

**ADDIS ABABA UNIVERSITY
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**VALIDATION OF THE BLEACH METHODS FOR THE
DIAGNOSIS OF PULMONARY TUBERCULOSIS**

BY

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Table of contents

Table of contents.....	III
List of Tables	V
List of Figures	VI
Abbreviations	VIII
Abstract.....	IX
1. Literature review.....	1
1.1. Introduction.....	1
1.2. Sputum smear microscopy – Direct smear	4
1.3. Bleach Methods	7
1.3.1. Centrifugation	7
1.3.2. Overnight Sedimentation (Sedimentation)	9
1.3.3. Short term digestion.....	10
1.4. Bleach in TB diagnosis	10
1.5. Other diagnostic methods	11
1.5.1. Culture.....	11
1.5.2. Florescence microscopy.....	13
1.4.3. Molecular techniques	14
2. Hypothesis.....	15
3. Objectives	15
3.1. General Objective	15
3.2. Specific Objectives	15
4. Materials and Methods.....	16
4.1. Study Design and Area	16
4.2. Study Period.....	16
4.3. Study Population.....	16
4.4. Sample Size.....	16
4.5. Selection of Participants	17
4. 5.1. Inclusion Criteria	17
4. 5.2. Exclusion Criteria	17
4.6. Orientation Workshop and Training	17
4.7. Ethical Considerations	18
4.8. Specimen Collection, Transportation and Handling.....	18
4.9. Specimen Processing	21
4.9.1. Direct smear	21
4.9.2. Bleach Methods	21
4.9.2.1. Centrifugation method	21
4.9.2.2. Overnight Sedimentation	21
4.9.2.3. Short term bleach digestion	22
4.10. AFB staining and Slide reading.....	22
4.11. Culture for Mycobacteria.....	23
4.12. Data management and Statistical analysis	23
5. Quality control	24
6. Results.....	25
6.1. Socio demographic characteristics.....	25

6.2. Laboratory results	27
7. Discussion	35
8. Conclusion	42
9. Recommendation	43
10. Limitations of the study	44
References.....	45

List of Tables	Page
Table 1 – Socio demographic characteristics of the study participants.....	26
Table 2 – Clinical symptoms of pulmonary TB in relation to the different methods.....	28
Table 3 – Comparison of culture results with direct smear and bleach methods.....	29
Table 4 – Sensitivity, specificity and predictive values of direct smear and bleach methods, with scanty smear read as negative.....	30
Table 5 – Smear positivity and grade result of direct smear and bleach methods.....	30
Table 6 - Smear positivity and grade result of direct smear and bleach methods considering scanty grade as one entity.....	31
Table 7 – Comparison of scanty results of different methods with culture result.	31
Table 8 – Sensitivity, specificity and predictive values of direct smear and bleach methods, with scanty smear read as positive	32
Table 9 – Comparison of yield of positivity of bleach methods and culture against direct smear.....	33

List of Figures

Page

Figure 1- Detection rates of acid fast bacilli in sputum samples.....27

List of Appendices	Page
APPENDIX I QUESTIONNAIRE	57
APPENDIX II LABORATORY DATA Acquisition Form (Direct smear).....	59
APPENDIX III LABORATORY DATA Acquisition Form (Short term).....	60
APPENDIX IV LABORATORY DATA Acquisition Form (Overnight sedimentation).	61
APPENDIX V LABORATORY DATA Acquisition Form (Centrifugation).....	62
APPENDIX VI LABORATORY DATA Acquisition Form (Culture).....	63
APPENDIX VII LABORATORY PROCEDURES.....	64
APPENDIX VIII CONSENT FORM (English version).....	68
APPENDIX IX CONSENT FORM (Amharic version).....	69
APPENDIX XI INFORMATION SHEET (Amharic Version).....	70
APPENDIX XII Declaration	73

Abbreviations

AFB	Acid-fast bacilli
AFS	Acid fast stain
AIDS	Acquired immunodeficiency syndrome
AHRI	Armauer Hansen Research Institute
ALERT	All Africa Leprosy TB, Rehabilitation, Research and Training Center
DMIP	Department of Microbiology, Immunology and Parasitology
DOTS	Directly Observed Treatment, Short course
EDHS	Ethiopian demography and health survey
FRPC	Faculty Research Publications Committee
HIV	Human immunodeficiency virus
IUATLD	International Union Against Tuberculosis and Lung Disease
MGIT	Mycobacteria growth indicator tube
MOHE	Ministry of Health of Ethiopia
NAA	Nucleic acid amplification
NaOCl	Sodium hypochlorite
NaOH	Sodium hydroxide
NTPs	National Tuberculosis Control Programs
PBS	Phosphate buffered saline
PLC	Private limited company
RCF	Relative centrifugal force
RHB	Regional Health Bureau
PTB	Pulmonary tuberculosis
SNNPRG	Southern Nations, Nationalities and Peoples Regional Government
SOP	Standard operating procedure
TB	Tuberculosis
VCT	Voluntary counselling and testing
WHO	World Health Organization
Yrs	years
ZN	Ziehl-Neelsen

Abstract

Bacteriological diagnosis of tuberculosis is largely dependent on direct microscopy of sputum smears. It is the most widely available diagnostic test, especially in developing countries where the other options are usually not practical due to the limited resources available. However, its sensitivity is not optimal when used in control programs, usually ranging from 8.8% to 46.4% in most African laboratories. The sensitivity of the technique is further undermined in areas with high HIV prevalence. If sensitivity could be improved, the method has the potential to become an even more valuable tool for National TB Control Programs. Treating the sputum with house bleach (NaOCl) has been reported to increase sensitivity of direct microscopy. Therefore, the aim of this study was to compare the diagnostic performance of the three different bleach methods reported so far: centrifugation, overnight sedimentation and short term bleach digestion (30 min), with conventional direct microscopy and validate against culture as a gold standard on a given group of specimens. This cross sectional study was conducted at Awassa Health Center and Bushulo Major Health Center in Awassa. Five hundred (500) consecutive new TB suspects presenting at the health facilities during July to December, 2006 were enrolled in the study. Direct smears were prepared and stained using Ziehl-Neelsen technique and specimens were decontaminated and inoculated onto Lowenstein-Jensen media for culture. The left over sputum samples were pooled, equal amount of bleach was added and then divided in three portions for the three bleach treatment techniques. Smears were prepared after centrifugation, overnight sedimentation and short term digestion. The direct and bleach treated smears were read blindly by two independent microscopists. Ten percent of the negative and 20% of all the positive smears were retained for quality control. Smears were graded using the WHO/IUATLD scale. Considering smears graded scanty (<10 Acid-fast bacilli per 100 fields) as negative, the direct smear was positive in 21%, short term digestion in 20%, overnight sedimentation in 23%, centrifugation in 31% and culture in 47%. The sensitivity of direct smear microscopy under the experimental condition was 41.9%. Among the bleach techniques, sensitivity was best for centrifugation (54.6%) followed by overnight sedimentation (43.7%) and short term digestion (38.9%). The bleach centrifugation method can clearly

increase the sensitivity of smear microscopy and would thus be a useful tool to improve case detection in the National Tuberculosis Programme.

Key words: tuberculosis, diagnosis, bleach methods, validation

1. Literature review

1.1. Introduction

Tuberculosis (TB) persists as a major cause of human mortality and morbidity, affecting almost a third of the world's population (Laurardo and Ashkin, 2000). Globally, TB causes more adult deaths than any other single curable infectious disease currently (Lawson *et al.*, 2006). There is clear evidence that the worldwide incidence of tuberculosis is increasing. It is estimated that between 2000 and 2020, nearly 1 billion people will be newly infected with tuberculosis, 200 million people will develop the disease, and 35 million will die from tuberculosis (WHO, 2001). Approximately 95% of cases and 98% of deaths occur in the developing world (Lawson *et al.*, 2006). TB remains a major public health problem worldwide (Ergete *et al.*, 2000). Inadequate case-detection and cure rates have been identified as reasons for a mounting global tuberculosis burden (Maher *et al.*, 1997).

TB has remained a common health problem in most developing countries even before the spread of HIV. Poverty, overcrowding, poor housing, natural and man made disasters were the main reasons for the sustained transmission of TB before the 1980's (Parry and Davis, 1996). However, the recent exponential increase in the number of TB cases in most sub-Saharan African countries is primarily due to the fast spread of HIV infection (Mancino *et al.*, 1997). In persons co-infected with the tubercle bacilli and HIV, the overall annual risk of developing active TB rises about 0.4% to 8% - that is, 20 times the risk for TB infection without HIV (Zumla *et al.*, 2000). The overall TB-HIV co-infection rate in sub-Saharan Africa was estimated 32% in 1997 (Dye *et al.*, 1999). There are few reports on TB-HIV co-infection in Ethiopia. The average prevalence of HIV in adult TB patients (15-49 yrs) is estimated to be 21% by WHO (Aderaye, 2007). A study conducted by Yassin *et al.* (2004), in five district hospitals of SNNPR, the prevalence of TB-HIV co-infection was 19%. In similar study by the same group in year 2004/5, but in 10 selected sentinel sites in SNNPR, the prevalence was 17.9% (unpublished data). From one year data obtained from Providers initiated HIV counseling and testing service at Awassa Health Center in year 2006/7, the prevalence of TB-HIV co-infection was 29.1%

(Awassa Health Center data). According to recent EDHS and the National Antenatal Care Surveillance System of Ethiopia (2006), HIV prevalence of the country was 1.4 % and 3.5% respectively and prevalence in the SNNPR was 0.2% and 2.3% respectively. From data obtained from VCT services at Awassa and Bushulo Health Centers in year 2006, the prevalence of HIV was 18.2% and 29.1% respectively (Awassa and Bushulo Health Centers data). The HIV co-infection alters not only the epidemiology of TB, but also the clinical presentation, laboratory findings, response to treatment and overall prognosis of the patients and ultimately the performance of TB control programme (Elliott *et al.*, 1993; Leung *et al.*, 1996; Karstaedt *et al.*, 1998; Smith *et al.*, 1994).

Bacteriological diagnosis of TB worldwide is largely dependent on direct microscopy of sputum smears after ZN staining (Angeby *et al.*, 2004). It is the most widely available diagnostic test especially in developing countries where the other options are usually not practical due to the limited resource available (Yassin, 2005). The technique needs no special equipment (Croflon *et al.*, 1999).

The Directly Observed Treatment, Short course (DOTS), the WHO's global strategy for controlling of TB epidemic also relies upon direct smear microscopy. This strategy recommends identification of tuberculosis cases by microscopic examination of sputum smears to identify acid-fast bacilli (Enarson *et al.*, 2000; Getahun *et al.*, 2007). The method is rapid, specific, and reasonably easy to perform; however, its sensitivity is not optimal when used in control programs, usually less than 60 % in most African laboratories (Yassin, 2005).

With the occurrence of multi-drug resistant TB, the risk of laboratory infection has become a major concern. Few laboratories in developing countries have adequate safety cabinets (Gebre *et al.*, 1995). Therefore, safety is of special concern in the handling of potentially infectious clinical specimens obtained from TB suspects. TB is listed as sixth among the most common laboratory-acquired infections (Pike, 1979). Tests that do not require bio-safety containment are most desirable (Foulds and O'brien, 1998).

The identification of sputum smear-positive patient is crucial for the control of tuberculosis, as these are the most contagious patients, particularly to their close contacts (Grzybowski *et al.*, 1975; Enarson and Rouillon, 1994). It is estimated that one undiagnosed smear positive patient infects between 10-20 contacts over a one year period if untreated (Styblo, 1991).

Sputum smear microscopy has limited value for the diagnosis of tuberculosis in children and does not, by definition, identify smear-negative tuberculosis (Steingart *et al.*, 2006). Although smear negative tuberculosis has a lower transmission potential, it contributes importantly to global tuberculosis and mortality (Perkins *et al.*, 2006).

The sensitivity of the smear microscopy is further undermined in areas with high HIV prevalence (Bruchfeld *et al.*, 2000; Lawson *et al.*, 2006) owing to the paucibacillary nature of pulmonary tuberculosis in patients with HIV infection (Siddiqi *et al.*, 2003) with reduced pulmonary cavity formation and low bacillary load (Hargreaves *et al.*, 2001). Therefore, HIV co-infected TB patients tend to produce fewer bacilli and smear examinations usually yield scanty bacilli or negative (Yassin, 2005). If sensitivity could be improved, the method has the potential to become an even more valuable tool for National TB Control Programs (NTPs) (Angeby *et al.*, 2004). Therefore, improvement of the diagnostic procedures for sputum microscopy for TB is urgently needed.

Acknowledging the poor performance of direct microscopy (Yassin, 2005), various groups have attempted to modify the smear diagnostic process to make it more sensitive. Methods aiming to concentrate the TB bacilli in sputum smears by digestion of sputum with household bleach and subsequent concentration of sputum (by centrifugation or overnight sedimentation) have received much attention (Gebre *et al.*, 1995; Habeenzu *et al.*, 1998; Van Deun *et al.*, 2000).

In addition to centrifugation and overnight sedimentation, short term digestion (30 min) was introduced as another bleach method (Yassin *et al.*, 2003). It works simply by digestion of sputum without centrifugation or sedimentation. The method was reported to

improve the quality and yield of smear microscopy. It was also reported that a single bleach digested sputum smears detected as many smear positive TB cases as are currently detected by three consecutive standard sputum smears with and without HIV infection (Lawson *et al.*, 2005; 2007; Douthwaite *et al.*, 2006). However, to our knowledge, the short term digestion has not been validated against culture. Therefore, it is critical to confirm these results against culture, which is the gold standard for diagnosis of TB. In addition, the previous studies had not compared the diagnostic performance of the three different bleach methods (centrifugation, overnight sedimentation (16 hours), short-term bleach digestion (30 minutes) with direct microscopy and culture on a given group of samples or patients. In order to address these gaps, the current study aimed at comparing the three bleach-processing methods with direct microscopy and with the gold standard, culture, so as to provide comprehensive information on which to base future recommendations.

1.2. Sputum smear microscopy – Direct smear

Mycobacteria are characterized by “acid-fastness” - once stained by basic dyes they can not be decolorized with acid alcohol ie, 95% ethyle alcohol containing 3% hydrochloric acid (acid-alcohol) quickly decolorizes all bacteria except the mycobacteria. This staining characteristic of MTB is due to the mycolic acid (waxy envelope) which resists decolorization by acid-alcohol. That is why the Ziehl-Neelsen technique is employed for MTB (Brooks *et al.*, 2001).

The sputum smear microscopy which uses the Ziehl- Neelsen staining technique is one of the earliest methods devised for detecting the tubercle bacillus and it remains a standard procedure (Division of Becton Dickinson and Company, 1998). It was developed in the 1880s and has remained essentially unchanged since then (Perkins *et al.*, 2006). The method is economical in both time and expense and is recommended for laboratories handling large numbers of sputum specimens (Githui *et al.*, 1993).

Sputum smear microscopy is well known for its widely variable sensitivity (Urbanek, 1985), ranging from 8.8% to 46.4% of culture verified cases when used in control programs (Aber *et al.*, 1980). In Honduras in 1997, the majority of TB patients were diagnosed on clinical grounds; only 44% of the reported cases of pulmonary TB were bacteriologically confirmed by acid-fast staining (Angeby *et al.*, 2000). In a study conducted in Ethiopia (Gebre *et al.*, 1995), a sensitivity of 30.8% was reported. A sensitivity of 54.2% was reported by Bruchfeld *et al.* (2000) in their work also in Ethiopia.

Several grading scales have been introduced in sputum microscopy, but the most popular is the scale recommended by WHO and IUATLD where smears are graded into 5 categories based on the number of acid-fast bacilli (AFB) seen under the microscope as negative (0 AFB/100 fields), scanty (<10 AFB/100 fields), “+” (10 to 99 AFB/100 fields), “+ +” (1 to 10 AFB/ field) or “+ + +” (>10 AFB/field) (Crofton *et al.*, 1999). Some National Tuberculosis Control Programmes revised these grading to suit the situation in their respective countries. For example in Ethiopia, smears are graded as negative, scanty (1 or 2 AFB/100 fields) or positive (≥ 3 AFB/100 fields) to reduce the time spent in counting bacilli for grading as the technicians are usually overburdened by performing other tests (MOHE, 2002) and further grading would not lead to change in the management of patients (Van Deun *et al.*, 2004). The other widely applied quantification scale is the American Thoracic Society (AST) scale with a cut-off for positivity of only 1 AFB/100 fields (American Thoracic Society, Center for Disease Control and Prevention, 2000). However, the IUATLD/WHO scale is used mainly in low-income, high-prevalence countries, and the AST scale is largely used in industrialized countries (Van Deun *et al.*, 2004)

The results of smear microscopy can be influenced by the type of specimens, thickness of the smear, extent of decolorization, type of counter stain used, and training and experience of the person examining the smear (ICMR Bulletin, 2002)

Direct smear microscopy detects those cases that are epidemiologically more important i.e., those that are most likely to transmit the infection to their close contacts. It is also highly specific, suitable for application in developing areas, relatively inexpensive and can be accomplished under field conditions (Foulds and O'brien, 1998). However, it is inherently insensitive since large numbers of organisms have to be present in the sputum to be detectable by direct microscopy (Aber *et al.*, 1980). Between 5,000 and 10,000 tubercle bacilli per milliliter of sputum are required for direct microscopy to be positive (WHO, 1998; Cheesborough, 2000; Wilkinson and Sturn, 1997) whereas culture requires only 10-100 viable bacilli (Siddiqi *et al.*, 2003). In addition to this, in direct smear only tiny amounts of material are examined—as little as 0.2 μ L even when viewing more than 100 microscopic fields (Perkins *et al.*, 2006)

The technique misses a substantial number of culture positive cases who are potentially infectious to others as smear-negative culture-positive disease accounts for 15-20 % of *M. tuberculosis* transmission (Wilkinson and Sturn, 1997). However, it is currently the only microbiological method for diagnosis and confirmation of pulmonary TB in developing countries where other methods are impractical due to the limited resources available. It is by far the fastest and cheapest method for the detection of AFB in sputum and it is highly specific (Yassin, 2005; Habeenzu *et al.*, 1998).

The low sensitivity of the method is a challenge when it is used in overburdened TB laboratories (Habeenzu *et al.*, 1998). In the study conducted in Kenya (Hawker *et al.*, 2001), of those patients with smear-negative presumed pulmonary TB by the local programme definition, 26% were smear-positive when re-examined carefully with two repeat sputum smears. The study suggested that, the high rates of smear-negative TB seen might in part be due to under-reading. This is probably because of the overwhelming burden of TB leading to over rapid and inaccurate sputum examination. The under diagnosis problem related to burden of work load which is observed in this finding is also a common problem in other developing countries' TB control programmes (Yassin, 2005).

In recent years, interest in improving the direct smear microscopy technique in developing countries has risen (Farina *et al.*, 2002). Several approaches are being made to enhance the sensitivity of smear microscopy (ICMR Bulletin, 2002).

1.3. Bleach Methods

1.3.1. Centrifugation

Among the different techniques that have been tried to increase the yield of smear microscopy, the oldest is the NaOCl sputum concentration technique described by Ellerman and Erlandsen in 1908. The technique was re-evaluated and compared with various other sputum digestants including Tegritol (sodium octyl sulphate) and Clorax (commercial alkaline sodium hydrochlorite solution) (Cited in Gopi *et al.*, 2004). These findings were not widely known nor widely used for years. However, after almost half a century many researchers re-visited the bleach techniques in the last decade and suggested that the performance of sputum smear microscopy can be significantly improved if sputum is liquefied with household bleach and then concentrated by centrifugation or overnight sedimentation (Aber *et al.*, 1980). In addition to liquefaction of sputum by NaOCl, solutions including sodium hydroxide, ammonium sulfate and dithiothreitol were shown by other investigations to assist the concentration of mycobacteria, improving microscopic diagnosis (Garay, 2000; Gebre *et al.*, 1995; Harries *et al.*, 1998b).

In an attempt to improve the sensitivity of direct microscopy by bleach methods, widely diverging experience (variable results) have been reported. They range from more than double positivity rates using centrifugation in Ethiopia (Gebre *et al.*, 1995; Miorner *et al.*, 1996) over still excellent results in Malawi (Allwood *et al.*, 1997) and Zambia (Habeenzu *et al.*, 1998) to no gain at all in South Africa (Wilkinson and Sturn, 1997). For instance, studies from Ethiopia, Zambia and Honduras documented a statistically significant improvement of the sensitivity with bleach concentration method ranging from 16-125% compared to the direct smears, with same specificity when culture was used as gold

standard (Habeenzu *et al.*, 1998; Gebre *et al.*, 1995; Coper and Nelsen, 1949; Bruchfeld *et al.*, 2000). The variation in the yield could partly be explained by the difference in the relative centrifugal force (RCF) used by the different studies and the duration of centrifugation. The optimum RCF and concentration time combination was suggested to be 3,000g to 3,800g for 15 min (Rickman and Moyer, 1980; Gebre *et al.*, 1995; Bhat, 1999).

Gebre *et al.* on their work in Ethiopia (1995) reported that the sensitivity of sputum microscopy can be significantly augmented after liquefaction of sputum with sodium hypochlorite (NaOCl), commonly known as household bleach, followed by centrifugation. In this work, NaOCl treatment and centrifugation increased the number of positive samples by 108-125% (average 114%) when it is compared with direct microscopy. The sensitivity of smears for AFB compared with culture was 30.8% when smears were prepared directly from sputum and 69.2% when smears were prepared after NaOCl treatment and centrifugation.

In the study in Zambia by Habeenzu *et al.* (1998), it was shown that the use of NaOCl increased the sensitivity from 43.4% to 76.3% with a specificity of 100 for both methods. The result of this study also showed that digestion of sputum with NaOCl and concentration of the bacilli by centrifugation significantly improved laboratory safety. The method is simple and the only extra reagent required, NaOCl, is readily available as household bleach throughout the sub-Saharan Africa and probably in other parts of the developing world. The cost is also negligible.

In study conducted in Black Lion University Hospital in Addis Ababa, Ethiopia (Bruchfeld *et al.*, 2000), it was reported that the overall increase in sensitivity using NaOCl and its subsequent concentration by centrifugation was from 54.2% to 63.1% and in HIV positive patients the sensitivity increased from 38.5% to 50.0%.

However, Wilkinson and Sturn (1997) found that the sensitivity of direct smear microscopy was not improved by sputum liquefaction and centrifugation; even some

smears which were initially positive by direct microscopy become negative after concentration. Similarly, Harries *et al.* (1998b) reported equally disappointing results.

1.3.2. Overnight Sedimentation (Sedimentation)

Where there is no centrifuge or there is no mains electricity, overnight sedimentation might be an alternative to achieve the concentration of the bacteria (Miorner *et al.*, 1996). A study in Ethiopia by Abdurrahman *et al.* (2000) has shown that overnight sedimentation of NaOCl treated sputum can also increase the yield of smear positivity. In the study, 22% of patients were AFB positive by direct smear examination, 37% following overnight sedimentation of NaOCl treated sputum and 41% when the treated sputum was centrifuged.

In another study conducted in Ethiopia (Miorner *et al.*, 1996), liquefaction of sputum with sodium hypochlorite followed by concentration of bacilli through overnight sedimentation significantly increased the sensitivity of the direct microscopy. However, a statistical comparison of results obtained after centrifugation with those obtained after sedimentation showed no significant difference between the two concentration methods. Similarly Van Deun *et al.* (2000) showed that although overnight sedimentation is more time consuming, it gives the same result in terms of increased sensitivity as that of centrifugation.

Overnight sedimentation method adds substantially to the sensitivity of direct microscopy, with out much extra input. Although it slightly increases the time of the investigation, the procedure is simple and can be applied easily in district laboratories with the basic equipment and staffing (Van Deun *et al.*, 2000). It has also been reported that the sensitivity of one NaOCl treated sample is higher than that of three consecutive direct smears. This is important, as there are often problems in receiving additional sputum samples for smear preparation. For example, in 1997 in Honduras 80% of the recommended second samples were not received (Angeby, *et al.*, 2000).

1.3.3. Short term digestion

Short term digestion was introduced recently as another bleach method (Yassin *et al.*, 2003). The method works by adding equal volume of bleach to a sputum sample and incubating it for 30 min. on top of the bench without centrifugation or sedimentation. It was reported to improve the quality and yield of smear microscopy by facilitating a clearer smear and it requires only a one day visit (Lawson *et al.*, 2007). A study conducted in Ethiopia by Yassin *et al.* (2003) showed that short-term digestion of sputum samples for 30 min improved the quality and yield of smear microscopy. Moreover, the study showed that a single bleach digested smear is as sensitive as three direct sputum smears for the diagnosis of new cases of PTB. Using the same method, Lawson *et al.* (2005; 2007) and Douthwaite *et al.* (2006) reported similar findings with that of Yassin *et al.* (2003) showing improvement of yield of direct microscopy in patients with and without HIV infection. These studies confirmed the earlier work in Ethiopia by Yassin *et al.* (2003) regarding the method and showed that the method improved the quality and yield of smear microscopy. However, the method has the diluting effect of the bacilli resulting in high scanty grade than the standard smear microscopy (Douthwaite *et al.*, 2006).

1.4. Bleach in TB diagnosis

It has been stated that bleach has great role in the diagnosis of TB. As a potent disinfectant, NaOCl kills mycobacteria. This will increase bio-safety in laboratories by minimizing the risk of laboratory infection, a risk that cannot be neglected, especially in laboratories with inadequate safety standards (Angeby *et al.*, 2000). It may even be more important in settings with a high incidence of HIV, where the probability of acquiring TB infection is high and thus be more susceptible to TB. It must be recognized, however, that if safety cabinets are not available, the bleach should be mixed with the sample in the container in which it has been deposited. To avoid aerosol formation one should not pour potentially infected sputum into a tube outside a safety cabinet (Angeby *et al.*, 2004). The antimicrobial activity of sodium hypochlorite is related to bacterial essential enzymatic

sites promoting irreversible inactivation by hydroxyl ions and the chloramination reaction. The chloramination reaction between chlorine and the amino group (NH) forms chloramines that interfere in cell metabolism. Chlorine (strong oxidant) presents antimicrobial action inhibiting bacterial enzymes leading to an irreversible oxidation of SH groups (sulphydryl group) of essential bacterial enzymes (Estrel *et al.*, 2002). Another aspect in favor of the bleach technique is that the bleach digests the sputum so that there will not be debris on the microscopy slide. This makes the reading simpler, and hopefully less time consuming than the recommended 5 min for reading of the direct smear (WHO, 1998). This partly contributes to the increased sensitivity of the NaOCl method (Farina *et al.*, 2002).

On the other hand, the bleach method has some built-in disadvantages. First, a bleach-treated sample cannot be used for mycobacterial culture, as the NaOCl kills *M. tuberculosis*. In case mycobacterial culture is asked for, another sample must be requested (Angeby *et al.*, 2004). As it was reported by Yassin *et al.* (2003), the smears prepared from digested sputum were thin and it was difficult to inspect the smears through naked eye. Therefore, extra care was required in labeling and staining the correct side of the slide.

1.5. Other diagnostic methods

1.5.1. Culture

Sputum culture is the gold standard for the diagnosis of tuberculosis and recommended for that purpose in all developed countries (Colebunders and Bastian, 2000). In resource poor settings, culture is recommended selectively and is mainly used for surveillance of drug sensitivity, to confirm treatment failure and relapse, and in pulmonary tuberculosis patients with repeated negative smear results (WHO, 2003; WHO, 1998; MOHE, 2005). Mycobacteria are slow-growing organisms and culture takes 3-8 weeks to get the results and needs reasonably sophisticated facilities and technical experience (WHO, 1998).

Sputum culture of HIV-infected patients needed more incubation time than that of patients without HIV infection (Johnson *et al.*, 1998), which is consistent with the low bacillary load seen in the sputum of HIV-infected patients (Brindle *et al.*, 1993). The conventional TB culture uses the solid egg-based Lowenstein-Jensen (LJ) media and agar-based Middlebrook 7H10 and 7H11 (Bruchfeld *et al.*, 2000; WHO, 2004). It is five to ten times more costly per sample than smear microscopy (WHO, 2004).

The recently introduced liquid based culture media like BACTEC MGIT 960 and BACTEC 460TB could yield more sensitive results in a few days (Graham *et al.*, 2002) and usually can shorten the recovery time of the mycobacteria by 15 days compared with the standard LJ medium (Hanna *et al.*, 1995).

The BACTEC mycobacteria growth indicator tube (MGIT) 960 culture system uses liquid culture media and fluorescence technology. The culture vials (MGIT tubes) contain silicone impregnated with an oxygen-sensitive fluorescent indicator. The large amount of oxygen initially present in the broth quenches fluorescence, but with rapid growth of mycobacteria, the oxygen becomes consumed and the indicator fluoresces brightly under 365-nm UV light. The level of fluorescence corresponds to the amount of oxygen consumed by the organisms in the inoculated specimens, and this, in turn, is proportional to the number of bacteria present. It uses Middlebrook 7H 9 broth medium (Bruce *et al.*, 1999) and the mean time for detection of growth of mycobacteria in MGIT was short and ranged from 8 days to 16 days. Moreover, the same infrastructure and technical expertise are needed as for the conventional culture method, and the MGIT is costly to install, which restricts its use, especially in peripheral facilities of resource-constrained settings (Getahun *et al.*, 2007).

The BACTEC 460-TB culture system is a radiometric method which is used to detect the growth of *M. tuberculosis* in 7H12 broth medium, which contains ¹⁴C-labeled substrates (fatty acids) as a single source of carbon. Positive growth leads to the consumption of this substrate with subsequent release of ¹⁴CO₂ into the atmosphere above the medium in the sealed vial. The BACTEC instrument detects the amount of ¹⁴CO₂ and records it as the

growth index. It improves the recovery of and decreases the time required to detect mycobacteria; however, this procedure is still labor-intensive and requires attention to special safety and regulatory issues regarding radioisotopes (Mendoza *et al.*, 1993).

Even though these liquid based culture systems (BACTEC MGIT 960 and BACTEC 460TB) are advantageous in rapid and easy detection of the mycobacterium, they are expensive and could not be available for routine purposes. There is also a relatively high rate of contamination in these liquid based culture systems than the conventional LJ culture. Usually the contamination rate for the Lowenstein-Jensen (LJ) culture was reported to be 2%-5% (Apers *et al.*, 2003) where as a higher rate of contamination (4 - 15%) was reported for these liquid based culture system (Aderaye, 2007).

1.5.2. Florescence microscopy

Fluorescence microscopy was introduced in the 1930s (Hagemann, 1938) and uses an acid-fast chlorochrome dye (eg, auramine O or auramine-rhodamine) with an intense light source such as a halogen or high-pressure mercury vapour lamp (Foulds and O'Brien, 1998). It is advantageous for those laboratories that handle large number of specimens (Steingart *et al.*, 2006). Fluorescence microscopy has been created with increased sensitivity (Kivihya-Ndugga *et al.*, 2003; Bell and Brown, 1962; Singh and Parija, 1998; Kubica, 1980; Githui *et al.*, 1993) usually 10% more sensitive than conventional microscopy, but there is concern that specificity may be lower (Steingart *et al.*, 2006). A potential shortcoming of fluorescence microscopy is the possibility of false-positive results because inorganic objects may incorporate fluorochrome dyes (Boyd and Marr, 1975; Richards *et al.*, 1941). Besides, the high cost needed for the fluorescence microscope limits its wider use in developing countries.

1.4.3. Molecular techniques

Newer molecular techniques such as DNA amplifications are rapid, but more of experimental use. Using direct detection of *Mycobacterium tuberculosis* by nucleic acid amplification (NAA) from respiratory samples, tuberculosis can be confirmed in less than 24 hours as opposed to 6 to 8 weeks. The sensitivity of NAA detection of MTB in smear-positive respiratory specimens is 96.9%, and the specificity is 100%, whereas the sensitivity and specificity in smear-negative specimens is 72.0% and 99.3%, respectively. It should be noted that NAA of MTB does not replace the need for routine mycobacterial culture and susceptibility testing. In addition to the direct detection of MTB, techniques such as probe-based technology and sequence analysis can be applied to cultured isolates to decrease the time to identification over routine biochemical analysis (Miller, 2007). Most of these techniques are expensive and are not feasible in areas where the majority of TB patients live.

Other rapid tests like antigen-specific assays that measure interferon gamma released from T cells through ELISA (QuantiFERON-TB) and enzyme-linked immunospot (ELISPOT) have been developed (Pai, *et al.*, 2004). Although ELISPOT was used to detect active tuberculosis disease in HIV infected adults (Chapman, *et al.*, 2002) and children, (Liebeschuetz *et al.*, 2004) these tests are generally known for their inability to distinguish between active disease and latent infection (Dheda *et al.*, 2005). Moreover, these tests need advanced and sophisticated infrastructure, so they are almost exclusively used in more developed countries. Even in such countries some of the methods have little use (Foulds and O'Brien, 1998). Serological techniques are not useful in control programmes due to lack of sensitivity and specificity (Daniel, 1989).

2. Hypothesis

Treatment of sputum with bleach prior to ZN staining will increase the sensitivity of direct smear microscopy by at least 30%, using culture as gold standard.

3. Objectives

3.1. General Objective

To compare the diagnostic performance of the three bleach methods:-centrifugation, overnight sedimentation and short-term digestion with direct microscopy and with the gold standard, culture.

3.2. Specific Objectives

- To determine the sensitivity, specificity, and predictive value of direct microscopy and bleach processed sputum smear microscopy against culture as the gold standard.
- To compare the yields of the routinely used direct microscopic with that of the bleach methods.

4. Materials and Methods

4.1. Study Design and Area

This was a cross sectional study conducted at Bushulo Major Health Center and Awassa Health Center (SNNPR state) in collaboration with AHRI. Bushulo Major Health Center is a missionary Health Center which is located in the outskirts of Awassa, the capital of SNNPR and Awassa Health Center is located at the center of the town which is 275 km south of Addis Ababa.

4.2. Study Period

The study was conducted during the period of June to December, 2006.

4.3. Study Population

Consecutive pulmonary TB patients who were sent to the laboratory for microscopic confirmation on sputum samples at Awassa Health Center and Bushulo Major Health Center during the study period between June 30 and December 6, 2006 were eligible for the study and were requested to provide informed consent.

4.4. Sample Size

The following assumption was used to calculate the sample size: the sensitivity of direct smear is about 40% and we hypothesize that bleach treatment will increase sensitivity by 30% ($40\% \times 30\% = 12\%$), that means to increase to 52% ($40\% + 12\%$) using bleach. Sample size of 380 for each group (direct and bleach) would be enough to detect the difference in the sensitivity among the two groups (40% and 52%) with 95% confidence and 90% power (and since the sample was divided and analysis was made from sputum

of the same patient, there was no need to double the 378), (using EPI6 EXE software, CDC, Atlanta, US). Therefore, the minimum sample size calculated is 378 and we maximized it to 500 to increase the precision of estimates.

4.5. Selection of Participants

4.5.1. Inclusion Criteria

The study included all new pulmonary TB suspects who were sent to the laboratory for microscopic confirmation on sputum samples and who could give written informed consent and child assent.

4.5.2. Exclusion Criteria

The following study groups were excluded from the study:

- those pulmonary TB suspects who have previously been treated for TB
- all patients currently on anti-TB treatment
- patients unable to produce sputum were excluded

4.6. Orientation Workshop and Training

A one day orientation workshop on the aim and objective of the study was carried out for the study team, health professionals and health officials. This created awareness on the study project among the study team and health professionals and helped in facilitating the work. This was followed by a three day training on the standard operating procedure of the study protocol for the study team members at both health centers and AHRI. This was an important step to make sure that all protocol details and methods were understood well and followed strictly.

4.7. Ethical Considerations

The routine AFB diagnostic service was not disrupted by this study. All bleach and culture results were reported to the physicians. All patients were managed according to the recommendations of the NTP. The patient's written consent and child's assent were obtained. This MSc research project was approved by the Department of Microbiology, Immunology and Parasitology (DMIP), ethically cleared by the Faculty Research Publications Committee (FRPC) and endorsed by the Faculty Academic commission. Approval/Permission was also obtained from AHRI/ALERT Ethics Review Committee and the Regional ethical review committee, of SNNPR. Full support and approval was obtained from Bushulo Major Health Center and Awassa Health Center prior to the reviews by the University, Regional and AHRI/ALERT ethics committees.

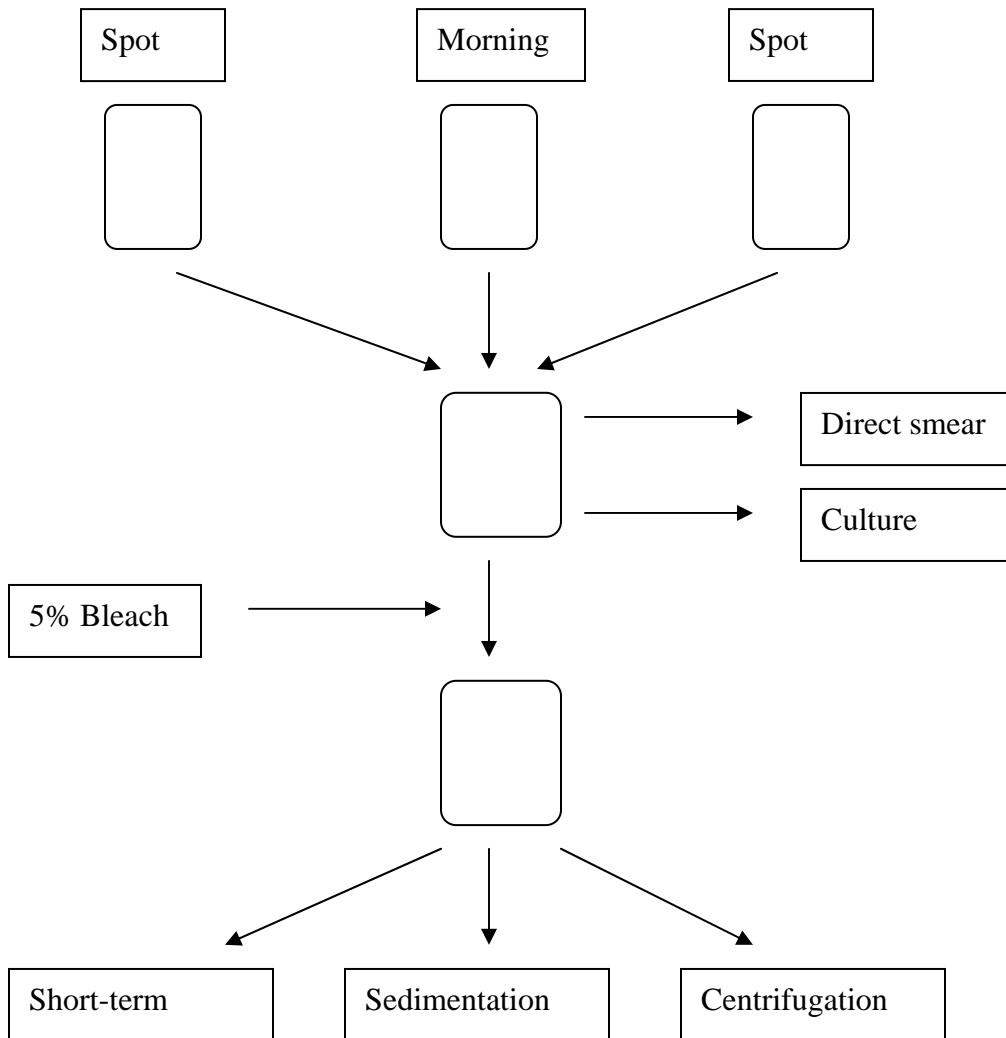
4.8. Specimen Collection, Transportation and Handling

All suspects were instructed as to how produce adequate sputum, not saliva. They were given labeled sputum cups for collection at the time points recommended by the national TB control programme (MOHE, 2002). The first on-the-spot sample was collected when the patient arrives at the laboratory. Two cups were provided to each study participant to bring the next day early morning and second on-the-spot specimens. The routine diagnostic smear was prepared from these samples according to the standard practice. The leftover sputum sample was retained for the study.

The leftover of the sputum samples from the spot, morning and second spot collections were pooled into one cup. From the pooled sputum, direct smear was prepared. About 1-2 ml sputum was taken for culture into a sterile screw-capped tube and stored at 4⁰C until transported to AHRI for culture (Specimen for culture was taken from the pool before adding bleach since bleach kills the bacilli). The sputum sample for culture was kept in a sterile tube and stored for less than 10 days until further processed. Re-distributing the

sputum into different tubes to be used for different tests was a difficult task due to the mucoid nature of the sputum; therefore, equal volume of household bleach (5% NaOCl - Ghion Industrial-Chemical Sector PLC, Addis Ababa, Ethiopia) was added to the pooled sample in order to liquefy the sputum. The bleach treated pooled sample was re-distributed (allocated) into three cups and was used for the corresponding three tests: centrifugation, overnight sedimentation and short-term digestion in order that the source material was the same for all the three tests.

Flow chart of specimen processing



NB. Specimen for Direct smear and Culture was taken before adding bleach to the pool.

4.9. Specimen Processing

4.9.1. Direct smear

Each sputum smear was prepared on new frosted end glass slides using a wooden applicator, air-dried, heat fixed and was stained using the standard ZN hot method (MOHE, 2002; Cheeborough, 2000).

4.9.2. Bleach Methods

4.9.2.1. Centrifugation method

The equally mixed solution (sputum and bleach) which was allocated in blue cups for centrifugation technique was then kept for 10-15 min at room temperature and was shaken at regular intervals. Then, equal volume of distilled water was added, and the sample was centrifuged at 3000 *g* for 15 min. The supernatant was discarded and the pellet was suspended in a few drops of the remaining fluid. Smears were prepared from the suspended sediment (Gebre *et al.*, 1995).

4.9.2.2. Overnight Sedimentation

The equally mixed solution (sputum and bleach) which was allocated in blue cups for sedimentation technique was then kept for 10-15 min at room temperature and was shaken at regular intervals. Then equal volume of distilled water was added to the top and mixed. The tubes were left to stand for sedimentation overnight, and next morning a small, thick smear ('bleach smear') was made from the sediment after decanting (Miorner *et al.*, 1996).

4.9.2.3. Short term bleach digestion

The equally mixed solution (sputum and bleach) which was allocated in blue cups for short term digestion was shaken by hand for 20 seconds and then left on Table-top for 30 minutes. A drop of the liquefied specimen was taken from the bottom of the conical test tube using pasture pipette and put on a new frosted end slide which was left to air dry (Yassin *et al.*, 2003).

4.10. AFB staining and Slide reading

After the sputum samples were processed and smears were prepared from each on labeled, new, frosted end slides, they were all stained using the standard ZN hot method (MOHE, 2002; Cheesborough, 2000). The same lot of staining solutions was used throughout. Quality control of the staining solutions was made by testing the lot with known positive and negative sputum smears. The slides were then read by two experienced laboratory technicians: The local laboratory technician read and graded the direct smears as routine using ordinary light microscopy (100X objectives). Another laboratory technician with equivalent experience in microscopy read the bleach-treated smears blind. The laboratory technician who read the bleach smears had no information on the methods used to treat the sputum samples and was also not aware of the direct smear microscopy results on these specimens. Similarly, the other technician had no information about the results of the bleached specimens. Both technicians used the IUATLD grading system (Crofton *et al.*, 1999).

All slides were stored after the reading. At the end of the study, all the slides were re-read by the previous two technicians but each technician reading the smears that had been read by the other earlier. Both technicians had no knowledge of the results of previous readings of each slide. The principal investigator re-labeled to blind the slides to be read by the two laboratory technicians. Once the results of the second (cross over) reading were available, the principal investigator compiled the results, identified discrepant results and checked the slides again together with the two laboratory technicians in order

to reach a consensus on the reading of each discrepant slide. A final decision was made based on the consensus and this result was used for the analysis. The culture was conducted at AHRI. The yield of smear positivity from direct smear, centrifugation, overnight sedimentation and short term digestion sputum smears was compared and validated against culture results.

4.11. Culture for Mycobacteria

Sputum was decontaminated with Petroff's method (Collee, *et al.*, 1989). In brief, an equal volume of 4% NaOH was added to the 1-2 mL of sputum transferred from the pooled specimens, as stated above, to screw-capped tubes for culture. Each tube was then tightened and vortexed for 15 min. The mixture was centrifuged at 3000 g for 15 minutes and the supernatant was decanted. The pellet was resuspended with 1-2 ml of PBS and neutralized immediately using 2N HCl. Two to four drops of the sediment were inoculated onto two slants of LJ media (one with glycerol and one with pyruvate). The culture was incubated at 37 °C and examined weekly for evidence of bacterial growth. A culture was considered negative if no bacterial colony was detected after 8 weeks of incubation.

4.12. Data management and Statistical analysis

The data was double entered into Epi-info version 2002 (US Center for Disease Control and Prevention, Version 3.3.2, Atlanta, GA, USA) by two data entry clerks and verified using the validation programme. Statistical analysis was done using STATA software (Stata Statistical Software: Version 8.2, College Station, TX: Stata corporation). Sensitivity, specificity and predictive values of the direct and bleach digested smears was calculated against culture results. Kappa statistics was used to determine the agreement between tests.

5. Quality control

Ten percent of negative and 20% of positive slides were randomly selected (using computer generated random numbers) from all the four different test sets and re-read by the principal investigator at AHRI. Fifty seven slides from direct smear, 53 from short term, 52 from overnight sedimentation and 50 from centrifugation with a total of 212 slides were re-read for quality control. The quality control readings corroborated the initial results in all cases except for one discrepant result in direct smear, short term and centrifugation methods and two in overnight sedimentation. All these 5 discrepant results were initially reported as negative but were reclassified as positive when re-read. A known reference strain (ATCC *M. gordonae*) of mycobacterium was used for quality control of the culture procedure.

6. Results

6.1. Socio demographic characteristics

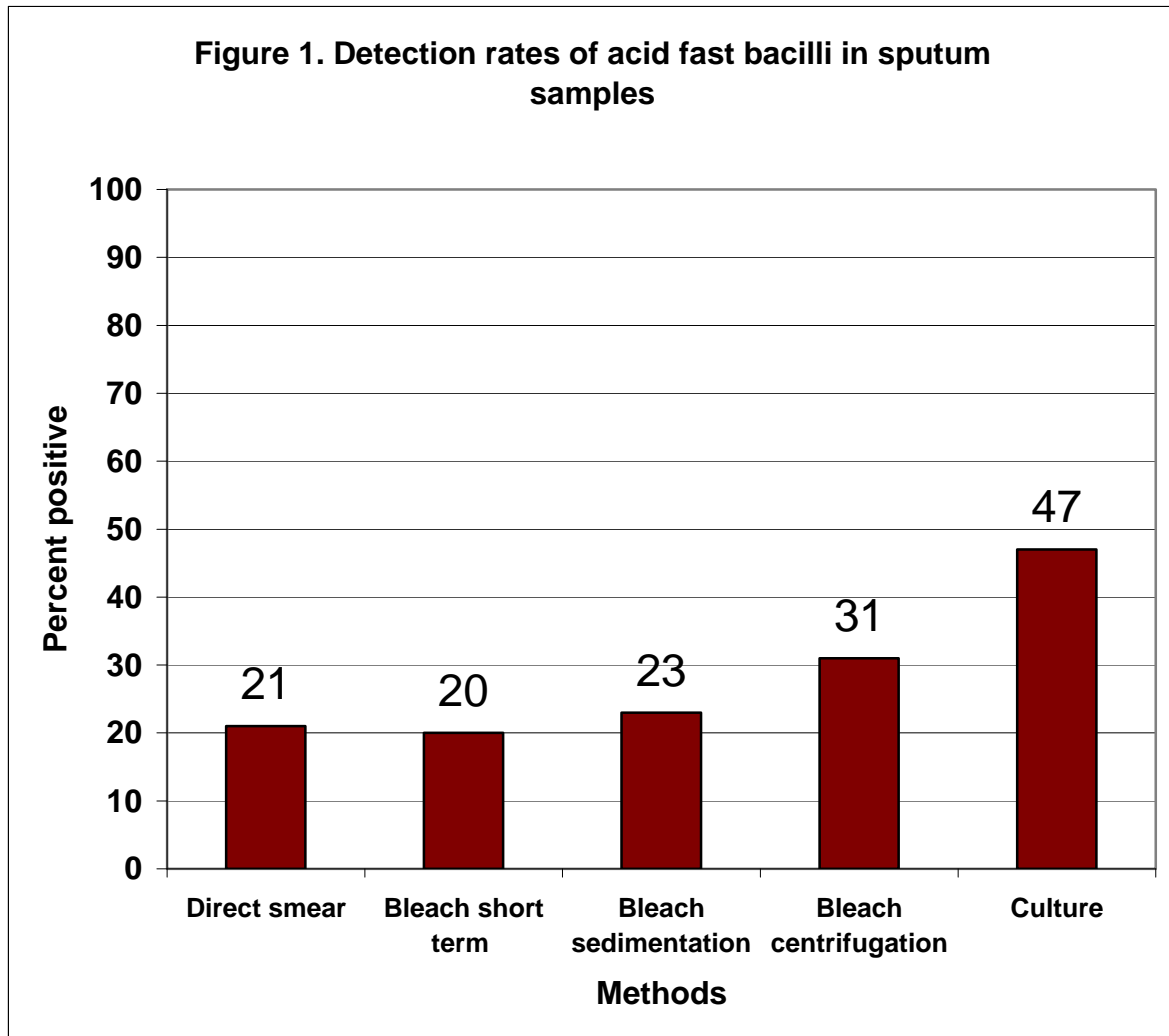
A total of 500 consecutive patients attending Awassa Health Center and Bushulo Major Health Center between June and December, 2006 with clinical symptoms compatible with PTB underwent sputum examination. Four patients were excluded from the analysis because of incomplete data in any of the five methods. Of 496 patients, 269 (54.3%) were male. The median age was 28 (range 8 – 90) years. The majority 53% (262/493) of the participants had elementary education and above, while 47% (231/493) were illiterate or had informal education. The majority, 31.8% (154/483) of the participants were housewives, 24.4% (118/483) were farmers and 18.6% (90/483) were students (Table 1).

Table 1. Socio demographic characteristics of TB suspects attending Awassa and Bushulo Health centers for laboratory confirmation of sputum samples (Awassa, Ethiopia, 2006)

Characteristics	N (%)
Sex (n=496)	
Male	269 (54.3)
Female	227 (45.7)
Age in years (n= 492)	
1-10	10 (2.0)
10-20	111 (22.6)
21-30	167 (33.9)
31-40	96 (19.5)
41-50	47 (9.6)
51-60	35 (7.1)
>60	26 (5.3)
Educational status (n= 493)	
Illiterate	198 (40.2)
Read and write	33 (6.7)
1-6 grade	99 (20.1)
7-8 grade	57 (11.6)
9-10/12	78 (15.8)
Collage and above	28 (5.7)
Occupation (n=483)	
Housewife	154 (31.8)
Farmer	118 (24.4)
Student	90 (18.6)
Civil servant/private employee	53 (10.9)
Daily laborer	31 (6.4)
Merchant	13 (2.7)
Unemployed	10 (2.0)
Others	15 (3.1)

6.2. Laboratory results

The results of smear microscopy and bleach methods are summarized in Figure 1. Of 496 patients, 20.6% were positive in direct smear, 19.8% in short term digestion, 23.4% in overnight sedimentation, 31.5% in centrifugation and 46.6% in culture. In the current study the analysis was done according to IUATLD grading system where scanty smears with less than 10 AFB per 100 fields were grouped as negative.



Analysis of clinical symptoms with positivity rate was presented in Table 2. Of those 376 patients who have fever, 89 (23.7%) were positive by direct smear, 81 (21.5%) by short term, 100 (26.6%) by overnight sedimentation, 133 (35.4%) by centrifugation and 187 (49.7%) by culture. The trend of positivity rate for other clinical symptoms of TB, namely, night sweat, weight loss and cough was similar to that of fever.

Table 2. Clinical symptoms in pulmonary TB in relation to yield of different methods for the diagnosis of pulmonary tuberculosis (Awassa, Ethiopia, 2006)

Clinical symptoms	Mycobacterial detection rates by methods				
	Direct smear	Short term	Sedimentation	Centrifugation	Culture
Fever (n=376)	89 (23.7%)	81(21.5%)	100 (26.6%)	133 (35.4%)	187 (49.7%)
Night sweat (n=409)	93 (22.7%)	89 (21.7%)	108 (26.4%)	139 (33.9%)	199 (48.7%)
Weight loss (n=403)	94 (23.3%)	87 (21.6%)	103 (25.6%)	139 (34.5%)	199 (49.4%)
Cough (n=449)	99 (22.0%)	96 (21.4%)	111 (24.7%)	148 (32.9%)	214 (47.7%)
All cases	102 (20.6%)	98 (19.8%)	116 (23.4%)	156 (31.5%)	231 (46.6%)

The sensitivity, specificity and predictive values were calculated against culture as the gold standard (Table 3) and presented in Table 4. The sensitivity of direct smear microscopy under the experimental condition was 41.9%. Among the bleach techniques, sensitivity was best for centrifugation (46.6%) and overnight sedimentation (34.0%), but least for short term digestion (28.2%). The current study showed an overall increase of 13% sensitivity over direct smear using the bleach method with centrifugation, which is statistically significant ($p < 0.0001$)

Table 3. Comparison of culture results with direct smear and bleach methods for the diagnosis of Pulmonary Tuberculosis in 496 patients with suspected pulmonary tuberculosis (Awassa, Ethiopia, 2006)

Direct smear	Culture		
	Positive	Negative	Total
Positive	97	5	102
Negative	134	260	394
Total	231	265	496
Short term digestion			
Positive	90	8	98
Negative	141	257	398
Total	231	265	496
Overnight sedimentation			
Positive	101	15	116
Negative	130	250	380
Total	231	265	496
Centrifugation			
Positive	126	30	156
Negative	105	235	340
Total	231	265	496

Table 4. Sensitivities, specificities and predictive values of direct smear and bleach methods for the diagnosis of pulmonary tuberculosis in 496 patients with suspected pulmonary tuberculosis (Awassa, Ethiopia, 2006)

Methods	Result (%)			
	Sensitivity	Specificity	PPV	NPV
Direct smear	41.9	98.1	95.1	65.9
Short term digestion	38.9	96.9	91.8	64.8
Overnight sedimentation	43.7	94.3	87.0	65.8
Centrifugation	54.6	88.7	80.8	69.1

PPV = positive predictive value; NPV = negative predictive value

The results of sputum smear and grade are shown in Table 5. Centrifugation technique detected more bacilli per microscope field than others with 11.5% smears graded as “+ + +” compared to 7% of overnight sedimentation, 6.5% of short term digestion and 3.8% of direct smear suggesting the bacilli were more concentrated in the bleach method.

Table 5. Smear positivity and grade result of direct smear and bleached methods for the diagnosis of pulmonary tuberculosis in 496 patients with suspected pulmonary tuberculosis (Awassa, Ethiopia, 2006)

Method	Microscopy results				
	Positive*				Negative
	+	++	+++	all **	
Direct smear	54 (10.9%)	29 (5.9%)	19 (3.8%)	102 (20.6%)	394 (79.4%)
Shot term	38 (7.7%)	28 (5.7%)	32 (6.5%)	98 (19.8%)	39 (80.2%)
Sedimentation	42 (8.5%)	39 (7.9%)	35 (7%)	116 (23.4%)	380 (76.6%)
Centrifugation	56 (11.3%)	43 (8.7%)	57 (11.5%)	156 (31.5%)	340 (68.6%)

* grading according to IUATLD standards ** scanty smears (less than 10 bacill/100 fields) are considered smear negative

Table 6 describes the results when the proportion of scanty grades is analyzed as a separate category (rather than pooled into negative results). As evident from the table, a higher proportion of scanty grades (10.7%) was observed in overnight sedimentation compared to 8.5% both in short term digestion and in centrifugation and 4.8% in direct smear. Of those 161 total scanty grades which were categorized as negative according to IUTLD/WHO grading system, 107 (66.5%) were culture positive (Table 7).

Table 6. Smear positivity and grade result of direct smear and bleached methods considering scanty grade as one entity for the diagnosis of pulmonary tuberculosis in 496 patients with suspected pulmonary tuberculosis (Awassa, Ethiopia, 2006)

Methods	AFB results		Positive results according to grading*			
	Negative	Positive	Scanty	+	++	+++
Direct smear	370 (74.6%)	126 (25.4%)	24 (4.8%)	54 (10.9%)	29 (5.9%)	19 (3.8%)
Short term	356 (71.7%)	140 (28.2%)	42 (8.5%)	38 (7.7%)	28 (5.7%)	32 (6.5%)
Sedimentation	327(65.9%)	169 (34.1%)	53 (10.7%)	42 (8.5%)	39 (7.9%)	35 (7.0%)
Centrifugation	298 (60.0%)	198 (40.0%)	42 (8.5%)	56 (11.3%)	43 (8.7%)	57 (11.5%)

* scanty smears are smears with less than 10 AFB per 100 fields; here considered positive

Table 7. Comparison of scanty result of different methods with culture result for the diagnosis of pulmonary tuberculosis in 161 patients with suspected pulmonary tuberculosis (Awassa, Ethiopia, 2006)

Methods	Scanty grade	Culture positive	Culture negative
Direct smear	24	21 (87.5%)	3 (12.5%)
Short term	42	33 (78.6%)	9 (21.4%)
Overnight sedimentation	53	32 (60.4%)	21 (39.6%)
Centrifugation	42	21 (50.0%)	21 (50.0%)
Total	161	107 (66.5%)	54 (33.5%)

Table 8. Sensitivities, specificities and predictive values of direct smear and bleach methods considering scanty (<10 AFB/100 fields) as positive for the diagnosis of pulmonary tuberculosis in 496 patients with suspected pulmonary tuberculosis (Awassa, Ethiopia, 2006)

Methods	Result (%)			
	Sensitivity	Specificity	PPV	NPV
Direct smear	51.1	96.9	93.6	69.5
Short term digestion	53.2	93.6	87.9	69.7
Sedimentation	57.6	86.4	78.7	70.0
Centrifugation	63.6	80.8	74.2	71.8

PPV = positive predictive value; NPV = negative predictive value

Considering scanty (<10AFB/100 fields) as positive, the sensitivity was 51.1%, 53.2%, 57.6% and 63.6% for direct smear, short term, overnight sedimentation and centrifugation respectively (Table 8). The overall 13% increase in sensitivity using centrifugation by considering scanty (<10AFB/100 fields) as positive is similar to that obtained considering scanty (<10AFB/100 fields) as negative.

Table 9. Comparison of yield of positivity of bleach methods and culture against direct smear for the diagnosis of pulmonary tuberculosis in 496 patients with suspected pulmonary tuberculosis (Awassa, Ethiopia, 2006)

Shot term digestion	Direct smear		
	Positive	Negative	Total
Positive	81	17	98
Negative	21	377	398
Total	102	394	496
Overnight sedimentation			
Positive	85	31	116
Negative	17	363	380
Total	102	394	496
Centrifugation			
Positive	92	64	156
Negative	10	330	340
Total	102	394	496
Culture			
Positive	97	134	231
Negative	5	260	265
Total	102	394	496

Table 9 summarizes the results obtained from direct microscopy as compared to the bleach methods and culture. Seventeen (4.3%) of 394 slides that were considered negative with the direct smear were rated positive by short term digestion, 31 (7.9%) by overnight sedimentation, 64 (16%) by centrifugation and 134 (34%) by culture. However, 21 (21%) of 102 slides positive by direct smear were negative by short term digestion, 17 (17%) by overnight sedimentation, 10 (10%) by centrifugation and 5 (5%) by culture.

Of those 394 patients who were negative by direct smears, 134 (34%) became positive in culture, similarly 141 (35%) of short term digestion, 130 (34%) of overnight sedimentation and 105 (31%) of centrifugation became positive in culture (Table 5). The agreements between the different methods were calculated using kappa statistics. The lowest agreement was observed between direct smear and centrifugation (agreement = 86.8 and kappa = 0.5) and relatively the highest was shown between short term and overnight sedimentation (agreement = 92.4 and kappa = 0.67).

7. Discussion

Sputum smear microscopy is the only diagnostic tool and widely used method in resource poor countries where other options are less practical due to the limited resources available. However, its sensitivity is usually less than 50% (ranging from 28% to 44.4%). Therefore, more sensitive techniques will need to be developed to improve TB case detection and early treatment of patients, particularly in resource-poor settings with high disease burden. Our current finding of 41.9% sensitivity of direct microscopy further corroborates the earlier reports. This result is relatively higher than the 30.8% sensitivity reported by Gebre *et al.* (1995) but lower than the 54.2% sensitivity reported by Bruchfeld *et al.* (2000) later from Ethiopia. It is in agreement with a sensitivity of 44% (Angeby *et al.*, 2000) in Honduras and 43.4% (Habeenzu *et al.*, 1998) in Zambia.

In the current study, yield of positivity was 21% on direct smear, 20% on short term digestion, 23% with overnight sedimentation and 31% with centrifugation. Confirmation with culture was possible for 47% of these same sputum samples. But, in a study in Ethiopia by Abdurahman *et al.* (2000), 22% of patients were AFB positive by direct smear examination, 37% following overnight sedimentation of NaOCl treated sputum and 41% when the treated sputum was centrifuged. Similarly, in another study in Ethiopia by Gebre-Selassie (2003), the rate of detection of AFB in sputum was 8.5%, 25.5% and 38.0% for direct smear, overnight sedimentation and centrifugation respectively. These tests were however not validated using culture as a gold standard.

The sensitivity of the centrifugation method in our current study was 54.8%. There was an overall increase of 13% sensitivity from direct smear and the difference is statistically significant ($p < 0.0001$). The result reported in our study is relatively lower than from those reported earlier: Gebre *et al.* (1995) and Bruchfeld *et al.* (2000) reported a sensitivity of 69.2% and 63.1% respectively where culture was used as reference. Habeenzu *et al.* from Zambia (1998) reported a sensitivity of 76.3% using fluorescence microscopy as reference. On the other hand, Wilkinson and Sturm (1997) reported, using culture as reference, that the sensitivity of direct smear microscopy was not as such

improved by sputum liquefaction and centrifugation (an increase of 1% from 43% to 44%). In their hands, even some smears which were initially positive by direct microscopy became negative after concentration. Similarly, Harries *et al.* (1998b) reported equally disappointing results. Although it is difficult to speculate why this could be so, details in the methods applied could be a possibility. Longer treatment of sputum with bleach has been reported to damage the bacilli to such an extent that staining characteristics are lost.

The specificities of centrifugation reported in previous studies were 100% (Habbenzu *et al.*, 1998; Gebre *et al.*, 1995) and 95.9% (Bruchfeld *et al.*, 2000), but in our case it was 88.7%. This relative decrease in specificity may be attributed to the detrimental action of the liquefying solution resulting in culture negativity as it was suggested by Perera and Arachchi (1999).

The specificity of microscopical examination for acid fast bacilli in identifying *M. tuberculosis* is determined by the ratio of the prevalence of *M. tuberculosis* complex to that of environmental mycobacteria in clinical specimens, and the extent of technical errors in the laboratory. Positive sputum smears due to environmental mycobacteria are very uncommon in many sub-Saharan African countries (Aber *et al.*, 1980; Braun *et al.*, 1992). We have not identified any such mycobacteria on culture of the specimens tested in this study.

The sensitivity of overnight sedimentation reported in our study was 43.4%, but that of centrifugation was 54.9%. From the current study it is observed that there is an increase in sensitivity of centrifugation over overnight sedimentation. However, in another study conducted in Ethiopia (Miorner *et al.*, 1996), there was a substantial increase in sensitivity to direct smear microscopy but, a statistical comparison of results obtained after centrifugation with those obtained after sedimentation showed no significant difference between the two concentration methods. Similarly Van Deun *et al.* (2000) showed that although overnight sedimentation is more time consuming, it gives the same result in terms of increased sensitivity as that of centrifugation. Technically,

centrifugation concentrates bacilli much more than simple sedimentation does and this could explain the higher sensitivity observed in our study.

Short term digestion is one of the bleach methods that has been introduced recently. It is not a direct concentration method as such unlike in the case of centrifugation or overnight sedimentation. Rather, it is a method that digests the sputum by exposure of the sputum to bleach for 30 minutes resulting in removal of debris and a clear field of smear microscopy. The method was reported to improve the quality and yield of smear microscopy by facilitating the preparation of clear smear. A study conducted in Ethiopia by Yassin *et al.* (2003) reported that short-term digestion of sputum samples for 30 min had 26% positivity compared to 17.5% of direct smear. But, in our current study, yield of positivity was 21% in direct smear and 20% in short term digestion. Because the samples were not validated by culture, it is difficult to compare the relatively low yield of direct microscopy in the former study, which might be due to a lower proportion of tuberculosis patients among those studied. Nevertheless, the difference with bleach digested sputum samples is strikingly different between the two reports. In another study on short term digestion, Lawson *et al.* (2006) reported that three direct smears identified 51% and one digested smear identified 50% of patients with PTB. Similarly, according to Lawson *et al.* (2007) report, one bleach digested smear identified 52% of the HIV positive and 63% of HIV negative patients with pulmonary TB, resulting in the same overall yield as three direct smears.

There is no report so far regarding the sensitivity of short term digestion in comparison to culture as gold standard and therefore it had not been possible to compare its diagnostic performance with other bleach methods. But, our current study reported the sensitivity of short term digestion to be 38.9% with specificity of 96.9%. This sensitivity is lower than that of direct smear (41.9%), overnight sedimentation (43.2%) and than that of centrifugation (54.9%). The lower sensitivity of the method might be attributed to the diluting effect of bleach, as an equal volume is added to the sputum sample, and as this is not further followed by any concentration (as it is done with the other methods of centrifugation or overnight sedimentation). Our results here do not suggest that short term

bleach digestion has any advantages in sensitivity over direct smears. However, we have not compared the possible relative advantage in rapidity and ease of smear reading.

We compared the diagnostic performance of these methods considering scanty (<10 AFB/100 fields) as positive and it was found that the sensitivity of direct smear was 51.1% and that of centrifugation was 63.6% with an over all 13% of increase which is statistically significant ($p < 0.0001$). This overall increase of 13% in sensitivity using centrifugation method was also similar with the result obtained when considering scanty (<10 AFB/100 fields) as negative. For the other bleach methods, i.e. overnight sedimentation and short term digestion, the sensitivity was 53.2% and 57.6% respectively. There is no similar study conducted so far evaluating the readings comparing scanty as negative and as positive and validating the interpretation with culture.

The IUATLD / WHO grading system recommends a threshold of 10 AFB per 100 fields before a slide is regarded as positive (i.e. only grades 1+ and above are considered positive). Recent work investigating the impact of lowering the positivity threshold in order to increase sensitivity of direct smear microscopy recommended a threshold of 4 bacilli or even 1 bacillus per 100 fields; as most of these smears are likely to be true positives in areas of high TB prevalence (Van Deun *et al.*, 2004). Similarly, the result of our study showed that of those 161 scanty slides, 107 (66.5%) were culture positive! As it is observed in the current study, two-thirds of these scanty results which were categorized as negative by the IUATLD/WHO scale were positive by culture. Similarly, in another study in Abuja, Nigeria (Lawson *et al.*, 2005), it was concluded that smears reported as scanty are more likely to be true than false-positive. These show that there is evidence against categorizing all scanty grades as negative. Therefore, the modification on the grading system made by the Ethiopian National TB Control Programme that lowers the cutoff for scanty grade to 1-2 AFB/100 microscopic fields and the positive to ≥ 3 AFB/100 fields is commendable as this helps retain those cases which could be misdiagnosed as negative.

Grading of positive slides is required to determine the load of AFB which may be helpful for the management of patients (Rajpal *et al.*, 2002). The IUATLD/WHO grading system recommends all AFB in scanty grades to be counted and reported in numbers per 100 fields. We had not originally included counting in our methods. To offset for this, therefore, 50% of the slides with scanty grades was taken randomly and counted by two independent laboratory technicians at the end of the study. The result showed that 22% of the slides had a bacilli count of ≥ 3 AFB / 100 fields.

In the current study, the centrifugation technique detected more bacilli per microscope field than others with 11.5% smears graded as “+ + +” compared to 7% of overnight sedimentation, 6.5% of short term digestion and 3.8% of direct smear suggesting the bacilli were more concentrated in the centrifugation method. Similarly, Gebre *et al.* (1995) showed a significant increase in the average number of AFB seen per microscopic field in the smears prepared after NaOCl treatment due to concentration of the bacilli. In their study, concentration through centrifugation yielded the highest average number of AFB per microscopic field.

In the current study, each of the bleach methods (except for short term digestion) detected more bacilli than direct smear, however 21 (21%) of 102 slides positive by direct smear were negative by short term digestion, 17 (17%) by overnight sedimentation and 10 (10%) by centrifugation. Similarly, in the study conducted by Wilkinson and Sturm (1997), 13 out of 42 (31%) specimens that were positive by direct smear were negative after concentrating by centrifugation. Again, in a study conducted by Harries *et al.* (1998b) it was reported that 3 out of 81 specimens that were positive by conventional Ziehl Neelsen examination became negative by concentrated smear. In these 2 studies, sputum was centrifuged at 1500 g and 1000 g, respectively, rather than at 3000-3800 g which is optimal for recovery of AFB (Rickman and Moyer, 1980; Gebre *et al.*, 1995; Bhat, 1999). Possible explanations were given for such disparity and these include: either liquefaction was incomplete or acid fast bacilli were disrupted by the process, or presumably because AFB were not effectively pelleted during centrifugation due to the low centrifugal force used. Similarly, in our case, the slides that were missed by short

term digestion might have suffered from the diluting effect of the method. We share the views of the previous authors in regard to the differences seen with the other two methods (in addition to the effect of low centrifugal force mentioned above).

We used Kappa statistics to determine the agreement of the tests. Kappa is intended to give a quantitative measure of the magnitude of agreement between observers (Viera and Garrett, 2005) and in our case between test methods. As it was shown from the results of the study, the lowest agreement was observed between direct smear and centrifugation (agreement = 86.7 and kappa = 0.5) and relatively the highest was shown between short term and overnight sedimentation (agreement = 92.4 and kappa = 0.7). It is logical to expect these low agreements between methods as the study generally showed a significant difference among the methods.

The bleach method was introduced 100 years ago (Cited in Ramsay *et al.*, 2006). However, it was not widely used until it was re-investigated and introduced by Miorner and co-workers in Ethiopia (Gebre *et al.*, 1995). One of its major advantages is that bleach digests the sputum so that there will not be debris on the microscopy slide, leaving a free field for bacterial detection, thus requiring less time than the recommended minimum of 5 minutes for reading a smear (WHO, 1998). In addition, reading is simplified by the higher density of bacilli per microscopic field obtained after concentration by centrifugation or overnight sedimentation. As a potent disinfectant, bleach has the advantage of limiting the risk of laboratory infection, a risk that can not be neglected with the emergence of multi-drug resistant TB (Gebre *et al.*, 1995; Miorner *et al.*, 1996). Household bleach is cheap and locally available, even in rural areas. However, bleach treated samples will not be suitable for culture as bleach kills the bacilli.

In developing countries, where culture facilities are unavailable, the relative importance of sputum concentration method cannot be overestimated (Aderaye, 2007). Angeby *et al.* (2004) recently reviewed studies of all bleach methods aimed at improving sputum smear microscopy. The reviewers concluded that there is enough evidence and enough local concern to promote the introduction of a bleach method as part of the DOTS strategy in

countries where culture is not performed routinely. However, Ramsay *et al.* (2006) suggested the need for solid evidence on the overall bleach methods, including information on the diagnostic performance of each bleach method before implementation.

We have compared the different bleach methods using the same sputum samples as source and validating all with culture. The need for centrifuges and electricity limits the use of the centrifugation method. Sedimentation was proposed as an alternative. However, overnight sedimentation of sputum with bleach appears to be no better than centrifugation and may lead to further delays in availability of results and requires patients to make repeated visits (Yassin, 2005). According to the current study, the diluting effect of short term digestion which is reflected in its low sensitivity reduces its value. Currently, many diagnostic centers in Ethiopia are already equipped with centrifuges and the necessary RCF can easily be achieved by a low cost table top centrifuge or even by a hand driven model (Gebre *et al.*, 1995). Recently, with the support made to strengthen the laboratories of the country through the HIV prevention programme, most laboratories are equipped with centrifuges and this could be a good opportunity for the National TB control programme.

To our knowledge, the current study is the first of its kind to compare all the reported bleach methods from a given group of samples and hence provides useful information on the currently available bleach methods in relation to direct smear and culture. The current study clearly showed a significant difference and disparity in diagnostic performance among the three different bleach methods. The study re-affirmed the significant increase in sensitivity with the centrifugation method, among the bleach methods, with an overall 13% increase; which can have a significant impact on improving the efficiency of TB control programmes. Therefore, it can be emphasized that the bleach method (particularly the method with centrifugation) can clearly increase the sensitivity of case detection and thus be a useful tool for the National Tuberculosis Programme. Thus, the introduction of the method in the routine diagnosis of pulmonary TB would benefit the Control Programme in increasing case detection.

8. Conclusion

The current study clearly demonstrated a difference in diagnostic performance among the three different bleach methods. The study confirmed a statistically significant increase of 13% overall sensitivity in using bleach with subsequent concentration by centrifugation. According to MOHE 2006 report, the case detection rate of TB of the country was 32% which is less than half of the expected 70% case detection rate of WHO for year 2005. Therefore, the *centrifugation* technique can be used as a useful tool in the control programme in increasing the reported low case detection rate and in helping to attain the expected case detection rate proposed by the WHO and the targets in the Millennium Development Goals. Further optimization of work flow under program conditions may be necessary to reduce the waiting time required by the bleach technique. Even though it requires additional days for the patient to get treatment and its sensitivity is less than that achieved by the centrifugation technique, overnight sedimentation can be used as a second alternative in the control programme.

9. Recommendation

The results obtained from the current study revealed the value of bleach methods in increasing the case detection. Therefore, it is recommended that the bleach technique (particularly of the centrifugation) should be piloted under field conditions and when confirmed as advantageous, implemented in the control programme for routine diagnosis of pulmonary TB. Further research on scanty results in relation to culture should be conducted particularly on patients co-infected with HIV.

10. Limitations of the study

The current study generated information on the diagnostic performance of the bleach methods for the diagnosis of pulmonary tuberculosis on TB suspects and this can serve as base line information. However, it lacks information which should be assessed in the future. These include:

- Information on diagnostic performance of the bleach method on those patients (suspects) with previous history of anti-TB treatment and current TB treatment; as these patients were excluded from the current study. Therefore, the diagnostic performance of bleach methods on these groups of people should be assessed in order to give comprehensive information.
- Information on the diagnostic performance of the bleach methods on TB patients with and without HIV infection as a separate entity .This would have importance to get information on its diagnostic performance particularly among TB/HIV co-infected patients.

References

Abdurrahman A, Beyene G, Wodajo N. (2000). Comparative study of Direct Sputum Microscopy with Different Sample Pre-Treatment Procedures for Examination of Acid Fast Bacilli. *Ethiop J health Science.* 10:19-23.

Aber VR, Allen BW, Mitchison DA, Ayuma P, Edwards EA, Keyes AB. (1980). Quality Control in tuberculosis bacteriology 1. Laboratory studies on isolated positive cultures and the efficiency of direct smear examination. *Tubercle.* 61: 123-33

Aderaye G. (2007). Pulmonary tuberculosis and *Pneumocystis jiroveci* pneumonia in HIV- infected patients in Ethiopia. US AB, Sweden

AIDS in Ethiopia. (2006). Sixth report. Federal Ministry of Health National HIV/AIDS Prevention and Control Office.

Allwood M D M, Lee YC, Salaniponi F M L, Nyangulu D S, Kuppusamy I, Squire S B. (1997). Case-finding with a single sputum sample and household bleach. [poster] *Int J Tuberc Lung Dis.* 1(suppl 1): S144

American Thoracic Society, Center for Disease Control and Prevention. (2000). Diagnostic standards and classification of tuberculosis in adults and children. *Am J Respir Crit Care Med.* 161: 1376-1395

Angeby K.A.K., Alvalado-Galvez C, Pineda-Garcia L, Hoffner S.E. (2000). Improved sputum microscopy for a more sensitive diagnosis of pulmonary tuberculosis. *Int J Tuberc Lung Dis.* 4: 648-687

Angeby KAK, Hoffner SE, Diwan VK. (2004). Should the 'bleach microscopy method' be recommended for improved case detection of tuberculosis? Literature review and key person analysis. *Int J Tuberc Lung Dis.* 8: 806-15

Apers L, Mutsvangwa J, Magwenzi J, Chigara N, Butterworth A, Mason P, Stuyft PV. (2003). A comparison of direct microscopy, the concentration method and the Mycobacteria Growth Indicator Tube for the examination of sputum for acid-fast bacilli. *Int J Tuberc Lung Dis.* 7: 376-381

Bell WJ, Brown PP. (1962). Fluorescence microscopy in the laboratory diagnosis and assessment of pulmonary tuberculosis. *Cent Afr J Med.* 8: 4–9

Boyd JC, Marr JJ. (1975). Decreasing reliability of acid-fast smear techniques for detection of tuberculosis. *Ann Intern Med.* 82: 489–92

Braun MM, Kilburn JO, Smithwick RW, Coulibaly IM, Coulibaly D, Silcox VA, Gnaore E, Adjorlolo G, De Cock, KM. (1992). HIV infection and primary resistance to antituberculosis drugs in Abidjan, Cote d'Ivoire. *AIDS.* 6: 1327-1330

Brindle RJ, Nunn PP, Githui W, Allen BW, Gathua S, Waiyaki P. (1993). Quantitative bacillary response to treatment in HIV-associated pulmonary tuberculosis. *Am Rev Respir Dis.* 147: 958–61

Brook GF, Butel JS, Morse SA. (2001). Jawetz, Melnic, and Adelberg's Medical Microbiology. 22nd edition, McGraw-Hill, USA.

Bruchfeld J, Aderaye G, Palme IB, Bjorvatn B, Kallenius G, Lindquist L. (2000). Sputum concentration improves diagnosis of tuberculosis in a setting with a high prevalence of HIV. *Trans R Soc Trop Med Hyg.* 94: 677-80

Chapman AL, Munkanta M, Wilkinson KA. (2002). Rapid detection of active and latent tuberculosis infection in HIV-positive individuals by enumeration of *Mycobacterium tuberculosis*-specific T cells. *Aids.* 16: 2285–93

Cheesborough M. (2000). District Laboratory Practice in Tropical Countries; Part 2, Cambridge University. Press; UK.

Colebunders R, Bastian I. (2000). A review of the diagnosis and treatment of smear-negative pulmonary tuberculosis. *Int J Tuberc Lung Dis.* 4: 97–107

Collee J.G, Duguid J.P, Fraser A.G., Marmion B.P. (1989). Staining of Acid-Fast bacilli. Mackie and McCartney Practical Medical Microbiology; Vol.2; 13th edition, Churchill Livingstone, UK.

Corper H, Nelsen C. (1949). Methods of Concentrating Acid-Fast bacilli. *Am J Clin Pathol.* 19: 169-173

Crofton J, Horne N, Miller F. (1999). Clinical Tuberculosis. Second ed. Macmillan Education Ltd., UK.

Daniel, TM. (1989). Rapid diagnosis of tuberculosis: laboratory techniques applicable in developing countries. *Review of Infectious disease.* 11: supplement 2, S471-S478

Dheda K, Udwardia ZF, Huggett JF, Johnson MA, Rook GA. (2005). Utility of the antigen-specific interferon-gamma assay for the management of tuberculosis. *Curr Opin Pulm Med* 11: 195–202

Division of Becton Dickinson and Company. (1998). Difco Manual; 11th edition, USA.

Dye C, Scheele S, Dolin P, Pathania V, Raviglione MC. (1999). Consensus statement. Global burden of tuberculosis: estimated incidence, prevalence, and mortality by country. WHO Global Surveillance and Monitoring Project. *JAMMA.* 282: 677-86

Douthwaite S, Yassin MA, Squire SB, Arbide I, Cuevas LE. (2006). Bleach-digested sputum smears for the diagnosis of tuberculosis in HIV-infected individuals. *Tropical Doctor*. 0: 000-000

Elliott AM, Hayes RJ, Halwiindi B. (1993). The impact of HIV on infectiousness of pulmonary tuberculosis: a community study in Zambia. *Aids*. 7: 981-7

Enarson DA, Rouillon A. (1994). The epidemiological bases of tuberculosis control. In: P.D.O. Davis (Ed) *Clinical tuberculosis*, P: 19-32, Chapman and Hall, London.

Enarson DA, Reider HL, Arnadottir T, Trebucq A. (2000). Management of Tuberculosis. A guide for low income countries. 5th edition., International Union Against Tuberculosis and Lung Disease, Paris.

Ergete W, Bekele A. (2000). Acid fast bacilli in aspiration smears from tuberculosis patients. *Ethiop. J. Health Dev.* 14: 99-104

Estrela C, Estrela CRA., Barbin EL, Spano JC, Marchesan MA, Pecora JD. (2002) Mechanism of action of Sodium Hypochlorite. *Braz Dent J.* 13: 113-117

Farina P, Mohammadi F, Zarifi Z, Tabatabee D.J, Ganavi J, Ghazisaeedi K, Farnia P.K, Gheidy M, Bahadori M, Masjedi M.R, Velayati A.A. (2002). Improving Sensitivity of Direct Microscopy for Detection of Acid-Fast Bacilli in Sputum: Use of Chitin in Mucus Digestion. *J Clin Microbiol.* 40: 508-511

Foulds J, O'Brien R. (1998). New tools for the diagnosis of tuberculosis: the perspective of developing countries. *Int J Tuberc Lung Dis.* 2: 778-783

Garay JE. (2000). Analyses of simplified concentration of sputum smear technique for pulmonary tuberculosis diagnosis in rural hospital. *Trop Doct.* 30: 70-2

Gebre N, Karlson U, Jonsson G, Macaden R, Wolde A, Assefa A. (1995). Improved microscopical diagnosis of pulmonary tuberculosis in developing countries. *Trans R Soc Trop Med Hyg.* 89: 191-193

Gebre-Selassie S. (2003). Evaluation of the concentration sputum smear technique for the laboratory diagnosis of pulmonary tuberculosis. *Trop Doct.* 33: 1-3

Getahun H, Harrington M, O'Brien R, Nunn P. (2007). Diagnosis of smear-negative pulmonary tuberculosis in people with HIV infection or AIDS in resource-constrained settings: informing urgent policy changes. *Lancet.* 369: 2042-9

Githui W, Kitui F, Juma ES, Obwana DO, Mwai J, Kwamanga D. (1993). A comparative study on the reliability of the fluorescence microscopy and Ziehl-Neelsen method in the diagnosis of pulmonary tuberculosis. *East Afr Med J.* 70: 263-6.

Gopi PG, Subramani R, Selvakumar N, Santha T, Eusuff SI, Narayanan PR. (2004). Smear examination of two specimens for the diagnosis of pulmonary tuberculosis in Tiruvallur district, south India. *Int J Tuberc Lung Dis.* 8: 824-8

Graham S, Das KG, Hidvegi RJ. (2002). Chest radiograph abnormalities associated with tuberculosis: reproductivity and yield of active cases. *Int Tuberc Lung Dis.* 6: 137-42

Grybowski S, Barnet GD, Styblo K. (1975). Contacts of cases of active pulmonary tuberculosis. *Bull Int Union Tuberc* 50: 90-106

Habeenzu C, Lubasi D, Fleming AF. (1998). Improved sensitivity of direct microscopy for the detection of acid-fast bacilli in sputum in developing countries. *Trans R Soc Trop Med Hyg.* 92: 415-416

Hagemann PKH. (1938). Fluoreszenzfärbung von tuberkelbakterien mitauramin. Munich Med Wschr. 85: 1066–68

Hanna BA, Bbrahimzadeh A, Bruce Elliott L, Morgan MA, Novak SM, Rusch-Gerdes S, Acio M, Dunbar DF, Holmes TM, Rexer CH, Savthyakumar C, Vannier A. (1999). Multicenter Evaluation of the BACTEC MGIT 960 System for Recovery of Mycobacteria. J Clin Microbiol. 37: 748-752

Hanna BA, Walters SB, Bonk SJ, Tick LJ. (1995). Recovery of mycobacteria from blood in mycobacteria growth indicator tube and Lowenstein-Jensen slant after lysis-centrifugation. J Clin Microbiol. 33: 3315–16

Hargreaves NJ, Kadzakumanja O, Whitty CJ, Salaniponi FM, Harries AD, Squire SB. (2001). ‘Smear-negative’ pulmonary tuberculosis in a DOTS programme: poor outcomes in an area of high HIV seroprevalence. Int J Tuberc Lung Dis. 5: 847–54

Harries AD, Chilewani N, Dzinyemba W. (1998b). Diagnosing tuberculosis in resource poor- poor setting: the value of sputum concentration. Transactions of the Royal Society of Tropical Medicine and Hygiene. 92-123

Hawken MP, Muhindi DW, Chakaya JM, Bhatt SM, Ng'ang'a LW, Porter JD. (2001). Under-diagnosis of smear-positive pulmonary tuberculosis in Nairobi, Kenya. Int J Tuberc Lung Dis. 5: 360-3.

ICMR BULLETIN. (2002). What is new in the diagnosis of tuberculosis? Part I: Techniques for diagnosis of tuberculosis. 1.32: 8.

Johnson JL, Vjecha MJ, Okwera A. (1998). Impact of human immunodeficiency virus type-1 infection on the initial bacteriologic and radiographic manifestations of pulmonary tuberculosis in Uganda. Makerere University-Case Western Reserve University Research Collaboration. Int J Tuberc Lung Dis. 2: 397–404

Karstaedt AS, Jones N, Khoosal M, Crewe-Brown HH. (1998). The bacteriology of pulmonary tuberculosis in a population with high human immunodeficiency virus seroprevalence. *Int J Tuberc Lung Dis.* 2: 312-6

Kivihya-Ndugga LE, van Cleeff MR, Githui WA. (2003). A comprehensive comparison of Ziehl-Neelsen and fluorescence microscopy for the diagnosis of tuberculosis in a resource-poor urban setting. *Int J Tuberc Lung Dis.* 7: 1163-71

Kubica GP. (1980). Correlation of acid-fast staining methods with culture results for mycobacteria. *Bull Int Union Tuberc.* 55: 117-24

Lauzardo M, Ashkin D. (2000). Phthisiology at the dawn of the new century. *Chest.* 117: 1455-73

Lawson L, Yassin MA., Ramsay A, Olajide I, Thacher TD, Davies PDO, Squire SB, Cuevas LE. (2006). Microbiological validation of smear microscopy after sputum digestion with bleach; a step closer to a one-stop diagnosis of pulmonary tuberculosis. *Journal of Tuberculosis.* 86: 34-40

Lawson L, Yassin M.A., Ramsay A, Emenyonum NE, Squire SB, Cuevas LE. (2005). Comparison of scanty AFB smears against culture in an area with high HIV prevalence. *Int J Tuberc Lung Dis.* 4: 371-376

Lawson L, Yassin M.A., Ramsay A, Emenyonum N.E., Thacher T.D., Davies P.D.O., Squire S.B., Cuevas LE. (2007). Short-term bleach digestion of sputum in the diagnosis of pulmonary tuberculosis in patients co- infected with HIV. *Tuberculosis.* tube. 02. 002

Leung AN, Brauner MW, Gamsu G. (1996). Pulmonary tuberculosis: comparison of CT findings in HIV-seropositive and HIV-seronegative patients. *Radiology.* 198: 687-91

Liebeschuetz S, Bamber S, Ewer K, Deeks J, Pathan AA, Lalvani A. (2004). Diagnosis of tuberculosis in South African children with a T-cell-based assay: a prospective cohort study. *Lancet*. 364: 2196–203

Mancino G, Placido R, Bacih S. (1997). Infection of human monocytes with *Mycobacterium Tuberculosis* enhances human immunodeficiency virus type 1 replication and transmission to T cells. *J Infect Dis*. 175: 1531-5

Maher D, Chaler P, Spinaci S, Harris AD. (1997). Treatment of tuberculosis: guidelines for national programmes. 2nd edition., WHO, Geneva.

Mendoza, M, Reyes LF, Pascual, M, Tan-Torres T. (1993). Culture Isolation of *Mycobacterium tuberculosis* using the Radiometric Bactec System Compared with the Conventional Lowenstein-Jensen Media. *Phil J Microbiol Infect Dis*. 22: 17-22

Miller MB. (2007). Molecular Diagnosis of Infectious Diseases. *NC Med J*. 68: 114-8

Ministry of Health Ethiopia. Manual. (2002). National Tuberculosis and Leprosy Control Programme Manual for Laboratory Technicians. 2nd edition. Addis Ababa, Ethiopia.

Ministry of Health Ethiopia. Manual (2005). National Tuberculosis and Leprosy Control Programme. 3rd edition. Addis Ababa, Ethiopia.

Miorner H, Ganlov G, Yohanes Z, Adane Y. (1996). Improved Sensitivity of Direct Microscopy for Acid-Fast Bacilli: Sedimentation as an Alternative to Centrifugation for Concentration of Tubercle Bacilli. *J Clin Microbiol*. 34: 3206 -320

Pai M, Riley LW, Colford JM. (2004). Interferon-gamma assays in the immunodiagnosis of tuberculosis: a systematic review. *Lancet Infect Dis*. 4: 761–76

Parry C, Davis PD. (1996). The resurgence of tuberculosis. *Soc Appl Bacteriol Symp Ser.* 25: 23S-26S.

Perera J, Arachchi D.M. (1999). The optimum relative centrifugal force and centrifugation time for improved sensitivity of smear and culture for detection of *Mycobacterium tuberculosis* from sputum. *Trans R Soc Trop Med Hyg.* 93: 405-9

Perkins MD, Roscigno G, Zumla A. (2006). Progress towards improved tuberculosis diagnostics for developing countries. *Lancet.* 942-43

Pike R M. (1979). Laboratory- associated infections: incidence, fatalities, causes, and prevention. *Ann Rev Microbial.* 33: 44-66

Rajpal S, Dhingra VK, Aggarwal. (2002). Sputum grading as predictor of treatment outcome in pulmonary tuberculosis. *Ind. J Tub.* 49: 139-141

Ramsay A, Squire SB, Siddiqi K, Cunningham J, Perkins MD. (2006). The bleach microscopy method and case detection for tuberculosis control. *Int J Tuberc Lung Dis.* 10: 256-258

Richards OW, Kline EK, Leach RE. (1941). Demonstration of tubercle bacilli by fluorescence microscopy. *Am Rev Tuberc.* 44: 255

Rickman TW, Moyer NP. 1980). Increased sensitivity of acid-fast smears. *Journal of Clinical Microbiology.* 11: 618-620

Shingadia D, Novelli. (2003). Diagnosis and treatment of tuberculosis in children. *Lancet Infect Dis.* 3: 642-32

Siddiqi K, Lambert ML, Walley J. (2003). Clinical diagnosis of smear- negative pulmonary tuberculosis in low- income countries: the current evidence. *Lancet Infect Dis.* 3: 288-96

Singh NP, Parija SC. (1998).The value of fluorescence microscopy of auramine stained sputum smears for the diagnosis of pulmonary tuberculosis. *Southeast Asian J Trop Med Public Health.* 29: 860–63

Smith RL, Yew K, Berkowitz KA, Aranda CP. (1994). Factors affecting the yield of acid-fast sputum smears in patients with HIV and tuberculosis. *Chest.* 106: 684-6

Steingart KR, Henry M, Vivienne Ng, Hopewell PC, Ramsay A, Cunningham J, Urbanczik, Perkins M, Aziz MA, Pai M. (2006). Fluorescence versus conventional sputum smear microscopy for tuberculosis: a systematic review. *Lancet Infect Dis.* 6: 570-81

Styblo K. (1991). Epidemiology of tuberculosis. Royal Netherlands Tuberculosis Association Selected Papers, The Hague 24: 1-129

Tuberculosis. (2005). HIV Medicine. <http://www.hivmedicine.com/txtbook/cr.htm>

Urbanczik R. (1985). Present position of microscopy and of culture in diagnostic mycobacteriology. *Zbl Bakt Hyg.* A260: 81-87

Van Deun A, Maug KA, Cooreman E, Hossain MA, Chambugan N, Rema V, Marandi H, Kawria A, Portaels. (2000). Bleach sedimentation method for increased sensitivity of sputum smear microscopy: does it work? *Int J Tuberc Lung Dis.* 4: 371-376

Van Denu A, Salim AH, Cooreman E, Daru P, Das A.P.K, Aung K.J.M, Rieder H.L. (2004). Scanty AFB smears: what’s in a name? *Int J Tuberc Lung Dis.* 8: 816-23

Viera AJ, Garrett JM. (2005). Understanding interobserver agreement: The Kappa Statistic. *Fam Med.* 37: 360-3

Wilkinson D, Sturm W. (1997). Diagnosing tuberculosis in a resource-poor setting: the value of sputum concentration. *Trans R Soc Trop Med Hyg.* 91: 420-421

World Health Organization. (1998). Laboratory Services in Tuberculosis Control. Parts I-III. WHO/tuberculosis/98.258. WHO, Geneva, Switzerland.

World Health Organization. (2001). Global tuberculosis control. WHO, Geneva, Switzerland.

World Health Organization. (2003). Treatment of Tuberculosis: guidelines for national programmes. WHO, Geneva, Switzerland.

World Health Organization. (2004). Toman's Tuberculosis: Case detection, treatment and monitoring-questions and answers. WHO/HTM/TB/2004.334. WHO Geneva, Switzerland.

Yassin M.A. (2005). Diagnosis of tuberculosis in developing countries in the era of high HIV transmission. Alternative approach. Rozenberg Publishers. Amsterdam.

Yassin M.A., Cuevas LE, Gebrexabher H, Squire SB. (2003). Efficacy and safety of short-term bleach digestion of sputum in case-finding for pulmonary tuberculosis in Ethiopia. *Int J Tubec Lung Dis* 7: 678-83

Yassin M.A., Takele L, Gebresenbet S, Girma E, Lera M, Lendebo E, Cuevas LE. (2004). HIV and Tuberculosis Co-infection in the Southern Region of Ethiopia: A Prospective Epidemiological Study. *Scand J Infect Dis.* 36: 670-673

Zumla A, Malon P, Henderson J, Grange JM. (2000). Impact of HIV infection on tuberculosis. *Postgrad Med J.* 76: 259-68

• Duration of cough (weeks)

2.2. How did you come to the health center? 1. Walking 2. Bicycle

3. House cart 4. Car 5. Others

2.3. How long did it take you to arrive here? (2 hrs walking=10 km)

2.4. Do you have previous visits for evaluation of cough? 1. Yes 2. No

2.5. How many visits did you make to the health center before this visit?

4. Education level

1. Illiterate 2. Read and write 3. 1-6

4. 7-8 5. 9-10/12 6. Collage and above

5. History of previous treatment TB 1. Yes 2. No

6. Current treatment for TB 1. Yes 2. No

APPENDIX II LABORATORY DATA Acquisition Form (Direct smear)

Validation of Bleach methods for the diagnosis of Pulmonary Tuberculosis:
Bushulo Major Health Center/Awassa Health Center, Awassa, Ethiopia

Addis Ababa University Medical Faculty Department of Microbiology,
Immunology and Parasitology

1. Study code

--	--	--

2. Date of specimen collection First on spot

--	--	--

Morning

--	--	--

Second day on spot

--	--	--

3. Type of specimen: 1. Pooled 2. Spot 3. Morning

2nd day spot

4. Nature of the sputum: 1. Mucopurulent 2. Bloody 3. Saliva

5. Adequacy of specimen: 1. Scanty 2. Adequate 3. Profuse

6. Direct smear: AFB 1. Positive 2. Negative

Grade: 1. Scanty 2. + 3. ++ 4.

Name of examiner _____

Signature _____ Date ___/___/___

Comments _____

APPENDIX III LABORATORY DATA Acquisition Form (Short term)

Validation of Bleach methods for the diagnosis of Pulmonary Tuberculosis:
Bushulo Major Health Center/Awassa Health Center, Awassa, Ethiopia

Addis Ababa University Medical Faculty Department of Microbiology,
Immunology and Parasitology

1. Study code

--	--	--

2. Date of specimen collection First on spot

--	--	--

Morning

--	--	--

Second day on spot

--	--	--

3. Type of specimen: 1. Pooled 2. Spot 3. Morning

2nd day spot

4. Nature of the sputum: 1. Mucopurulent 2. Bloody 3. Saliva

5. Adequacy of specimen: 1. Scanty 2. Adequate 3. Profuse

6. Short term: AFB 1. Positive 2. Negative

Grade: 1. Scanty 2. + 3. ++ 4. +++

Name of examiner _____

Signature _____ Date ___/___/___

Comments _____

APPENDIX IV LABORATORY DATA Acquisition Form (Overnight sedimentation)

Validation of Bleach methods for the diagnosis of Pulmonary Tuberculosis:
Bushulo Major Health Center/Awassa Health Center, Awassa, Ethiopia

Addis Ababa University Medical Faculty Department of Microbiology,
Immunology and Parasitology

1. Study code

--	--	--

2. Date of specimen collection First on spot

--	--	--

Morning

--	--	--

Second day on spot

--	--	--

3. Type of specimen: 1. Pooled 2. Spot 3. Morning

2nd day spot

4. Nature of the sputum: 1. Mucopurulent 2. Bloody 3. Saliva

5. Adequacy of specimen: 1. Scanty 2. Adequate 3. Profuse

6. Overnight sedimentation: AFB 1. Positive 2. Negative

Grade: 1. Scanty 2. + 3. ++ 4. +++

Name of examiner _____

Signature _____ Date ___/___/___

Comments _____

APPENDIX V LABORATORY DATA Acquisition Form (Centrifugation)

Validation of Bleach methods for the diagnosis of Pulmonary Tuberculosis:
Bushulo Major Health Center/Awassa Health Center, Awassa, Ethiopia

Addis Ababa University Medical Faculty Department of Microbiology,
Immunology and Parasitology

1. Study code

--	--	--

2. Date of specimen collection First on spot

--	--	--

Morning

--	--	--

Second day on spot

--	--	--

3. Type of specimen: 1. Pooled

2. Spot

3. Morning

2nd day spot

4. Nature of the sputum: 1. Mucopurulent

2. Bloody

3. Saliva

5. Adequacy of specimen: 1. Scanty

2. Adequate

3. Profuse

6. Centrifugation: AFB 1. Positive

2. Negative

Grade: 1. Scanty

2. +

3. ++

4. +++

Name of examiner _____

Signature _____

Date ___/___/___

Comments _____

APPENDIX VI LABORATORY DATA Acquisition Form (Culture)

Validation of Bleach methods for the diagnosis of Pulmonary Tuberculosis:
Bushulo Major Health Center/Awassa Health Center, Awassa, Ethiopia

Addis Ababa University Medical Faculty Department of Microbiology,
Immunology and Parasitology

1. Study code

--	--	--

2. Date of specimen collection First on spot

--	--	--

Morning

--	--	--

Second day on spot

--	--	--

3. Type of specimen: 1. Pooled 2. Spot 3. Morning

2nd day spot

4. Nature of the sputum: 1. Mucopurulent 2. Bloody 3. Saliva

5. Adequacy of specimen: 1. Scanty 2. Adequate 3. Profuse

6. Culture 1. Positive 2. Negative

7. Date of inoculation

--	--	--	--	--	--

8. Date of report

--	--	--	--	--	--

Name of examiner _____

Signature _____ Date ___/___/___

Comments _____

APPENDIX VII LABORATORY PROCEDURES

1. Ziehl-Neelson staining (Hot method)

Procedure

- Fix the air dried smear with heat
- Cover the smear with carbol fuchsin
- Heat the stain until vapor just begins to rise. N.B. do not over heat
- Allow the heated stain to remain for 5 minutes
- Wash off the stain with clean water
- Cover the smear with 3% acid- alcohol and leave it until solution runs clear
- Wash well with clean water
- Cover the smear with methylene blue for 2 minutes
- Wash off the stain with clean water
- Wipe the back of the slide clean and place in draining rack for the smear to air dry
- Examine the smear microscopically

Reporting according to IUATLD/WHO scale

No. of AFB seen	Field to be examined	Grade
0	Per 100 field	Negative
1 to 9 AFB	Per 100 field	Scanty
10 to 99 AFB	Per 100 field	+
1 to 10 AFB	Per field	++
> 10 AFB	Per field	+++

2. Bleach methods

2.1. Centrifugation

Procedure

- Mix the pooled sputum sample with an equal amount of commercially available sodium hypochlorite (5 % NaOCl)
- Transfer the contents to 15 ml plastic conical tubes (blue cup tubes-15 ml).
- Incubate the mixture for 10-15 min at room temperature and will be shaken at regular interval.
- Add equal amount of distilled water to the sample,
- Centrifuge at 3000 g for 15 min.
- Discard the supernatant and the pellet will be suspended in a few drops of the remaining fluid.
- Prepare smears from the suspended sediment for ZN staining

2.2. Over night Sedimentation

Procedure

- Mix the pooled sputum sample with an equal amount of commercially available sodium hypochlorite 5 % NaOCl.
- Transfer the contents to 15 ml plastic conical tubes (blue cup tubes-15 ml).
- Incubate the mixture for 10-15 min at room temperature and will be shaken at regular interval.
- Add equal amount of distilled water to the sample,
- Leave the tube to stand for sedimentation overnight,
- Decant the supernatant,
- Make a small, thick smear (bleach smear) from the sediment in the next morning for ZN staining

2.3. Short term bleach digestion

Procedure

- Mix the pooled sputum sample with an equal amount of commercially available sodium hypochlorite 5 % NaOCl
- Shake the mixture by hand for 20 seconds
- Leave the mixture on Table-top for 30 minutes
- Take a drop of the liquefied specimen using a Pasteur pipette and put on a slide which will be left to air dry

3. Culture

Procedure

- Transfer 1-2 ml of sputum to screw-capped tubes
- Add equal volume of 4% NaOH
- Vortex the well tightened tube for 15 min
- Centrifuge the tube at 3000g for 15 min
- Decant the supernatant
- Add 1-2 ml of PBS to the tube and vortex gently
- Neutralize immediately using 2N HCL
- Inoculate the LJ media with 2-4 drops of the sediment
- Incubate the inoculated LJ media at 37°C

APPENDIX-VIII CONSENT FORM (English version)

Code number-----

Study subject initials -----

Name of the study subject -----

I have been informed about the study, which plans to validate the bleach methods for the diagnosis of pulmonary TB. The objective and the application of the study were explained to me. I am also informed that all information contained with in the questionnaire is to be kept confidential. Moreover, I have also been well informed of my right to keep hold of information, decline to cooperate and drop out of the study if I want and that none of my actions will have any bearing at all on my overall health care and hospital access.

It is therefore with full understanding of the situations that I agreed to give the informed consent voluntarily to the researcher to use sputum specimen for the investigation. I also agreed that the organisms isolated might be stored and investigated further on similar grounds. Moreover I have had the opportunity to ask questions about the project and I have received clarifications to my satisfaction.

I was also told that results would be reported timely to the requesting physicians for appropriate treatment. I agree that I am contributing to the improvement of the method by participating in this project.

I-----hereby give my consent for giving of the requested information and specimen for the purpose of validation of bleach methods.

Signature (Participant):_____ Witness (Illiterate):_____

Signature (Nurse, PI):_____

Date_____

APPENDIX IX CONSENT FORM (Amharic version)

የአማርኛ የስምምነት ቅፅ

የምስጢር ቁጥር _____

የተሳ ፊው ሙሉ ስም _____

የቤተሰብ ወይም አሳዳጊ ሙሉ ስም _____

ዝምድና _____

ኔ ስሜ ከለይ የተጠቀሰው የቲቢ ህሙማንን ለመመርመር የሚያገለግሉ የላቦራቶሪ ምርመራ ዓይነቶችን የመመርመር ብቃት ለማረጋገጥ ይረዳ ዘንድ ሊደረግ በ ቀደው ጥናት ላይ ንድሳተፍ ተጠይቄ ስለጉዳዮም ለመረዳት በቂ መረጃ አግኝቻለሁ። ስለሆነም የአክ ናሙና ወስዶ መመርመር አስፈላጊ ስለሆነ ና በተጨማሪም ለወደፊትም ለተመሳሳይ ምርመራ ከጥናቱ የተረፈውን ናሙና ለጥናት ቢያውሉት ተቃውሞ ንደሌለኝና ናሙናውን በመስጠት ልተባበር ሙሉ ፈቃደኛ መሆኔን ገልጫለሁ። ከ ኔም ሆነ ከልጄ /ከማሳድገው/ ጋት የአክ ናሙና ተወስዶ ለጥናት ቢውል ምንም ችግር ንደማይፈጥር ተረድቼ በጥናቱ ልሳተፍ ወይም ልጄ /ለማሳድገው/ጋት ንዲሳተፍ / ንድትሳተፍ ሙሉ ፈቃደኛ መሆኔን ገልጻለሁ። ከምርምሩ መሳተፍ ወይም አለመሳተፍ መብቴ የተጠበቀ መሆኑንና ላለመሳተፍ ብወስን በአዋሳ ጤና ጣቢያ/ቡቩሎ ከፍተኛ ጤና ጣቢያ በሚደረግልኝ ህክምና ላይ ምንም ተፅዕኖ ንደማይኖረው ተረድቼአለሁ።

ስለዚህ የጥናቱን ጠቃሚነት አምኔበት የስምምነት ቃሉን የሰጠሁት በፍጽም ፈቃደኝነት ነው። በመጨረሻም ኔም ሆነ ልጄ ከጥናቱ ውጤት ተጠቃሚም ልንሆን ንደምንችል ተገልጾልኝ በመሳተፍና በፈቃደኝነት በመተባበሪ ወገኖቼን ልረዳ በመቻሌ ደስተኛ መሆኔን ገልጬ አንዳንድ ማብራሪያ የሚፈልጉ ጥያቄዎችን ጠይቄ መልስ ተሰጥቶኛል።

የውል ተቀባይ ፊርማ _____ የውል ሰጪ ፊርማ _____

ምስክር /ማንበብና መፃፍ ለማይችል/ _____

ቀን _____

APPENDIX XI INFORMATION SHEET (Amharic Version)

**የቲቢ ህሙማንን ለመመርመር የሚያገለግሉ የላቦራቶሪ ምርመራ
ዓይነቶችን የመመርመር ብቃት ለማረጋገጥ የሚደረግ ጥናት
ማብራሪያ ቅጽ**

አዋሳ ጤና ጣቢያ / ቡሽሎ ከፍተኛ ጤና ጣቢያ -አዋሳ- ኢትዮጵያ
ለጥናቱ ተሳ ፊዎች/ለወላጆች/ለአሳዳጊዎች
(ከ18 ዓመት ድሜ በ ች ለሆኑ ተሳ ፊዎች)

ዋና ተመራማሪ:- ያሬድ መርዕድ
ምርምሩን የሚያካሂዱበት ተቋም:- አዋሳ ጤና ጣቢያ ና ቡሽሎ ከፍተኛ
ጤና ጣቢያ

1. የጥናቱ ዓላማ:-

ጥናቱ የሚካሄደው የቲቢ ህሙማንን ለመመርመር የሚያገለግሉ የላቦራቶሪ ምርመራ ዓይነቶችን የመመርመር ብቃት በማነጻጸርና በማረጋገጥ ለአሰራር የተሽለዉን ለመጠቀም

2. የተሳትፎ ሁኔ

በጥናቱ ሊሳተፉ ከተስማሙ

1. ሐኪሙ ወይም ዋናው ተመራማሪ ለሚጠይቁት ጥያቄዎች መልስ መስጠት

ለምሳሌ፡- የራስዎን/የልጅዎን ዕድሜ/ ሳሉ መቼ ንደተከሰተ/
ይህን ህክምና ለማግኘት ምን ያህል ርቀት ተገዘዉ ንደመጡ .
. .

2. ለምርመራው የሚሆን ትንሽ የአክ ናሙና መስጠት

3. ሊከሰቱ ስለሚችሉ ስጋቶችና የምቶት መጓደሎች

ለጥናቱ በሚሰጡት ናሙና ምክንያት ምንም አይነት ችግር አይከሰትም ምክንያቱም የናሙና አሰጣጡ ሂደት ለተለመደው የቲቢ የላቦራቶሪ ምርመራ ከሚሰጡት የተለየ ባለመሆኑ ና የሚያደርሰውም ጉዳት ባለመኖሩ ነዉ.

4. ጥቅሞች

የምርመራው ዉጤት የቲቢ ከሚዎች በተለይም ደሃው የህብረተሰብ ክፍል የላቦራቶሪ ምርመራን ለማግኘት በተደጋጋሚ ወደ ጤና ድርጅቶች የሚመላለሱበትን ጊዜ ለማሳጠር ይጠቅማል። ይህም በጤና ድርጅቶችና በህመማን ይወጣ የነበረዉን ወጪ የሰው ሀይልና ጊዜን ይቀንሳል። በተጨማሪም የላቦራቶሪ ምርመራ የሚያደርገውን ባለሙያ የስራ ተነሳሽነት ይጨምራል።

5. ምስጢር ስመጠበቅ

ለሁሉም ጥያቄዎች የሰጡንን መልሶች በምስጢር ንደምንይዝ ሊያውቁ ይገባል። የተወሰደው ናሙና ለጥናት ብቻ የሚውል ነው። የ ርስዎም ሆነ የልጅዎ ማንነት በስም አይገለጽም።

6. በጥናቱ ያለመሳተፍ ወይም ራስን የማግለል መብት፡-

- የ ርስዎም ሆነ የልጅዎ ተሳትፎ በፈቃደኝነት ላይ የተመሰረተ ነው

- በጥናቱ ውስጥ መግባት አለመፈለግም በጤና ንክብካቤዎ ላይ ምንም ዓይነት ተፅዕኖ አይኖረውም
- ርስዎ በጥናቱ ላይ መሳተፍዎትን ለመሰረዝ ከፈለጉ በማንኛውም ሰዓት መሰረዝ ይችላሉ። ይህንንም ከወሰኑ ማንም ምክንያቱን ንዲገልጹ ሊያስገድድዎት አይችልም።

7. መረጃ ስለ ማግኘት

ይህ ጥናት በአህሪ (አለርት) ሥነ ምግባር ቅኝት ግምገማ ኮሚቴ በአዲስ አበባ ዩኒቨርስቲ [ሜዲካል](#) ፋክልቲ የድህረ ምረቃ ክፍል በክልሉ ጤና ቢሮ በአዋሳ ጤና ጣቢያና በቡቨሎ ክፍተኛ ጤና ጣቢያ ድጋፍ አግኝቷል። የ ነዚህ ኮሚቴዎች ሥራ የጥናቱን ተሳ ፊዎች ከጉዳት መጠበቃቸውን ማረጋገጥ ነው። ማንኛውም ዓይነት ጥያቄ ካለዎት፣ ርግጠኛ ሆነው ራስዎንም ሆነ ልጅዎትን ለጥናቱ ለማሳተፍ ስኬወስኑ ድረስ ተገቢ የሆነውን ማንኛውም መረጃ የመጠየቅ መብት አለዎት።

Signature

.....

Place

Addis Ababa

Date

...../...../.....