=10	-26	<100	80	FALSE

Appendix2: Panel slide reading form

Date: _____ Batch number: _____ Slide Number: _____

Name of the reader_____

	A	B	C	D	E	F	G	H	I	J	Total
1											
2											
3											
4											
5											
6											
7											
8											
9											
10											
Grand total											

Appendix 3: Panel Testing Recording and Feedback Format

Central Laboratory Use Only:
Test Slide set # _____
Date Sent: _____
Date results received: _____

Peripheral laboratory _____

Date PT received by your laboratory_____ (DD/MM/YY)

Date PT results returned to Central Laboratory _____ (DD/MM/YY)

Name of the technician reading the smears _____

Note: If more than one technician performs AFB microscopy in the laboratory, each technician should read all 6 smears and record their results on a separate form. Technicians should not discuss results or share forms until all results have been sent back to the central laboratory. Forms for all technicians should be sent to the central laboratory for evaluation.

		Reference Laboratory Only		
Slide Number	**Result**	**Expected Result**	**Error Type**	**Points**

Feedback				
Total Points:			Pass/Fail:	
HFP	HFN	LFP	LFN	QE
Recommended Action				

Appendix 4: Formula for preparing the 3+ Dilution

The target concentration for the 3+ dilution is 50 AFB/field where:

A = Concentration of the positive stock solution

B = the amount of positive stock solution added to the 3+ dilution tube (total volume = 1 mL)

C = the amount of negative stock solution added to the 3+ dilution tube (total volume = 1 mL)

All dilutions are prepared in a total volume of 1 mL

$$\mathbf{B =} \frac{\text{Target concentration x 1 mL}}{\text{Concentration of positive stock}} \mathbf{=} \frac{\text{50 AFB/Field x 1 mL}}{\text{A}}$$

Calculate the amount of the negative stock: C = 1 mL – B = _____mL

Every care was taken to vortex each dilution for 30 seconds before preparing slides

Appendix 5: Assessment of AFB smear microscopy in 52 hospitals and health centres in Ethiopia.

Site Name	Smear	Result	Expected Result	Error Type	Score	Total score	%	Performance
Adama Hospital	T_1	3+	2+	Correct	10			
	T_2	1+	scanty	Correct	10			
	T_3	No AFB	No AFB	Correct	10			
	T_4	4+	3+	Correct	10			
	T_5	3+	No AFB	HFP	0			
	T_6	2+	1+	Correct	10	50/60	83%	Acceptable
Addis Ababa Reg Lab	T_1	2+	2+	Correct	10			
	T_2	scanty	scanty	Correct	10			
	T_3	No AFB	No AFB	Correct	10			
	T_4	2+	3+	Correct	10			
	T_5	No AFB	No AFB	Correct	10			
	T_6	1+	1+	Correct	10	60/60	100.00%	Acceptable
Adigrat Zonal HL	T_1	3+	2+	Correct	10			
	T_2	1+	scanty	Correct	10			
	T_3	No AFB	No AFB	Correct	10			
	T_4	3+	3+	Correct	10			
	T_5	No AFB	No AFB	Correct	10			
	T_6	1+	1+	Correct	10	60/60	100.00%	Acceptable
Adma Regional Lab	T_1	3+	2+	Correct	10			
	T_2	scanty	scanty	Correct	10			
	T_3	No AFB	No AFB	Correct	10			
	T_4	3+	3+	Correct	10			
	T_5	No AFB	No AFB	Correct	10			
	T_6	1+	1+	Correct	10	60/60	100%	Acceptable
Air Force (Bishoftu) HL	T1	2+	2+	Correct	10			
	T2	scanty	scanty	Correct	10			
	T3	No AFB	No AFB	Correct	10			
	T4	3+	3+	Correct	10			
	T5	No AFB	No AFB	Correct	10			
	T6	scanty	1+	Correct	10	60/60	100%	Acceptable
Alert Hospital.	T_1	2+	2+	Correct	10	60/60	100%	Acceptable

LAB								
	T_2	9/100	scanty	Correct	10			
	T_3	No AFB	No AFB	Correct	10			
	T_4	2+	3+	Correct	10			
	T_5	No AFB	No AFB	Correct	10			
	T_6	1+	1+	Correct	10			
Amanuel HL	T1	3+	2+	Correct	10			
	T2	scanty	scanty	Correct	10			
	T3	NoAFB	No AFB	Correct	10			
	T4	3+	3+	Correct	10			
	T5	NoAFB	No AFB	Correct	10			
	T6	2+	1+	Correct	10	60/60	100%	Acceptable
Ambo HL	T_1	3+	2+	Correct	10			
	T_2	1+	scanty	Correct	10			
	T_3	scanty	No AFB	LFP	5			
	T_4	3+	3+	Correct	10			
	T_5	No AFB	No AFB	Correct	10			
	T_6	1+	1+	Correct	10	55/60	91.60%	Acceptable
Arbaminch HL	T1	pos	2+					
	T2	pos	scanty					
	T3	pos	No AFB					
	T4	no result	3+	----------				
	T5	no result	No AFB	----------				
	T6	no result	1+	----------		------	------	Not acceptable
Asela HL	T1	3+	2+	Correct	10			
	T2	scanty	scanty	Correct	10			
	T3	NoAFB	No AFB	Correct	10			
	T4	3+	3+	Correct	10			
	T5	NoAFB	No AFB	Correct	10			
	T6	1+	1+	Correct	10	60/60	100%	Acceptable
Assaita Health Center	T1	3+	2+	Correct	10			
	T2	scanty	scanty	Correct	10			
	T3	No AFB	No AFB	Correct	10			
	T4	3+	3+	Correct	10			
	T5	No AFB	No AFB	Correct	10			
	T6	2+	1+	Correct	10	60/60	100%	Acceptable
Assossa HL	T1	2+	2+	Correct	10			
	T2	1+	scanty	Correct	10			
	T3	No AFB	No AFB	Correct	10			
	T4	3+	3+	Correct	10			
	T5	No AFB	No AFB	Correct	10	60/60	100%	Acceptable

	T6	1+	1+	Correct	10			
Attat HL	T1	3+	2+	Correct	10			
	T2	scanty	scanty	Correct	10			
	T3	No AFB	No AFB	Correct	10			
	T4	2+	3+	Correct	10			
	T5	No AFB	No AFB	Correct	10			
	T6	1+	1+	Correct	10	60/60	100%	Acceptable
Awash Health Center	T1	3+	2+	Correct	10			
	T2	scanty	scanty	Correct	10			
	T3	No AFB	No AFB	Correct	10			
	T4	3+	3+	Correct	10			
	T5	No AFB	No AFB	Correct	10			
	T6	2+	1+	Correct	10	60/60	100%	Acceptable
Hawassa referal hospital	T1	3+	2+	Correct	10			
	T2	1+	scanty	Correct	10			
	T3	No AFB	No AFB	Correct	10			
	T4	4+	3+	Correct	10			
	T5	No AFB	No AFB	Correct	10			
	T6	2+	1+	Correct	10	60/60	100.00%	Acceptable
Axum Zonal HL	T1	3+	2+	Correct	10			
	T2	1+	scanty	Correct	10			
	T3	No AFB	No AFB	Correct	10			
	T4	3+	3+	Correct	10			
	T5	No AFB	No AFB	Correct	10			
	T6	1+	1+	Correct	10	60/60	100%	Acceptable
Bahir Dar reg lab	T1	3+	2+	Correct	10			
	T2	12/100	scanty	Correct	10			
	T3	No AFB	No AFB	Correct	10			
	T4	3+	3+	Correct	10			
	T5	No AFB	No AFB	Correct	10			
	T6	1+	1+	Correct	10	60/60	100%	Acceptable
Bella Defence HL	T1	2+	2+	Correct	10			
	T2	scanty	scanty	Correct	10			
	T3	No AFB	No AFB	Correct	10			
	T4	2+	3+	Correct	10			
	T5	No AFB	No AFB	Correct	10			
	T6	1+	1+	Correct	10	60/60	100.00%	Acceptable
Black Lion HL.	T1	3+	2+	Correct	10			
	T2	1-4 AFB	scanty	Correct	10			
	T3	No AFB	No AFB	Correct	10			
	T4	3+	3+	Correct	10			
	T5	No AFB	No AFB	Correct	10	60/60	100%	Acceptable

	T6	2+	1+	Correct	10			
Chiro HL	T1	3+	2+	Correct	10			
	T2	1+	scanty	Correct	10			
	T3	No AFB	No AFB	Correct	10			
	T4	3+	3+	Correct	10			
	T5	No AFB	No AFB	Correct	10			
	T6	3+	1+	QE	5	55/60	91.60%	Acceptable
Dessie HL	T1	2+	2+	Correct	10			
	T2	1+	scanty	Correct	10			
	T3	No AFB	No AFB	Correct	10			
	T4	2+	3+	Correct	10			
	T5	scanty	No AFB	LFP	5			
	T6	1+	1+	Correct	10	55/60	91.60%	Acceptable
Dessie RL	T1	3+	2+	Correct	10			
	T2	scanty	scanty	Correct	10			
	T3	No AFB	No AFB	Correct	10			
	T4	3+	3+	Correct	10			
	T5	No AFB	No AFB	Correct	10			
	T6	1+	1+	Correct	10	60/60	100%	Acceptable
Dil Chora HL	T1	1+	2+	Correct	10			
	T2	scanty	scanty	Correct	10			
	T3	No AFB	No AFB	Correct	10			
	T4	2+	3+	Correct	10			
	T5	No AFB	No AFB	Correct	10			
	T6	scanty	1+	Correct	10	60/60	100%	Acceptable
Dubti RL	T1	3+	2+	Correct	10			
	T2	scanty	scanty	Correct	10			
	T3	No AFB	No AFB	Correct	10			
	T4	3+	3+	Correct	10			
	T5	No AFB	No AFB	Correct	10			
	T6	2+	1+	Correct	10	60/60	100%	Acceptable
Federal police	T1	3+	2+	Correct	10			
	T2	1+	scanty	Correct	10			
	T3	NO AFB	No AFB	Correct	10			
	T4	3+	3+	Correct	10			
	T5	NO AFB	No AFB	Correct	10			
	T6	2+	1+	Correct	10	60/60	100%	Acceptable
Felegehiwot ref HL	T1	2+	2+	Correct	10			
	T2	No AFB	scanty	LFN	5			
	T3	No AFB	No AFB	Correct	10			
	T4	3+	3+	Correct	10			
	T5	No AFB	No AFB	Correct	10			
	T6	1+	1+	Correct	10	55/60	91.6%	Acceptable

Fiche HL	T1	2+	2+	Correct	10			
	T2	5/100	scanty	Correct	10			
	T3	No AFB	No AFB	Correct	10			
	T4	3+	3+	Correct	10			
	T5	No AFB	No AFB	Correct	10			
	T6	2+	1+	Correct	10	60/60	100%	Acceptable
Gambela Hospital	T1	3+	2+	Correct	10			
	T2	scanty	scanty	Correct	10			
	T3	No AFB	No AFB	Correct	10			
	T4	3+	3+	Correct	10			
	T5	No AFB	No AFB	Correct	10			
	T6	1+	1+	Correct	10	60/60	100%	Acceptable
Goba HL	T1	3+	2+	Correct	10			
	T2	1+	scanty	Correct	10			
	T3	No AFB	No AFB	Correct	10			
	T4	3+	3+	Correct	10			
	T5	No AFB	No AFB	Correct	10			
	T6	2+	1+	Correct	10	60/60	100%	Acceptable
Gonder University Hosp	T1	3+	2+	Correct	10			
	T2	1+	scanty	Correct	10			
	T3	No AFB	No AFB	Correct	10			
	T4	3+	3+	Correct	10			
	T5	No AFB	No AFB	Correct	10			
	T6	1+	1+	Correct	10	60/60	100%	Acceptable
Harrar Regional lab	T1	2+	2+	Correct	10			
	T2	scanty	scanty	Correct	10			
	T3	No AFB	No AFB	Correct	10			
	T4	3+	3+	Correct	10			
	T5	No AFB	No AFB	Correct	10			
	T6	1+	1+	Correct	10	60/60	100%	Acceptable
Hawassa regeonal lab	T1	3+	2+	Correct	10			
	T2	5/100	scanty	Correct	10			
	T3	No AFB	No AFB	Correct	10			
	T4	3+	3+	Correct	10			
	T5	No AFB	No AFB	Correct	10			
	T6	2+	1+	Correct	10	60/60	100%	Acceptable
Jimma University HL	T1	3+	2+	Correct	10			
	T2	1+	scanty	Correct	10			
	T3	No AFB	No AFB	Correct	10			
	T4	no result	3+	----------	---			
	T5	no result	No AFB	----------	---	---	-----	Not acceptable

	T6	no result	1+	----------	---			
Karamara HL	T1	1+	2+	Correct	10			
	T2	scanty	scanty	Correct	10			
	T3	neg	No AFB	Correct	10			
	T4	3+	3+	Correct	10			
	T5	neg	No AFB	Correct	10			
	T6	1+	1+	Correct	10	60/60	100%	Acceptable
Lemlem Karl HL	T1	2+	2+	Correct	10			
	T2	scanty	scanty	Correct	10			
	T3	No AFB	No AFB	Correct	10			
	T4	2+	3+	Correct	10			
	T5	No AFB	No AFB	Correct	10			
	T6	1+	1+	Correct	10	60/60	100%	Acceptable
Mekele HL	T1	3+	2+	Correct	10			
	T2	1+	scanty	Correct	10			
	T3	No AFB	No AFB	Correct	10			
	T4	3+	3+	Correct	10			
	T5	No AFB	No AFB	Correct	10			
	T6	2+	1+	Correct	10	60/60	100%	Acceptable
Menelik HL	T1	4+	2+	QE	5			
	T2	3+	scanty	QE	5			
	T3	No AFB	No AFB	Correct	10			
	T4	4+	3+	Correct	10			
	T5	No AFB	No AFB	Correct	10			
	T6	2+	1+	Correct	10	50/60	83.30%	Acceptable
Metu Karl HL	T1	3+	2+	Correct	10			
	T2	1+	scanty	Correct	10			
	T3	No AFB	No AFB	Correct	10			
	T4	no result	3+	----------	---			
	T5	no result	No AFB	----------	---			
	T6	no result	1+	----------	---	---	----	Not acceptable
N. Command - Mekelle HL	T1	3+	2+	Correct	10			
	T2	scanty	scanty	Correct	10			
	T3	NO AFB	No AFB	Correct	10			
	T4	2+	3+	Correct	10			
	T5	NO AFB	No AFB	Correct	10			
	T6	1+	1+	Correct	10	60/60	100%	Acceptable
Nekemte HL	T1	2+	2+	Correct	10			
	T2	1+	scanty	Correct	10			
	T3	scanty	No AFB	LFP	5			
	T4	3+	3+	Correct	10			
	T5	NoAFB	No AFB	Correct	10			
	T6	1+	1+	Correct	10	55/60	91.60%	Acceptable

Nekemte RL	T1	2+	2+	Correct	10			
	T2	scanty	scanty	Correct	10			
	T3	scanty	No AFB	LFP	5			
	T4	3+	3+	Correct	10			
	T5	No AFB	No AFB	Correct	10			
	T6	1+	1+	Correct	10	55/60	91.60%	Acceptable
S.E Command (Harar) HL	T1	3+	2+	Correct	10			
	T2	1+	scanty	Correct	10			
	T3	No AFB	No AFB	Correct	10			
	T4	3+	3+	Correct	10			
	T5	No AFB	No AFB	Correct	10			
	T6	2+	1+	Correct	10	60/60	100%	Acceptable
Saint Paul's HL	T1	3+	2+	Correct	10			
	T2	8AFB/100	scanty	Correct	10			
	T3	No AFB	No AFB	Correct	10			
	T4	1+	3+	QE	5			
	T5	No AFB	No AFB	Correct	10			
	T6	1+	1+	Correct	10	55/60	91.60%	Acceptable
Shashemene HL	T1	2+	2+	Correct	10			
	T2	9/100	scanty	Correct	10			
	T3	No AFB	No AFB	Correct	10			
	T4	1+	3+	QE	5			
	T5	No AFB	No AFB	Correct	10			
	T6	4/100	1+	Correct	10	55/60	91.60%	Acceptable
Shire Zonal HL	T1	3+	2+	Correct	10			
	T2	1+	scanty	Correct	10			
	T3	No AFB	No AFB	Correct	10			
	T4	3+	3+	Correct	10			
	T5	No AFB	No AFB	Correct	10			
	T6	2+	1+	Correct	10	60/60	100%	Acceptable
St. Peter's HL	T1	2+	2+	Correct	10			
	T2	1+	scanty	Correct	10			
	T3	No AFB	No AFB	Correct	10			
	T4	3+	3+	Correct	10			
	T5	No AFB	No AFB	Correct	10			
	T6	scanty	1+	Correct	10	60/60	100%	Acceptable
Yekatit HL	T1	3+	2+	Correct	10			
	T2	1+	scanty	Correct	10			
	T3	No AFB	No AFB	Correct	10			
	T4	2+	3+	Correct	10			
	T5	No AFB	No AFB	Correct	10			
	T6	scanty	1+	Correct	10	60/60	100%	Acceptable

Yergalem HL	T1	3+	2+	Correct	10			
	T2	scanty	scanty	Correct	10			
	T3	neg	No AFB	Correct	10			
	T4	2+	3+	Correct	10			
	T5	neg	No AFB	Correct	10			
	T6	1+	1+	Correct	10	60/60	100%	Acceptable
Zweditu HL	T1	3+	2+	Correct	10			
	T2	1+	scanty	Correct	10			
	T3	No AFB	No AFB	Correct	10			
	T4	3+	3+	Correct	10			
	T5	No AFB	No AFB	Correct	10			
	T6	2+	1+	Correct	10	60/60	100%	Acceptable
Gordi memorial HL	T1	2+	2+	Correct	10			
	T2	1+	scanty	Correct	10			
	T3	No AFB		Correct	10			
	T4	3+	3+	Correct	10			
	T5	No AFB	No AFB	Correct	10			
	T6	2+	1+	Correct	10	60/60	100%	Acceptable
Ras Desta Damtew HL	T1	2+	2+	Correct	10			
	T2	scanty	scanty	Correct	10			
	T3	No AFB	No AFB	Correct	10			
	T4	3+	3+	Correct	10			
	T5	No AFB	No AFB	Correct	10			
	T6	1+	1+	Correct	10	60/60	100%	Acceptable
Tigray Regional lab	T1	3+	2+	Correct	10			
	T2	1+	scanty	Correct	10			
	T3	No AFB	No AFB	Correct	10			
	T4	3+	3+	Correct	10			
	T5	No AFB	No AFB	Correct	10			
	T6	2+	1+	Correct	10	60/60	100%	Acceptable

Scoring system (APHL 2002)

Set of 6 slides each slide worth 10 points, total possible score =60.
HFP and HFN scores 0
LFP, LFN and QE scores 5
Acceptable score = 80%

Appendix 6: Evaluation of the proficiency of peripheral lab technicians before and after Training

Before training

code	score	Minor errors	Major errors
1	100	3	
2	80	1	1
3	100	2	
4	100	3	
5	60	1	2
6	100		
7	80	1	1
8	80	2	1
9	100	2	
10	60		2
11	80	2	1
12	100		
13	100	2	
14	100	2	
15	100	1	
16	100	3	
17	100	2	
18	60		2
19	100	2	
20	100		
21	80		1
22	80	2	1
23	80		1
24	100	2	
25	100	1	
26	80	2	1
27	40		3
28	100		
29	100	1	

After training

Code	score	Minor errors	Major errors
1	100		
2	100	2	
3	100	1	
4	100	1	
5	100		
6	100		
7	100		
8	60	2	2
9	80	1	1
10	80		1
11	100	1	
12	100		
13	100	2	
14	100	2	
15	100	1	
16	100	1	
17	100	1	
18	100	1	
19	100	2	
20	100	3	
21	100	1	
22	100	2	
23	100	2	
24	100	1	
25	100	1	
26	100	1	
27	100		
28	100		
29	100	1	

Classification of errors (APHL, 2002)

Result of technician	**Result of controller**				
	negative	**1-9 AFB/100 f**	**1+**	**2+**	**3+**
negative	**Correct**	**LFN**	**HFN**	**HFN**	**HFN**
1-9 AFB/100 f	**LFP**	**Correct**	**Correct**	**QE**	**QE**
1+	**HFP**	**Correct**	**Correct**	**Correct**	**QE**
2+	**HFP**	**QE**	**Correct**	**Correct**	**Correct**
3+	**HFP**	**QE**	**QE**	**Correct**	**Correct**

Correct	No errors	
QE	Quantification error	Minor error
LFN	Low False Negative	Minor error
LFP	Low False Positive	Minor error
HFN	High False Negative	Major error
HFP	High False Positive	Major error