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ABSTRACT

The lack of simple, accurate and rapid diagnostics is a major hindrance to TB control efforts, especially in developing countries including Ethiopia where sputum smear microscopy is the mainstay of diagnosis. Therefore, the need for rapid and accurate diagnostics is crucial. The new Speed-oligo Direct *Mycobacterium tuberculosis* (SO-DMT) assay is a novel assay which is based on multiplex PCR combined with dipstick hybridization that allows amplification of 16S rRNA and IS*6110* to detect genus *Mycobacterium* and *Mycobacterium tuberculosis* complex from respiratory samples, respectively. The objective of the study is to evaluate the performance of the new SO-DMT assay in relation to conventional microscopic and culture methods and Xpert MTB/RIF assay for the detection of *M. tuberculosis* complex directly from sputum samples in SNNPR, Ethiopia. A total of 145 sputum samples were included in the evaluation of SO-DMT assay. One sample per patient was decontaminated by NALC-NaOH method to perform Ziehl-Neelsen stain, culture and SO-DMT assay. One hundred nine of the sputum specimens were also tested by Xpert MTB/RIF assay. The sensitivity, specificity, positive predictive value and negative predictive value of SO-DMT assay for detection of MTBC were 96%, 97.8%, 99% and 91.7%, respectively with reference to culture. The corresponding values after resolution of discrepant results with culture and clinical data were 96% (100% in smear-positives and 89.5% in smear-negatives), 100%, 100% and 91.7% (100% in smear-positives and 90.5% in smear-negatives), respectively. The concordance between SO-DMT assay results and culture had a Cohen's kappa index of 0.921 (SE, 0.035), indicating excellent concordance. The sensitivity of both SO-DMT and Xpert MTB/RIF assays was 94.8% (100% in smear-positives and 87.1% in smear-negatives). The specificity of SO-DMT and Xpert MTB/RIF assays were 100% and 93.8% (100% in smear-positives and 93% in smear-negatives), respectively. Therefore, SO-DMT has a good sensitivity and specificity in smear-positive and smear negative specimens. It's use can avoid the need to wait for culture results. This assay might be a good alternative to real-time PCR assays for laboratories not equipped with real-time PCR instruments.

Key words: Löwenstein-Jensen culture, SO-DMT assay, Xpert MTB/RIF assay, Ziehl-Neelsen staining

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LIST OF ABBREVIATIONS

AFB	Acid-Fast Bacilli
AMDT	Amplicor *Mycobacterium tuberculosis* Direct Test
AMK	Amikacin
BCG	Bacillus Calmette Guérin
bp	Base Pair
CAP	Capreomycin
CDC	Centers for Disease Control and Prevention
CI	Confidence Interval
Cs	Cycloserine
DOTS	Directly Observed Treatment Short-Course
EMB	Ethambutol
ETO	Ethionamide
FMOH	Federal Ministry of Health
IAC	Internal Amplification Control
INH	Isoniazid
IS*6110*	Insertion Sequence 6110
KAN	Knamycin
LFX	Levofloxacin
LJ	Löwenstein-Jensen
LR	Likelihood Ratio
MBP64	*Mycobacterium tuberculosis* Protein 64
MDR	Multidrug Resistant

MOTT	Mycobacteria Other Than Tuberculosis
MTB	*Mycobacterium tuberculosis*
MTBC	*Mycobacterium tuberculosis* Complex
NAAT	Nucleic Acid Amplification Test
NALC	N-acetyl-L-cysteine
NPV	Negative Predictive Value
ODAC	Oleic Acid, Albumin, Dextrose and Catalase
PANTA	Polymyxin B, Amphotericin B, Nalidixic Acid, Trimethoprim and Azlocillin
PCR	Polymerase Chain Reaction
PPD	Purified Protein Derivate
PPV	Positive Predictive Value
PZA	Pyrazinamide
rDNA	Ribosomal Deoxyribonucleic Acid
RIF	Rifampicin
RT-PCR	Real Time-Polymerase Chain Reaction
SDA	Strand Displacement Amplification
SE	Standard Error
SNNPRS	Southern Nations, Nationalities and People's Regional State
SO-DMT	Speed-oligo Direct *Mycobacterium tuberculosis*
SPSS	Statistical Package for Social Sciences
STM	Streptomycin
TB	Tuberculosis

TMA	Transcription Mediated Amplification
TST	Tuberculin Skin Test
US FDA	United States Food and Drug Administration
XDR	Extensively Drug Resistant
WHO	World Health Organization
ZN	Ziehl-Neelsen

1. INTRODUCTION

Tuberculosis (TB) is one of the oldest infectious diseases. It was discovered in 1882 by Robert Koch (Sakula, 1982). It is an airborne, infectious disease caused by mycobacteria belonging to *Mycobacterium tuberculosis* complex (MTBC) which primarily attacks the lungs. It remains an important cause of morbidity, mortality and economic loss in the world (LoBue *et al.,* 2010). According to the World Health Organization (WHO) report there were almost 8.6 million new cases and 1.3 million TB deaths in 2012 (WHO, 2013).

TB is also one of the most common causes of death in human immunodeficiency virus (HIV) positive adults living in less-developed countries (Corbett *et al.*, 2003). A most serious aspect of the problem is the emergence of strains resistant to multiple drugs which are more difficult and expensive to treat, especially in settings where TB control programs could not adequately monitor treatment regimens for multidrug-resistant (MDR)-TB (Shah *et al.,* 2007). This situation has been further complicated by the recent emergence of extensively drug-resistant (XDR)-TB to which treatment options are limited (Migliori *et al.,* 2008).

Rapid diagnosis of TB is one of the cornerstones of global TB control as it allows therapeutic interventions (Alcaide and Coll, 2011). For the detection of *M. tuberculosis*, microscopic examination of acid-fast stained smears and culture are still the methods of choice in most microbiological diagnostic laboratories. However, none of these methods are really satisfactory due to poor sensitivity of microscopic examination (45% to 89%)

and the slow growth of *M. tuberculosis* on culture often leads to a delay in TB diagnosis (Soini and Musser, 2001; Davies and Pai, 2008). A delay in diagnosing TB lead to increased morbidity and mortality in patients, as well as the spread of TB and associated drug resistances to the community (LoBue *et al.*, 2010). Therefore, the most effective means of protection is early diagnosis and treatment of the disease. Consequently, several molecular methods have been recently introduced for rapid detection and identification of the MTBC from clinical specimens (Piersimoni and Scarparo, 2003).

Speed-oligo Direct *Mycobacterium tuberculosis* (SO-DMT: Vircell SL, Santa Fe, Granada, Spain) is a novel assay which is based on multiplex polymerase chain reaction (PCR) and dipstick hybridization detecting genus *Mycobacterium* and MTBC, directly from clinical samples. This study was aimed to assess the performance of the SO-DMT assay for the diagnosis of tuberculosis from sputum specimens and compare its results with those of microscopic examination by Ziehl-Neelsen (ZN) acid-fast stain, conventional Löwenstein-Jensen (LJ) culture and Xpert MTB/RIF assay.

1.1. Statement of the problem

The lack of simple, accurate and rapid diagnostics is a major hindrance to TB control efforts, especially in developing countries including Ethiopia where sputum smear microscopy is the mainstay of diagnosis. Therefore, the need for innovative and newer diagnostic tools such as the SO-DMT assay is high that can improve case detection and initiate and accelerate treatment. Even though, the introduction of this new technology and demonstration studies are currently underway and preliminary data appear to be

promising, such technology has not been used as part of a screening strategy for TB case detection in Ethiopia. Furthermore, its operational feasibility as a point-of-care diagnostic technology for improving case detection based at local TB clinics has not yet been assessed.

1.2. Significance of the study

The evaluation of the incremental diagnostic success of SO-DMT assay will have a significant impact and future policy implications on implementation of this new diagnostic tool in improving TB case detection efforts at the local clinics. It can also be used as a base reference for establishment of further improved health network platforms at the local and national level that could have immediate solutions for rapid and improved case detection with accelerated treatment.

2. LITERATURE REVIEW

2.1. Historical background

Tuberculosis is believed to be one of the oldest human diseases. It is caused by mycobacteria belonging to MTBC (Gutierrez *et al.*, 2005). An early progenitor of *M. tuberculosis* was probably contemporaneous and coevolved with early hominids in East Africa, three million years ago. The modern members of *M. tuberculosis* are thought to have originated from a common ancestor about 15,000-35,000 years ago (Gutierrez *et al.*, 2005). Studies of skeletal remains have also revealed that tuberculosis has existed for thousands of years in Egypt (Zink *et al.*, 2003; Zink *et al.*, 2007). In addition, documents exist to show the presence of the disease in India and China as early as 3,300 and 2,300 years ago, respectively (Daniel, 2006).

Around 460BC, Hippocrates has described TB as phthisis (consumption) which is the most widespread disease of the time. He also considered the disease to be contagious (Sakula, 1982). At the beginning of the 17th century, the TB epidemic in Europe was known as the “great white plague”. In the field of tuberculosis, the first major breakthrough was by the Jean-Antoine Villemin, who in 1865 demonstrated that TB could be passed from humans or cow to rabbit or guinea-pig, and that the sputum of a consumptive could infect a rabbit (Sakula, 1982). As years passed the cause of the TB remained in the dark. Eventually, Robert Koch discovered the tubercle bacillus in 1882 (Sakula, 1982). Koch’s discovery was a breakthrough leading to other important findings such as staining and culture techniques, which contributed to the fight against TB (Sakula, 1982).

In the 19th century, the introduction of the sanatorium cure provided the first widely practiced approach to anti-TB treatment. Patients were isolated in sanatoria and given treatments such as injecting air into the chest cavity. Attempts were also made to decrease lung size by surgery called thoracoplasty (Palomino *et al.*, 2007). At the beginning of the 20th century, Albert Calmette and Camille Guérin, obtained the Bacillus Calmette Guerin (BCG) vaccine from isolated attenuated *Mycobacterium bovis* strain. BCG was first administered to humans as oral vaccine in 1921 and still widely applied today (Palomino *et al.*, 2007).

During the first half of the 20th century, no effective treatment was available. However, in 1944 streptomycin (STM) was first introduced for TB treatment (Zhang and Yew, 2009). A considerable improvement in the disease was observed in patients on STM therapy, but soon after the first months, treatment failed as a consequence of development of resistance to the drug (Gillespie, 2002). At the end of 1944, para-aminosalicylic acid was produced but to which *M. tuberculosis* could easily again developed resistance. Then isoniazid (INH) became available in 1952 (Palomino *et al.*, 2007). Clinicians soon recognized that if all these drugs were given simultaneously, drug resistance did not emerge (Iseman, 1994).

2.2. Epidemiology

2.2.1. Global epidemiology

Tuberculosis remains a major global health problem. It causes illness among millions of people each year and ranks as the second leading cause of death from an infectious

disease worldwide, after the HIV (WHO, 2013). There were an estimated 8.6 million new cases detected in 2012 and nearly 1.3 million people died of tuberculosis, equivalent to 122 cases per 100,000 population. Most of the estimated number of cases in 2012 occurred in Asia (58%) and Africa (27%) and smaller proportions of cases occurred in the Eastern Mediterranean Region (8%), the European Region (4%) and the Region of the Americas (3%). The 22 high burden tuberculosis countries account for 81% of all estimated cases worldwide. Among the 22 high burden countries, the five countries with the largest number of incident cases in 2012 were India, China, South Africa, Indonesia and Pakistan, respectively. India and China alone accounted for 26% and 12% of global cases, respectively. As shown in Figure 1 the number of incident TB cases relative to population varies widely among countries (WHO, 2013).

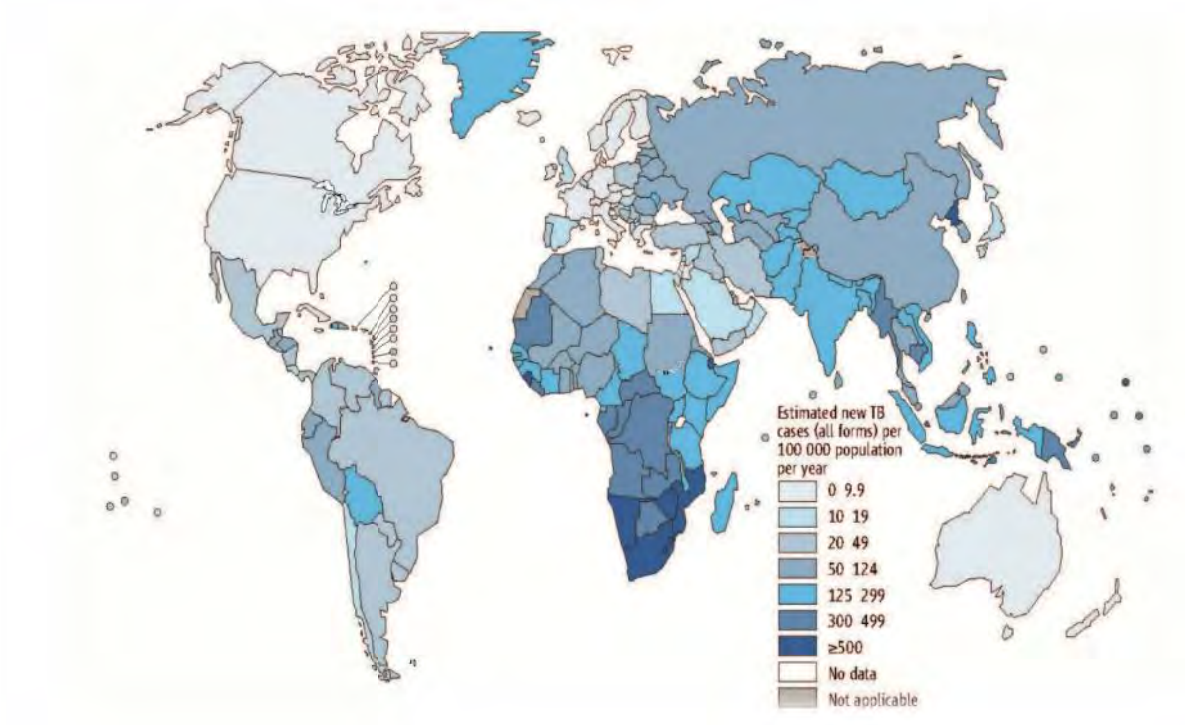


Figure 1. Estimated TB incidence rate in 2012 (WHO, 2013).

Tuberculosis epidemic is further compounded by the HIV/AIDS pandemic which weakens the immune system and hence favors progression into active TB disease (Corbett *et al.*, 2003). Of 8.6 million incident cases in 2012, an estimated 1.1 million (13%) were HIV positive. The proportion of TB cases co-infected with HIV was highest in countries in the African Region. Overall, 37% of TB cases were estimated to be co-infected with HIV in this region, which accounted for 75% of TB cases among people living with HIV worldwide (WHO, 2013). Poor TB control policies, inadequate resources for early diagnosis and treatment have contributed to the burden of TB in these developing countries, particularly in the sub-Saharan region (Getahun *et al.*, 2010).

In several countries, HIV has been associated with epidemic outbreaks of TB. Many of the reported outbreaks involved multidrug-resistant strains, which respond poorly to standard therapy the growing burden of TB (Corbett *et al.*, 2003). MDR-TB is defined as a disease caused by *M. tuberculosis* resistant to at least rifampicin (RIF) and INH (Iseman, 1993; Jain and Dixit, 2008). Globally, 3.6% of new cases and 20.2% of previously treated cases were estimated to have MDR-TB. The highest levels are in eastern Europe and central Asia where in many countries, more than 20% of new cases and more than 50% previously treated cases have MDR-TB (WHO, 2013). More recently, the emergence of XDR forms a serious obstacle to the global TB control. XDR-TB is defined as MDR-TB resistant to any fluoroquinolone, and at least one of the three following injectable drugs capreomycin (CAP), kanamycin (KAM) and amikacin (AMK) used for MDR-TB treatment (CDC, 2006). XDR-TB has been identified in 92 countries.

The average estimated proportion of MDR-TB cases with XDR-TB is 9.2% (WHO, 2013).

2.2.2. The TB situation in Ethiopia

TB still remains one of the major unresolved health problems in Ethiopia. It has long been recognized as a major public health problem since the 1950s (FMOH, 2009) and the country has been implementing the WHO recommended directly observed treatment short-course (DOTS) strategy since 1990s (FMOH, 2009). The Ministry of Health hospital statistics data showed that TB is one of the leading causes of morbidity, the fourth most common cause of hospital admission and the second most common cause of hospital death in Ethiopia (FMOH, 2009). The country ranked seventh in the list of 22 high burden countries severely affected by tuberculosis with an estimated incidence rate of 247 cases per 100,000 populations (WHO, 2013). TB mortality rate is estimated at 18 per 100,000 populations per year (WHO, 2013). The high prevalence of HIV infection, widespread poverty and overcrowding has created an environment which made tuberculosis a formidable threat in Ethiopia (FMOH, 2009).

Southern Nations, Nationalities and People's Regional State (SNNPRS) is one of the Federal States of Ethiopia. The burden of TB in the southern region of Ethiopia is attributed to HIV co-infection, TB drug resistance and poverty (Cambanis *et al.*, 2005). Important factors which further complicate the control of TB in the region include poor implementation of TB control programs, poor health access service, delays in diagnosis and treatment, inadequate treatment supervision and patient follow-up, ineffective

management of patients and the significance of completing treatment by patients (Shargie *et al.*, 2006; Shargie and Lindtjørn, 2007; Datiko and Lindtjørn, 2009).

2.3. Mycobacterium

Genus *Mycobacterium* belongs to *Mycobacteriacae* family, *Actinomycetales* order and *Actinomycetes* class (Shinnick and Good, 1994). All mycobacteria are acid-fast, aerobic, non-motile, non-capsulated and non-sporing. They have a high content of guanine plus cytosine (61 to 71%) in the genomic deoxyribonucleic acid (DNA). All *Mycobacterium* species share a characteristic cell wall thicker than in many other bacteria, which is hydrophobic, waxy and rich in mycolic acids, which make them acid and alcohol fast (Shinnick and Good, 1994). The genus *Mycobacterium* comprises more than 120 different species (Tortoli, 2006). The genus includes saprophytic species that are widespread in nature as well as the causative pathogens of the major human disease complexes tuberculosis and leprosy (Kayser *et al.*, 2005).

Mycobacterium leprae also known as Hansen`s bacillus, was discovered in 1873 by Gerhard Armauer Hansen in the skin nodules of patients with leprosy. It was the first bacterium to be identified as a cause of a human disease. It cannot be grown on nutrient media or in cell cultures. For this reason, the microscopy is the common way to detect leprosy (Kayser *et al.*, 2005).

Mycobacteria that are neither tuberculosis nor leprosy bacteria are categorized as atypical mycobacteria, non-tuberculosis mycobacteria or mycobacteria other than tuberculosis

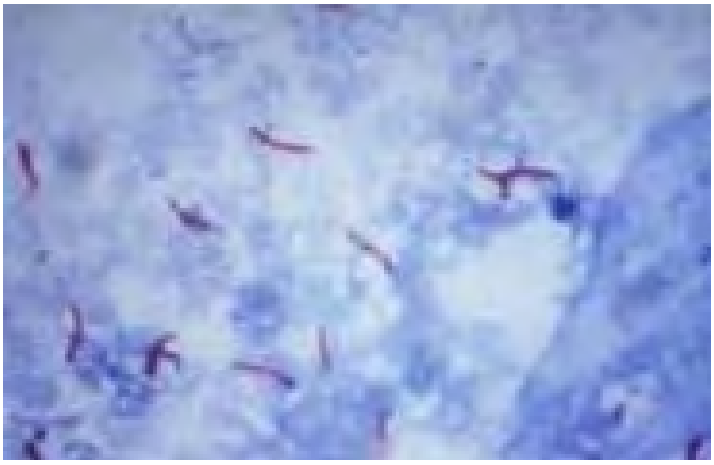
(MOTT). MOTT is frequent inhabitants of the natural environment such as water, soil, etc and also contributes to human and animal mucosal flora. The majority of MOTT is living ubiquitously in nature. However, MOTT infections are generally rare. Their occurrence is encouraged by compromised cellular immunity and frequent occurrence is observed together with certain malignancies, in immunesuppressed patients and in AIDS patients (Kayser *et al.*, 2005).

2.3.1. *Mycobacterium tuberculosis* complex (MTBC)

Mycobacterium tuberculosis complex is the etiological agents that cause TB. The MTBC comprises *M. canettii, M. tuberculosis*, *M. africanum, M. pinnipedii, M. microti, M. caprae, M. bovis* and *M. bovis* BCG (Gutierrez *et al.*, 2005). The human pathogens include *M. tuberculosis, M. africanum* and *M. canettii* and the animal adapted species include *M. bovis* (bovine), *M. caprae* (goats), *M. pinnipedii* (pinnipeds) and *M. microti* (rodents) (Mostowya and Behr, 2005). The MTBC consists of bacteria that genetically share identical 16S ribosomal ribonucleic acid (rRNA) sequence and greater than 99.9% nucleotide identity (Sreevatsan *et al.*, 1997; Brosch *et al.*, 2002). However, they differ in phenotypic traits such as host preference, virulence and epidemiology (Mostowya and Behr, 2005). Of these species, *M. tuberculosis* is the main bacterium inducing disease in humans (Shinnick and Good, 1994).

2.3.2. *Mycobacterium tuberculosis*

Mycobacterium tuberculosis is the causative agent of most cases of tuberculosis. It is a gram positive, non-motile, non-sporing, aerobic, catalase positive, straight or slightly



destruction by macrophages and intracellulary multiply in the alveoli of the lungs (Smith, 2003). This is due to their ability to inhibit formation of the phagolysosome (Kayser *et al.*, 2005). This may eventually kill the macrophages causing the release of the tubercle bacilli into the bloodstream. If alive, the bacilli may disseminate throughout the systemic circulation and affect other body tissues and organs, including areas in which TB disease is most likely to develop such as the apices of the lungs, the kidneys, the brain, the bones and through the lymphatic system to regional lymph nodes (LoBue *et al.*, 2010).

After several weeks later, T-lymphocytes begin to proliferate and secrete lymphokines. The lymphokines activate the macrophages that trigger cellular immunity. The immune response kills most of the bacilli and the remaining bacilli are confined through the formation of granulomas. At this point, latent TB infection has been established, which may be detected using the tuberculin skin test (TST) or interferon-gamma release assay (LoBue *et al.*, 2010). Latent TB infection is a form of disease in which the tubercle bacilli remains dormant inside the person's body without any symptoms or without the active form of TB (Saleem and Azher, 2013).

Most infected people contain the infection by efficient immune response. In some people, the tubercle bacilli overcome the defenses of the immune system and begin to multiply, resulting in the progression from LTBI to TB disease (LoBue *et al.*, 2010). Persons who have TB disease are usually infectious and may spread the bacteria to other people (Saleem and Azher, 2013). TB disease may occur soon after or many years after infection. Unless treated, approximately 3-5% of persons who have been infected with *M.*

tuberculosis will develop TB disease in the first 2 years after infection and another 2-5% will develop disease at some time later in life. Thus, approximately 5-10% of persons with normal immune systems who are infected with *M. tuberculosis* will develop TB disease at some point in their lives (LoBue *et al.*, 2010). TB disease most commonly affects the lungs and it is called pulmonary TB. On the other hand, extra-pulmonary TB disease occurs in places other than the lungs, including the larynx, the lymph nodes, the pleura, the brain, the liver, the kidneys or the bones and joints (Palomino *et al.*, 2007). It is usually a long term effect of dissemination occurring at the time of initial infection but developing perhaps many years later (Davies and Pai, 2008).

HIV, malnutrition, smoking and immunosuppressive treatments are risk factors for the development of an active disease, since these weaken the cell-mediated immune system of infected person (Davies and Pai, 2008). Overcrowded and enclosed places such as hospitals, schools, day nurseries, refugee camps and prisons increase the risk of transmission of TB (Palomino *et al.*, 2007; Getahun *et al.*, 2010).

Pulmonary TB symptoms are cough, sometimes with haemoptysis, fever, fatigue, weight loss, night sweats, dyspnoea, which may develop late as a considerable part of the lung is destroyed and chest pain (Davies and Pai, 2008). These symptoms are used for diagnosis of TB but it is difficult to diagnose on the basis of signs and symptoms alone because the symptoms mimics with other respiratory problems (Davies and Pai, 2008).

2.5. Diagnosis of tuberculosis

The clinical diagnosis of TB is based on patient history, chest radiography, symptoms, physical examination and a positive TST (Davies and Pai, 2008). The TST is the oldest diagnostic test for TB. In this test, purified protein derivate (PPD) is applied intracutaneously and the localized immune response is measured at the site of antigen application. A positive reaction appears within 48 to 72 hr as an inflammatory reaction at least 10 mm in diameter at the site of antigen application (Kayser *et al.*, 2005). The TST is indicative of mycobacterial infection, but does not discriminate between latent and active disease. Additionally, PPD is not specific for *M. tuberculosis*; therefore, it can lead to false positive results in persons vaccinated with BCG and those exposed to some of the environmental or non-tuberculosis mycobacteria (Huebner *et al.*, 1993).

2.5.1. Bacteriological diagnosis of TB

Laboratory diagnosis of TB has traditionally been based on smear microscopy, culture and phenotypic identification (Alcaide and Coll, 2011). Ziehl-Neelsen acid-fast staining is a rapid, simple and less expensive method for the demonstration of the acid-fast mycobacteria (Alcaide and Coll, 2011). However, it has a low sensitivity, requiring 5000-10,000 bacilli per ml to become positive (Kansal *et al.*, 2014). The diagnosis of TB becomes even more problematic in extra-pulmonary forms of TB and in HIV-infected patients. It cannot also differentiate live bacilli from inactive or dead bacilli (Palomino, 2005).

A definitive diagnosis of active TB infection depends on the isolation and identification of mycobacteria from the clinical specimen by culture (Alcaide and Coll, 2011). Generally, media used to culture mycobacteria fall into three categories: egg-based solid media, agar-based solid media and liquid media. Conventional methods for mycobacterial culture utilize media containing agar-based Middlebrook or egg-based Löwenstein-Jensen media. The culture technique is regarded as the gold standard for TB diagnosis due to its good sensitivity (Palomino, 2005). Cultures of mycobacteria require only 10-100 organisms to detect *M. tuberculosis* (Davies and Pai, 2008; Kansal *et al.*, 2014). However, the slow growth (3-8 weeks) of the tubercle bacillus is a major obstacle to rapid disease diagnosis (Palomino, 2005).

The introduction of broth based growth systems such as Bactec 460 TB system and mycobacteria growth indicator tube (MGIT, Becton Dickinson Microbiology Systems) have significantly reduced the time to detection. MGIT contains a modified Middlebrook 7H9 broth to which oleic acid, albumin, dextrose and catalase (OADC) enrichments and polymyxin B, amphotericin B, nalidixic acid, trimethoprim and azlocillin (PANTA) antibiotic supplements are added in conjunction with a fluorescence quenching-based oxygen sensor at the bottom of the tube (Pfyffer *et al.*, 1997; Chew *et al.*, 1998). Bacterial growth utilizes oxygen in the medium and the indicator fluoresces. Cultures can be read rapidly with a 365 nm ultraviolet Wood's lamp or MGIT instrument (Palaci *et al.*, 1996; Bemer *et al.*, 2002). However, several days (12.7 days on average) are still required to obtain the final laboratory result (Palomino, 2005).

After a positive culture is obtained, species identification of mycobacteria has traditionally been based on different biochemical tests and phenotypic characteristics such as growth rate, pigmentation and colonial morphology that allow classification of a particular strain to a group of well defined mycobacteria. These methods are simple to perform and do not require sophisticated equipment. Nevertheless, they are laborious, cumbersome and give ambiguous results. The methods are inadequate for the effective control of TB (Palomino, 2005). Therefore, alternative methods that provide rapid, sensitive and accurate results for detection of active TB in clinical specimens are essential for the successful diagnosis of TB patients.

2.5.2. Molecular diagnosis of TB

In recent years, nucleic acid amplification tests (NAATs) have become more common in the diagnosis of TB leading to considerable improvement of both speed and accuracy of diagnosis of tuberculosis. These tests specifically amplify the genetic material of the causative organisms to detectable levels through the PCR (Soini and Musser, 2001). NAATs include a wide variety of 'in house' methods with multiple protocols of nucleic acid extraction and PCR amplification of different genetic targets, such as IS*6110*, *rpo*B, 16S ribosomal DNA (rDNA) or MBP64. Although these 'in house' amplification tests have generally improved in recent years, the recommendation is to use commercial tests that have a greater level of standardization, reproducibility and automation. NAATs have some disadvantages, for example, problems with inhibitors, sensitivity in smear-negative samples and DNA extraction (Alcaide and Coll, 2011). Therefore, such assays do not completely replace the conventional laboratory approach to the diagnosis of active

disease (Davies and Pai, 2008). NAATs can be used for direct detection of TB in clinical specimens, the identification of the species in grown-up cultures, as well as the detection of genetic mutations related with the resistance to main antibiotics (Soini and Musser, 2001).

2.5.3. Direct genotypic detection of mycobacteria in clinical specimens

NAATs amplify *M. tuberculosis* specific nucleic acid sequences with a nucleic acid probe, enabling direct detection of *M. tuberculosis* in clinical specimens (Davies and Pai, 2008). These methods provide an early detection with high sensitivity that shows clear advantages over the traditional methods that have mentioned above. Some of commercially available NAATs used for direct detection of *M. tuberculosis* in clinical specimens are shown in Table 1.

The Amplicor *Mycobacterium tuberculosis* Test (Roche Diagnostic System Inc., Basel, Switzerland) is a DNA based test that amplifies a specific segment, which is a 584 bp fragment of the 16S rRNA gene using genus-specific primers, followed by hybridization to oligonucleotide probes and detection by colorimetric reaction (Dalovisio *et al.*, 1996). Cobas Amplicor is an automated version of this test. It is approved in 1996 by the US Food and Drug Administration (FDA) for direct detection of *M. tuberculosis* in AFB smear-positive respiratory samples (Soini and Musser, 2001). The turnaround time of this assay is 6-7 hr. A literature has shown the overall specificity for Amplicor ranges from 91.3% to 100% (Piersimoni and Scarparo, 2003). Overall sensitivity ranges from 83% to

96.7% for respiratory samples, from 90% to 100% in smear-positive samples and 50% to 95.9% in smear-negative samples (Piersimoni and Scarparo, 2003).

The Amplified *Mycobacterium tuberculosis* Direct Test (AMTD; Gen-Probe Inc., San Diego, CA, USA) is a rapid isothermal (42ºC) method which is based on the amplification of 16S rRNA (Piersimoni and Scarparo, 2003). In this assay, rRNA is released from the target cells by sonication and then a promoter-primer binds to the rRNA target. Reverse transcriptase creates DNA copy of the target. Then the initial RNA strand is degraded from the complementary DNA-RNA, and a second primer binds to the cDNA and extended, leading to the formation of double-stranded cDNA, which is then transcribed by DNA-directed RNA polymerase to produce more rRNA molecules. The new transcripts serve as templates for reverse transcription and further amplification. The RNA amplicons are detected with an acridinium ester-labeled DNA probe in a solution hybridization assay (Abe *et al.*, 1993; Soini and Musser, 2001). The reaction is performed in a single tube, which helps to reduce carryover contamination. The enhanced MTD test is approved by US FDA for detection of *M. tuberculosis* in both AFB smear positive and negative respiratory specimens (Soini and Musser, 2001). The literature reported that overall specificity of AMTD ranges from 92.1% to 100% in respiratory specimens. The overall test sensitivity ranges from 85.7 to 97.8%. Sensitivity ranges from 91.7% to 100% for smear-positive and 65.5% to 92.9% for smear-negative samples (Piersimoni and Scarparo, 2003). Although the turnaround time is 2.5 hr, AMTD lacks an internal amplification control (Alcaide and Coll, 2011).

The BD ProbeTec MTB test (Becton Dickinson, Sparks, MD) was introduced in 1998 as a semi-automated technique for rapid detection of MTBC in respiratory samples. It is an isothermal (52.5ºC) amplification process based on the strand-displacement amplification technique that uses enzymatic replication of target sequences in IS*6110* and the 16S rRNA gene. The amplified product is detected by luminometer (Bergmann and Woods, 1998; Palomino, 2005; Palomino, 2009). The BD ProbeTec ET is an improved version, which has an internal control to detect the presence of inhibitors (Palomino, 2009). A literature report has shown that it has a sensitivity of 98.5% to 100% in smear-positive samples and 33.3% to 85.7% in smear-negative ones, and specificity of 98.9% to 100% in respiratory samples (Piersimoni and Scarparo, 2003). The turnaround time of the test is 3.5 to 4 hr. The system is not yet approved by the US FDA (Palomino, 2009).

2.5.3.1. Xpert MTB/RIF assay

Real-time PCR (RT-PCR) techniques are based on simultaneous amplification of different DNA targets and fluorimetric detection by labeled probes. These tests have a number of important advantages, especially their rapidity and fewer cross contamination problems due to the processes after DNA extraction occur in a single tube (Palomino, 2005). The GeneXpert MTB/RIF (Cepheid, Sunnyvale, CA) is a recently introduced RT-PCR based assay which is endorsed by the WHO for the rapid diagnosis of TB directly from clinical specimens. It is fully automated and integrates sample preparation, DNA amplification and detection (WHO, 2011). It is designed to amplify a 192 bp segment of the MTBC *rpo*B gene using 5 overlapping molecular beacon probes that span the entire 81 bp rifampicin resistance determining region. The Xpert MTB/RIF assay also contains

an internal control in which the detection of lyophilized *Bacillus globigii* spores serve as an internal processing and amplification control (Miller *et al.,* 2011). The assay provides results directly from sputum in less than 2 hr. It has been reported that the overall specificity ranges from 92% to 100%. Sensitivity ranges from 95% to 100% for smear-positive and 43.4% to 84.6% for smear-negative respiratory samples (Bateson *et al.*, 2013).

Table 1. Commercially available NAATs for the detection of the MTBC (Bateson *et al.*, 2013).

NAAT name	**Manufactu-rer**	**Amplification method**	**Sensitivity range**		**Specificity range**
			Smear +ve samples	**Smear -ve samples**	
Amplicor MTB test	Roche Molecular Systems	PCR	90–100	50–95.9	91.3–100
Amplified MTD test	Gen-Probe Inc	TMA	91.7–100	65.5–92.9	92.1–100
BD ProbeTec ET	Becton Dickinson	SDA	98.5–100	33.3–85.7	98.9–100
Xpert MTB/RIF	Cepheid	PCR	95–100	43.4–84.6	92–100

NAAT = nucleic acid amplification test; PCR = polymerase chain reaction; TMA = transcription mediated amplification; SDA = strand displacement amplification; +ve = positive; -ve = negative; MTB = *Mycobacterium tuberculosis*; RIF = rifampicin

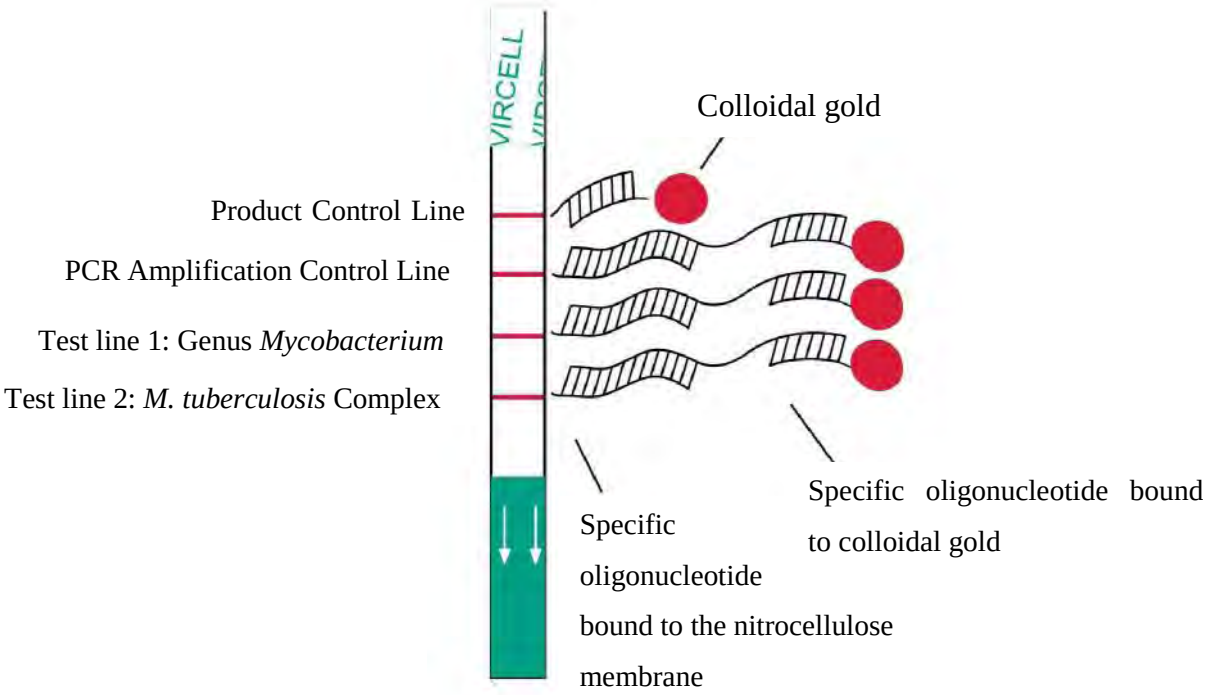
2.5.3.2. Speed-oligo Direct *Mycobacterium tuberculosis* assay

Speed-oligo Direct *Mycobacterium tuberculosis* assay (SO-DMT; Vircell SL, Santa Fe, Granada, Spain) is a novel assay, based on multiplex PCR dipstick hybridization detecting MTBC and genus *Mycobacterium* in direct respiratory specimens. It allows a quick diagnosis of infections produced by mycobacteria and differentiates between MTBC and MOTT. The amplification is based on the amplification of insertion sequence IS*6110* and 16S rRNA gene sequences. An internal amplification control which is human gene *RNase P* is included in the test to check the correct extraction of DNA, the absence of carry-over of amplification inhibitors in the sample and the correct amplification set-up. The control consists of a DNA fragment and a specific oligo pair for its amplification (Lara-Oya *et al.*, 2013). According to the manufacturers the kit was able to detect up to 2 genome copies per reaction on the MTBC test line and 20 genome copies per reaction on the *Mycobacterium* genus test line (Lara-Oya *et al.*, 2013).

As mentioned earlier, SO-DMT assay targets include 16S rRNA gene sequences and the repetitive IS*6110* of the MTBC genome. The 16S rRNA is an approximately 1500 nucleotides sequence encoded by the 16S rDNA. The sequence of the 16S rRNA gene is less likely to be mutated and therefore it is highly conserved. It is common to the whole genus *Mycobacterium*. In addition, the spacer region between 16S rRNA contains hypervariable regions which are particularly useful to resolve within-species variation (Dvorská *et al.*, 2001; Therese *et al.*, 2009).

The other SO-DMT target region is IS*6110* which is unique to members of the MTBC. It is a repetitive mobile genetic element and varies in copy numbers in the genome of MTBC. The high copy number of the IS*6110* increases the sensitivity of detection. It was reported that the IS*6110* sequence has 1,361 bp long and has imperfect inverted repeats (Thierry *et al.*, 1990). Most epidemiological studies have taken advantage of this element and used it as a diagnostic marker for the MTBC. SO-DMT assay also uses IS*6110* sequence as a diagnostic marker for the MTBC. It is known to be highly conserved sequence among MTBC (Lara-Oya *et al.*, 2013).

The principle of the assay is summarized as follow. Generally, the SO-DMT assay comprises four steps: DNA extraction, amplification with specific oligo pairs, detection of the amplified products and result reading. The detection is performed by means of a cassette. When the amplification is accomplished, the amplification control and the specific test amplicons are denatured and allowed to run through the cassette. The specific and control amplicons react with the complementary probes coupled to colloidal suspension of gold nanoparticles. Subsequently, the complex of specific and control amplicons with the colloidal gold conjugates flows through the membrane until the complex finds the specific probes (two different test lines, one specific for the MTBC and another for the genus *Mycobacterium*, and an amplification and extraction control line), where a second hybridization takes place (Figure 3). The double hybridization avoids the detection of unspecific amplified fragments providing excellent test specificity. The product control line appears because of the hybridization of the gold probe excess with a complementary oligonucleotide absorbed on the membrane.


Colloidal gold
Product Control Line
PCR Amplification Control Line
Test line 1: Genus *Mycobacterium*
Test line 2: *M. tuberculosis* Complex
Specific oligonucleotide bound to colloidal gold
Specific oligonucleotide bound to the nitrocellulose membrane

2.6. Treatment of tuberculosis

The goals of treatment are to ensure cure without relapse, to prevent death, to stop transmission and to prevent the emergence of drug resistance (Frieden *et al.,* 2003). Treatments with multiple drugs and for long enough are essential in order to eliminate the bacteria, inhibit the emergence of drug resistance and prevent relapses (Mitchison, 2000). First line drugs are recommended for the initial treatment of TB. These include INH, RIF, EMB, PZA and STM. The use of these drugs greatly facilitated the decrease in the incidence of TB worldwide (Gillespie, 2002). In Ethiopia, the current recommended drug regimen for category I pulmonary TB case is 6 months multidrug regimen with two phases. This regimen consists of 2 months RIF-INH-EMB-PZA and 4 months EMB-INH. For category II (retreatment regimen) tuberculosis case is 2 months STM (RMP-INH-EMB-PZA) plus 1 month (RMP-INH-EMB-PZA) plus 5 months (EMB3 (RMP-INH)3), respectively (FMOH, 2008).

Management of patients with MDR-TB involves the use of second-line drugs. In Ethiopia, the standard treatment regimen for MDR-TB is 6 months (EMB-PZA-KAM (AMK)-LFX-ETO-CS) plus 12 months (EMB-PZA-LFX-ETO-CS) (FMOH, 2009). Unfortunately, these drugs have few limitations such as less effective, more expensive and slightly more toxic (Sharma and Mohan, 2004; Migliori *et al.*, 2008). Thus, until the next major achievements are available the most essential means of TB control are rapid identification and effective cure of infectious cases (Palomino *et al.*, 2007).

3. OBJECTIVES

3.1. General objective

- To evaluate the performance of the new Speed-oligo Direct *M. tuberculosis* assay in relation to conventional microscopic and culture methods for the detection of *M. tuberculosis* complex directly from sputum samples.

3.2. Specific objectives

- To determine the performance of SO-DMT assay for the diagnosis of MTBC in smear-positive and smear-negative specimens using culture and clinical data as a reference.
- To compare the SO-DMT and Xpert MTB/RIF assays in the diagnosis of MTBC in smear-positive and smear-negative specimens.
- To identify the presence of acid-fast bacilli in sputum specimens using Ziehl-Neelsen microscopy.
- To detect the presence of MTBC in smear-positive and smear-negative sputum specimens on LJ culture.

4. MATERIALS AND METHODS

4.1. Study site

The study was conducted in SNNPR Government Health Bureau Public Health Laboratory, Hawassa, Ethiopia between November 2013 and September 2014 to prospectively investigate the performance of SO-DMT assay. The sputum specimens were sent to the laboratory from different diagnostic centers in southern Ethiopia for further TB diagnosis and management. The samples were also taken to Addis Ababa Administration Health Bureau Health Research and Laboratory Service, Addis Ababa, Ethiopia for further diagnosis of tuberculosis with SO-DMT and Xpert MTB/RIF assays.

4.2. Study participants and clinical specimens

The sputum specimens were obtained from 145 patients who fulfilled the selection criteria. The age range of the patients was from 18 to 75 years, most of whom (80.6%) were in the age range of 18-47 years. Out of 145 patients, 96 (66.2%) patients were males and the remaining 49 (33.8%) were females. Sixty five (44.8%) of them were new patients, whereas the rest eighty (55.2%) were previously treated patients. One specimen per patient was collected in a sterile disposable 50 ml screw capped universal centrifuge tube by the appropriate laboratory technicians. Specimens delivered to SNNPR Government Health Bureau Public Health Laboratory late were refrigerated at 2-8°C and processed the following morning or frozen at temperature below -20ºC for longer periods of time. They were transported in ice box to Addis Ababa Administration Health Bureau

Health Research and Laboratory Service to perform SO-DMT and Xpert MTB/RIF assays.

4.2.1. Inclusion criteria and exclusion criteria

Patients with age of 18 and above with symptoms of pulmonary TB were included in the study. Patients without TB symptoms and aged below 18 were excluded.

4.3. Sample size

The study was conducted on 145 sputum specimens obtained from patients during the study period.

4.4. Specimen processing

Sputum specimens were split into two aliquots, one of which was used for Xpert MTB/RIF assay and the other aliquot was decontaminated by the N-acetyl-L-cysteine (NALC)-sodium hydroxide (NaOH) method and the pellet was examined for acid-fast bacilli by microscopy, culture and SO-DMT assay.

4.4.1. NALC-NaOH method

The sputum sample was digested in a solution containing 1 g of NALC combined with 2.9% sodium citrate buffer and 4% NaOH (1:1) in a 50 ml falcon tube (Kent and Kubica, 1985). After digestant was added to the tubes, the caps of the tubes were tightened and mixed for 5 sec to homogenize the sputum using a vortex placed in a biological safety

cabinet and each tube was inverted to insure that NALC-NaOH solution contacts all inside surfaces of the tubes and caps. The mixture was allowed to stand for 15 min at room temperature and the reaction was stopped by addition of sterile distilled water to a final volume of 50 ml mark on the tube. The tubes were centrifuged at 3000xg for 15 min at 4°C. After centrifugation, the supernatant fluid was carefully poured into a splash proof discard can containing disinfectant. Finally, the resulting sediment was resuspended in 1 to 2 ml sterile distilled water and used for smear preparation by ZN staining, culturing on LJ media and DNA extraction for SO-DMT assay.

4.5. Smear preparations and examination of sputum specimens

Smears were prepared from resuspended sediment by spreading a drop over an area 1 × 2 cm on the microscope slide. Then the slide was allowed to air dry and heat fixed by passing the slide 3 to 4 times through a Bunsen burner. After smear preparation, the slide was stained by ZN method (Kent and Kubica, 1985). The slide was flooded with concentrated carbol fuchsin and heated to steaming with a burner flame. The stain was left for 5 min and washed with water. Next an acid-alcohol solution was placed on the slide and allowed to decolorize for 2 min. Then the slide was washed with water and counterstained with a methylene blue solution for 2 min. The smear was washed again with water and allowed to dry. Finally, the smears were examined under the oil immersion objective lens of the microscope (x1,000) (Kent and Kubica, 1985). Both smear positive and negative specimens were used for subsequent testing by LJ culture and the SO-DMT assay.

4.6. Mycobacterium isolation and identification

The pellet of decontaminated sputum was inoculated into two bottles of LJ media for *M. tuberculosis* isolation and a third bottle of LJ medium for isolation of *M. bovis* isolation. The inoculated media were then incubated at 37°C in a slanted position, with the screw caps slightly loosened to ensure even distribution of inoculum. After 24-48 hr, screw caps were tightened and tubes were further incubated. The incubated tubes were examined visually for growth every day for the first week and every week thereafter for the total of 8 weeks. LJ media with no evidence of mycobacterial growth after 8 weeks were recorded as negative and discarded appropriately. The isolates grown were used for identification. Identification of *M. tuberculosis* was based on growth rate, growth temperature, pigment production and colony morphology. All mycobacterial colonies were confirmed for the presence of an AFB by using ZN stain (Kent and Kubica, 1985).

4.7. Xpert MTB/RIF assay

The Xpert MTB/RIF assay was run according to Cepheid's Xpert MTB/RIF instructions. Xpert MTB/RIF was included for comparison after 36 samples were tested by SO-DMT assay due to this Xpert MTB/RIF was performed in only 109 samples. A sputum sample was mixed with treatment reagent in a 2:1 ratio. The mixture was then shaken by hand and incubated for 15 min at room temperature, with intermittent manual shaking. It was then 2 ml solution was transferred to the Xpert MTB/RIF test cartridge using a Pasteur pipette. The test cartridge contains the wash buffer, reagents for DNA extraction and PCR amplification, and fluorescent detection probes. Then the cartridge was loaded onto the GeneXpert machine for analysis. After the cartridge was placed in the instrument

module, the automated processes include the following: specimen filtering, sonication to lyse the bacilli and released DNA collection and combination with the PCR reagents, amplification and detection. Results are reported as positive or negative for *M. tuberculosis* and as susceptible or resistant for RIF.

4.8. Speed-oligo Direct *Mycobacterium tuberculosis* assay

All the DNA extraction, amplification, reading and interpretation were done according to Vircell's SO-DMT assay instructions.

4.8.1. DNA extraction

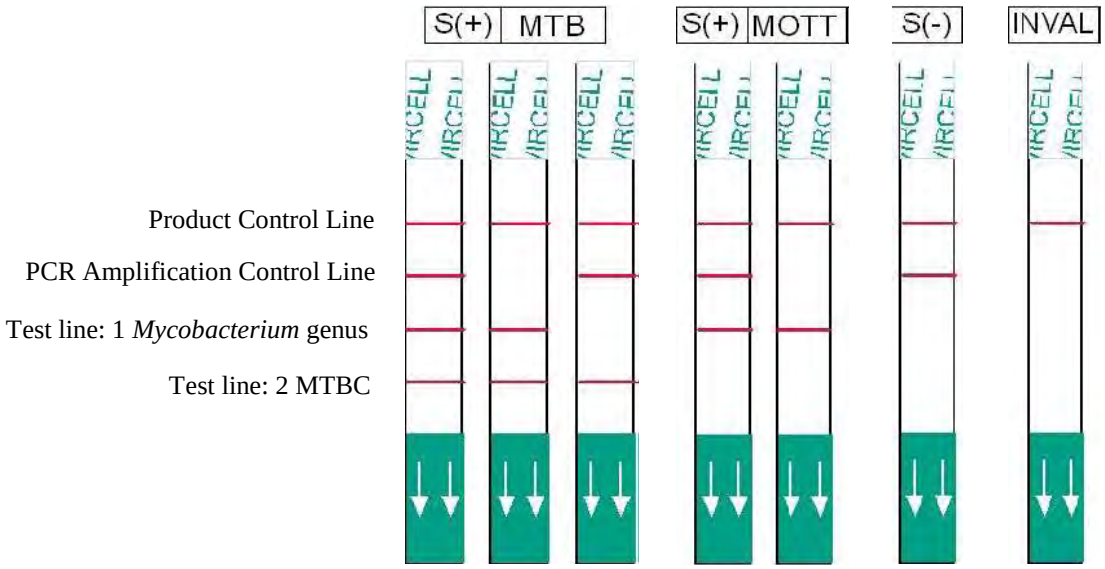
DNA is extracted from previously decontaminated resuspended sediment using the reagents included in the kit. For DNA extraction, 200 µl of previously decontaminated, neutralized and concentrated sputum sample was transferred to a screw capped tube and centrifuged for 10 min at 12,000×g in a microcentrifuge placed in a biological safety cabinet. After centrifugation, the supernatant was removed carefully with pipette and re-suspended in 300 µl of lysis solution. Then the solution again was centrifuged for 10 min at 12,000×g in a microcentrifuge. Next the supernatant was removed carefully with pipette and the sediment re-suspended in 100 µl of lysis solution and the suspension transferred to the extraction vial. Then microorganisms in the suspension were inactivated for 20 min at 95°C by thermoblock. This suspension was shaken vigorously with a cell disrupter at 3000 rpm for 5 min. Lastly, the sample was stored at 2-8°C until it was used in the amplification reaction for direct PCR amplification or stored frozen at -20°C for longer storage period.

4.8.2. PCR amplification

For single specimen PCR amplification, a pair of vials with PCR mix was provided with SO-DMT cassette by Vircell. In the reagent preparation area, 22 µl of PCR mix reconstitution solution was added into a pair of PCR mix vials for each sample, plus one for the positive and one for the negative controls and vortexed thoroughly for 2-3 sec. Then 10 µl of the extracted DNA sample was added to each vial containing PCR mix reconstitution solution by avoiding taking glass beads and then 10 µl of positive control and negative control were added to the corresponding tubes. Then the PCR tubes were inserted into a thermal cycler for amplification. The thermal cycler was programmed for 40 cycles with initial denaturation at 92°C for 1 min. Each cycle was performed with denaturation at 92°C for 20 sec, annealing at 55°C for 20 sec and an extension at 72°C for 20 sec. At the end of the last cycle, the mixtures were incubated at 72°C for 1 min.

4.8.3. Cassette detection

SO-DMT cassette with probes bound to colloidal gold and to the membrane was used for PCR product detection. The PCR product was denaturated for 3 min at 95°C in a thermal cycler and immediately 32 µl of PCR product was added to each cassette on the thermoblock. The cassette was pre-heated on thermoblock at 55°C simultaneously to the previous denaturation step to heat the running solution. After the addition of the PCR product to the cassette, the cassette was incubated for 20 min. Finally, the result was obtained from the presence of visible lines by a visual interpretation based on the presence or absence of the red color on control and test lines using interpretation card provided with the SO-DMT cassette.


S(+) MTB
S(+) MOTT
S(-)
INVAL
Product Control Line
PCR Amplification Control Line
Test line: 1 *Mycobacterium* genus
Test line: 2 MTBC
MTBC positive sample
MOTT positive sample
Negative sample
Invalid assay

Microbiology Department, Hospital Universitari Germans Trias i Pujol, Barcelona, Spain to check the inter laboratory reproducibility of the performance of SO-DMT assay results.

4.9. Discrepancy and statistical analysis

4.9.1. Discrepancy analysis

The patient's medical record was reviewed to resolve the discordant between culture and SO-DMT results.

4.9.2. Statistical analysis

The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), positive likelihood ratio (LR+) and negative likelihood ratio (LR-) of the SO-DMT assay were calculated with a 95% confidence interval (CI) with reference to culture or/and clinical data. The concordance of the SO-DMT assay with culture and clinical data and Xpert MTB/RIF assay was determined by Cohen's Kappa statistics, considering significance *P*-value<0.05. The data was analyzed by using a diagnostic calculator (http://www.medcalc.org/calc/diagnostic_test.php) and Statistical Package for the Social Sciences (SPSS) 20.0.

Sensitivity is the probability of getting a positive test result in subjects with the disease. Specificity is the probability of getting a negative test result in subjects without disease. PPV is the probability of having the disease of interest in a subject with positive result. NPV is the probability of getting subject without the disease with a negative test result.

LR+ tells us how much more likely the positive test result is to occur in subjects with the disease compared to those without the disease. LR- tells us how much less likely the negative test result is to occur in subjects with the disease than in a subject without disease (Okeh and Ogbonna, 2013).

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4.10. Ethical considerations and bio-safety issues

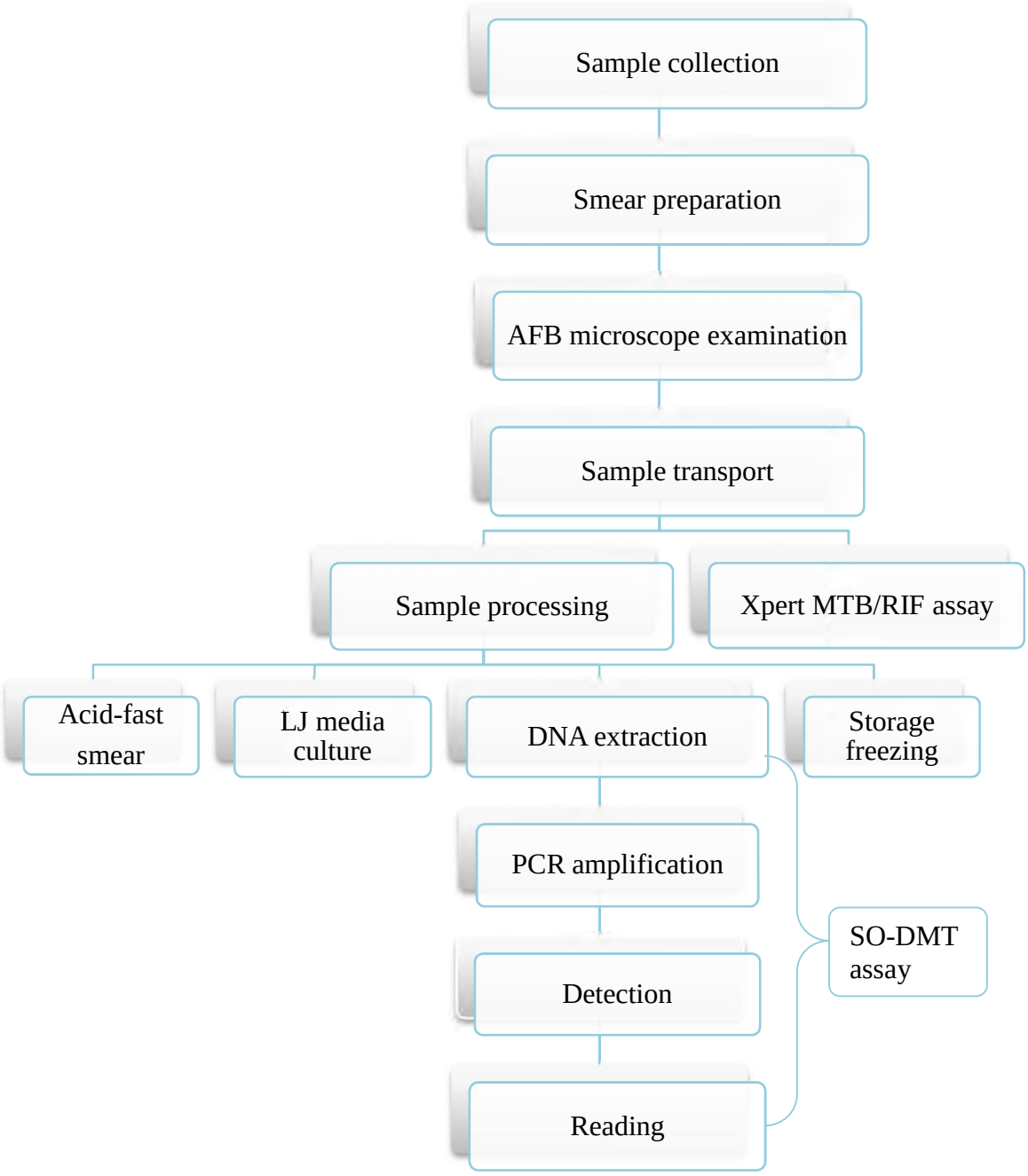
4.10.1. Ethical considerations

The study proposal was reviewed and approved by the Ethical Committee of the College of Natural Sciences, Addis Ababa University. Confidentiality was assured in all steps of the study. Analysis was done on data without revealing the identity of the study participants.

4.10.2. Bio-safety issues

In this study, all procedures were achieved using the safety laboratory recommended by WHO (WHO, 2012). The exchange of specimens between the diagnostic centers and the laboratory was carried out according to the standard regulations. Analysis of the specimens in the experimental site was performed with extreme care to avoid the possible risk of infection. Sample processing, DNA extraction, culture inoculation and identification were performed carefully to avoid the possible risk of aerosol formation and were carried out in a biological safety cabinet. Particular care was taken when tubes were being opened, closed or shaken and when materials were being centrifuged. All waste materials from the tuberculosis laboratory were decontaminated before disposal or removed from the laboratory area.

4.11. General workflow of the study



AFB = Acid-fast bacilli; LJ = Lowenstein-Jensen; SO-DMT = Speed-oligo Direct *Mycobacterium tuberculosis*; MTB = *Mycobacterium tuberculosis*; PCR = Polymerase chain reaction

Figure 6. Flow chart showing some of the laboratory procedures.

Figure 7. Colonies of *M. tuberculosis* grown on LJ media.

Table 2. Results of AFB smear and culture.

AFB specimens	AFB result	Culture results		Sensitivity	Specificity
		Positive	Negative		
AFB+ specimens	69 (47.6%)	62 (42.8%)	7 (4.8%)	62%	84.4%
AFB- specimens	76 (52.4%)	38 (26.2%)	38 (26.2%)		
Total	145 (100%)	100 (69%)	45 (31%)		

AFB+ = acid-fast bacilli positive; AFB- = acid-fast bacilli negative

5.3. Detection of *Mycobacterium tuberculosis* complex by SO-DMT assay

Of 145 sputum specimens that were studied, 97 (67%) were positive for MTBC by SO-DMT assay and the remaining 48 (33%) specimens were negative. No positive result was obtained by SO-DMT assay for mycobacteria other than *M. tuberculosis* in this study.

One culture negative sample was found to be positive by SO-DMT assay and four culture positive specimens were negative by SO-DMT assay (Table 3). Therefore, there were only five (3.5%) discordant results obtained between SO-DMT assay and LJ culture results. The sensitivity, specificity, PPV and NPV of SO-DMT assay for the detection of MTBC relative to culture were 96% (96/100 [95% CI, 90.1 to 98.9]), 97.8% (44/45 [95% CI, 88.2 to 99.6]), 99% (96/97 [95% CI, 94.4 to 99.8]) and 91.7% (44/48 [95% CI, 80 to 97.6]), respectively (Table 3). The positive and negative likelihood ratios of SO-DMT assay relative to culture were 43.2 and 0.04, respectively. These indicate that SO-DMT

assay is a good assay in ruling in and out the diagnosis. In general, the agreement between the new SO-DMT assay and culture was excellent as the kappa coefficient was calculated to be 0.921 (SE, 0.035) and it was statistically significant (P<0.05).

Table 3. Results of culture and SO-DMT assay.

	Culture results	**SO-DMT assay results**		**Total**	**Sn**	**Sp**	**PPV**	**NPV**
		Positive	**Negative**					
Overall	**Positive**	96	4	100	96%	97.8%	99%	91.7%
	Negative	1	44	45				
AFB+	**Positive**	62	0	62	100%	85.7%	98.4%	100%
	Negative	1	6	7				
AFB-	**Positive**	34	4	38	89.5%	100%	100%	90.5%
	Negative	0	38	38				

AFB+ = acid-fast bacilli positive; AFB- = acid-fast bacilli negative; SO-DMT = Speed-oligo Direct *Mycobacterium tuberculosis* assay; Sn = sensitivity; Sp = specificity; PPV = positive predictive value; NPV = negative predictive value

Out of 69 smear-positive specimens, 63 (91.3%) were positive by SO-DMT assay and the rest 6 (8.7%) smear-positive specimens were negative. From 76 smear-negative sputum samples, 42 (55.3%) specimens were also negative by SO-DMT assay, but the remaining 34 (44.7%) were identified as MTBC positive by SO-DMT assay (Table 3).

The SO-DMT assay gave a positive MTBC result for all of 62 (100%) specimens which were both smear and culture positive specimens. SO-DMT assay was also detected

MTBC in 34 (89.5%) of 38 culture positive smear-negative specimens. Additionally, SO-DMT assay detected MTBC in 1 (14.3%) of 7 smear-positive and culture negative specimens, but failed to detect mycobacteria in 4 (10.5%) specimens which were smear-negative and culture positive specimens. SO-DMT assay showed a negative test for all of 38 (100%) smear-negative culture negative specimens and in 6 (85.7%) of 7 smear-positive and culture negative specimens (Table 3).

The overall sensitivity, specificity, PPV and NPV of SO-DMT assay on smear-positive specimens were 100% (62/62 [95% CI, 94.2 to 100]), 85.7% (6/7 [95% CI, 42.2 to 97.6]), 98.4% (62/63 [95% CI, 91.4 to 99.7]), 100% (6/6 [95% CI, 54.1 to 100]), respectively. The same values for smear-negative specimens were 89.5% (34/38 [95% CI, 75.2 to 97]), 100% (38/38 [95% CI, 90.7 to 100]), 100% (34/34 [95% CI, 89.6 to 100]), 90.5% (38/42 [95% CI, 77.4 to 97.3]), respectively (Table 3).

5.4. Discrepancies between SO-DMT assay and culture results

There were 5 (3.5%) specimens discordant between culture and SO-DMT assay results (Table 3). Of these 5 specimens, one specimen was SO-DMT assay positive and culture negative. After the patient's medical record was reviewed, the patient with culture negative and SO-DMT assay positive was found on antituberculosis treatment and it was reclassified as true positive. On the other hand, 4 specimens were positive by culture and yielded negative results with SO-DMT assay. All of these were considered as false-negative results of SO-DMT assay (Table 4).

The overall sensitivity, specificity, PPV and NPV values of SO-DMT assay were 96% (97/101 [95% CI, 90.2 to 98.9]), 100% (44/44 [95% CI, 91.9 to 100]), 100% (97/97 [95% CI, 96.2 to 100]) and 91.7% (44/48 [95% CI, 80 to 97.6]) after resolution of discrepant results by culture and clinical data, respectively (Table 4). The LR+ and LR- of SO-DMT assay after discrepancy analysis were infinity and 0.04, respectively. In general, the agreement between new SO-DMT assay and culture was excellent as the kappa coefficient was calculated to be 0.936 (SE, 0.031). It was statistically significant (P<0.05).

Table 4. Results of culture, clinical data and SO-DMT assay.

	Culture and clinical data	**SO-DMT assay results**		**Total**	**Sn**	**Sp**	**PPV**	**NPV**
		Positive	**Negative**					
Overall	**Positive**	97	4	101	96%	100%	100%	91.7%
	Negative	0	44	44				
AFB+	**Positive**	63	0	63	100%	100%	100%	100%
	Negative	0	6	6				
AFB-	**Positive**	34	4	38	89.5%	100%	100%	90.5%
	Negative	0	38	38				

AFB+ = acid-fast bacilli positive; AFB- = acid-fast bacilli negative; SO-DMT = Speed-oligo Direct *Mycobacterium tuberculosis* assay; Sn = sensitivity; Sp = specificity; PPV = positive predictive value; NPV = negative predictive value

The overall sensitivity, specificity, PPV and NPV of SO-DMT assay on smear-positive specimens after analysis of discrepancies were 100% (63/63 [95% CI, 94.3 to 100]),

100% (6/6 [95% CI, 92 to 100]), 100% (63/63 [95% CI, 94.3 to 100]) and 100% (6/6 [95% CI, 92.2 to 100]), respectively. The corresponding results on smear-negative specimens were 89.5% (34/38 [95% CI, 75.2 to 97]), 100% (38/38 [95% CI, 90.7 to 100]), 100% (34/34 [95% CI, 89.6 to 100]) and 90.5% (38/42 [95% CI, 77.4 to 97.3]), respectively (Table 4).

5.5. Results of SO-DMT and Xpert MTB/RIF assays

Out of 109 specimens tested for Xpert MTB/RIF and SO-DMT assays, 70 (64.2%) were Xpert MTB/RIF and SO-DMT assays positive, 5 (4.6%) were Xpert MTB/RIF positive and SO-DMT negative, 3 (2.8%) were Xpert MTB/RIF negative and SO-DMT positive and 31 (28.4%) were Xpert MTB/RIF and SO-DMT negative. The agreement between Xpert MTB/RIF and SO-DMT assays was very good with a Cohen's kappa index of 0.832 (SE, 0.057) with P-value < 0.05.

Table 5 shows results of culture, SO-DMT assay and Xpert MTB/RIF assay. Out of 109 specimens, 76 (69.7%) were culture positive and the remaining 33 (30.3%) were culture negative. Of 76 culture positive specimens, 72 (94.7%) were Xpert MTB/RIF positive and the remaining 4 (5.3%) culture positive specimens were Xpert MTB/RIF negative. Thirty (90.9%) of 33 culture negative specimens were Xpert MTB/RIF negative, but 3 (9.1%) were Xpert MTB/RIF positive. Out of 76 culture positive specimens 72 (94.7%) were SO-DMT positive and 4 (5.3%) were SO-DMT negative. Of 33 culture negative specimens, 32 (97%) were SO-DMT negative and one (3%) was SO-DMT positive.

Table 5. Results of SO-DMT and Xpert MTB/RIF assays.

Specimen	**Culture positive**		**Culture negative**		**Sn (%)**	**Sp (%)**	**PPV (%)**	**NPV (%)**
	SO-DMT+	**SO-DMT-**	**SO-DMT+**	**SO-DMT-**				
	72	4	1	32	94.7	97	98.6	88.9
AFB+	45	0	1	4	100	80	97.8	100
AFB-	27	4	0	28	87.1	100	100	87.5
	Xpert+	**Xpert-**	**Xpert+**	**Xpert-**				
	72	4	3	30	94.7	91	96	88.2
AFB+	45	0	1	4	100	80	97.8	100
AFB-	27	4	2	26	87.1	93	93	87
	Clinical data and culture positive		**Clinical data and culture negative**					
	SO-DMT+	**SO-DMT-**	**SO-DMT+**	**SO-DMT-**				
	73	4	0	32	94.8	100	100	88.9
AFB+	46	0	0	4	100	100	100	100
AFB-	27	4	0	28	87.1	100	100	87.5
	Xpert+	**Xpert-**	**Xpert+**	**Xpert-**				
	73	4	2	30	94.8	93.8	97.3	88.2
AFB+	46	0	0	4	100	100	100	100
AFB-	27	4	2	26	87.1	93	93	86.7

AFB+ = acid-fast bacilli positive; AFB- = acid-fast bacilli negative; SO-DMT+ = Speed-oligo Direct *Mycobacterium tuberculosis* assay positive; SO-DMT- = Speed-oligo Direct *Mycobacterium tuberculosis* assay negative; Sn = sensitivity; Sp = specificity; PPV = positive predictive value; NPV = negative predictive value; Xpert+ = Xpert MTB/RIF positive; Xpert- = Xpert MTB/RIF negative

The sensitivity, specificity, PPV and NPV of the SO-DMT assay were 94.7% (72/76), 97% (32/33), 98.6% (72/73) and 88.9% (32/36) with reference to culture, respectively, where as the corresponding values for Xpert MTB/RIF assay were 94.7% (72/76), 91% (30/33) , 96% (72/75) and 88.2% (30/34), respectively (Table 5).

Out of 77 positive specimens, 73 (94.8%) were positive for Xpert MTB/RIF and SO-DMT assays with reference to culture and clinical data. The rest 4 (5.2%) specimens were Xpert MTB/RIF and SO-DMT negative. Of 32 negative specimens, 30 (93.8%) were negative with Xpert MTB/RIF, whereas 2 (6.2%) were positive for Xpert MTB/RIF. All 32 (100%) negative specimens were also negative by SO-DMT assay (Table 5).

The overall sensitivity, specificity, PPV and NPV of the SO-DMT assay for diagnosis of tuberculosis were 94.8% (73/77), 100% (32/32), 100% (73/73) and 88.9% (32/36) with reference to culture and clinical data, respectively. On the other hand, the corresponding values for Xpert MTB/RIF assay were 94.8% (73/77), 93.8% (30/32), 97.3% (73/75) and 88.2% (30/34), respectively (Table 5).

5.6. Turnaround time of SO-DMT and Xpert MTB/RIF assays

In this study, the turnaround time for SO-DMT assay was about 2 hr and 45 min (45 min for DNA extraction, 1 hr and 40 min for DNA amplification and 20 min for PCR product detection) and 2 hr for Xpert MTB/RIF assay.

6. DISCUSSION

The global increasing incidence of TB together with the lack of diagnostic tools capable of detecting all cases successfully call for urgent efforts to develop improved diagnostic tests that meet all the requirements of rapidity and sensitivity (Bateson *et al.*, 2013). The new SO-DMT assay is one of such assays recently introduced to detect MTBC DNA directly from clinical specimens.

In the present study, the performance of new SO-DMT assay was compared to culture. There were only five specimens discordant among culture and SO-DMT results (Table 3). One specimen with smear and SO-DMT positive but culture negative was from the patient on anti-tuberculosis treatment and it was reclassified as true positive. Dorman *et al*., (2012) has demonstrated that the presence of active TB by molecular diagnosis and culture negative is presumably due to antibiotic mediated suppression of bacterial growth. Different studies have also explained that some specimens from patients whose sputum specimens were culture negative but molecular diagnostic positive for TB had a past history of tuberculosis or clinical response to recent anti-tuberculosis chemotherapy (Lindbråthen *et al.*, 1997; Moore and Curry, 1998; Mazzarelli *et al.*, 2003).

On the other hand, 4 specimens were positive by culture and yielded negative results with the SO-DMT assay and all were taken as false negative results after reviewing the clinical data (Table 4). These might be due to loss of mycobacteria during sample processing. Lara-Oya *et al*., (2013), has given possible explanations that the presence of a low number of copies of IS*6110* in MTBC strains and deficient lysis of the mycobacterial

wall as attributers for false negative results. Another reason could be the natural clumping of the mycobacteria make difficult to get uniform sampling even with vortexing and specimen lysing procedures (Beavis *et al.*, 1995; Roth *et al.*, 1997). Other literatures have also showed the presence of PCR inhibitors as attributer for false negative results (Beavis *et al.*, 1995; Piersimoni and Scarparo, 2003). However, PCR inhibitors in the false negative results observed in the present study are unlikely to occur since SO-DMT assay contains an internal DNA extraction processing and amplification control (*RNase P* gene) which should lead to an invalid result if inhibitors are present in the sample.

The overall sensitivity, specificity, PPV and NPV of the SO-DMT assay for diagnosis of MTBC with culture and clinical data were 96%, 100%, 100% and 91.7%, respectively (Table 4), whereas the corresponding results of the SO-DMT assay with culture were 96%, 97.8%, 99% and 91.7% (Table 3). The sensitivity of the SO-DMT assay remained unchanged with the initial 96% of sensitivity after analysis of discrepancies. However, the specificity has showed an increment of 2.2%, which is from 97.8% to 100%. Different studies on NAATs have shown that an increase in sensitivity ranging from 3% to 4.2% and specificity ranging from 1% to 30% after analysis of discrepancies among AFB, culture and molecular diagnostics (Bergmann and Woods, 1998; Moore and Curry, 1998; deLuna *et al.*, 2006).

As would be expected, the sensitivity of SO-DMT assay for smear-negative specimens (89.5%) was lower than that of smear-positive specimens (100%) (Table 4). Generally, the low sensitivity of NAATs for the diagnosis of smear-negative compared to smear-

positive specimens is due to a low number of AFB in smear negative specimens. All false negative results in the present study were from smear-negative specimens (Table 3). It has been explained that the majority of false negative results that are observed by currently available NAATs in clinical specimens collected from patients with active TB were from smear-negative specimens (Piersimoni and Scarparo, 2003). After discrepancy analysis the sensitivity of SO-DMT assay remained the same 100% and 89.5% for both smear positive and negative specimens, respectively (Table 4). This is due to no changes observed in the initial and overall sensitivity values after analyzing the discrepancy. However, the specificity has showed an increment of 14.3%; that is, from initial 85.7% to overall 100% for smear-positive specimens. Different studies have also shown that an increment in the sensitivity and specificity of smear positive and negative specimens after analysis of discrepancies between AFB, culture and NAATs (Bergmann and Woods, 1998; Garrino *et al.*, 1999).

As explained earlier, the new SO-DMT assay, relative to culture and clinical data, has showed a sensitivity of 96% for detection of *M. tuberculosis* complex directly from sputum samples (Table 4). The results of the present study indicated higher sensitivity value than previously reported two studies. In a study performed by Lara-Oya *et al*., (2013) using the SO-DMT assay on 538 respiratory specimens the sensitivity was found to be 75% for *Mycobacterium* and 86% for MTBC detection. In a study performed by Antonenka *et al*., (2013) on 121 respiratory specimens (sputa and bronchial secret) found overall sensitivity of 58.9%. The higher sensitivity observed in the present study might be attributable to the higher number of culture and smear positive specimens (62) observed

compared to those previous studies (Table 3). Lara-Oya *et al*., (2013) obtained 24 culture positive and smear negative specimens and Antonenka *et al*., (2013) included 18 culture positive and smear negative specimens and MOTT specimens.

The other reasons for high sensitivity result of the present study compared to those previous SO-DMT assay evaluation studies might be because of two major improvements recently done on this newly designed SO-DMT assay. In the previous version of the assay amplification reaction is carried out in a single PCR tube for a single test (Lara-Oya *et al.*, 2013), but the new SO-DMT assay has a pair of PCR tubes and amplification is carried out in a pair of PCR tubes for a single test. So the multiplex reaction has more Cl_2Mg, dNTPs, Taq polymerase, PCR primers and DNA extracts that make the assay more sensitive. The other improvement made on this new SO-DMT assay is increasing the volume of denatured PCR products added to the hybridization step. In the previous SO-DMT assay procedure only 5 µl of denatured amplified product is added for hybridization and detection (Lara-Oya *et al.*, 2013), but in this improved new SO-DMT assay the volume of denatured amplified product added for hybridization is 6 times more than previous assay procedure. Therefore, there will be more amplicons to hybridize with probes on the strip and increase sensitivity of the new SO-DMT assay.

The overall sensitivity of SO-DMT assay observed in this study is within the range of other similar commercial NAATs reported by different researchers for the diagnosis of MTBC in respiratory specimens. Amplified MTD Test has reported to have sensitivity of 85.7% to 97.8% (Piersimoni and Scarparo, 2003) and BD ProbeTec MTB test sensitivity

of 60.7% to 100% (Piersimoni and Scarparo, 2003; Parsons *et al.*, 2011). Amplicor MTB Test has shown a sensitivity of ranging from 83% to 96.7% (Piersimoni and Scarparo, 2003).

The specificity of SO-DMT assay for detection of MTBC was 100% with reference to culture and clinical data (Table 4). This was the same to previously reported value by Antonenka *et al*., (2013) who has reported a specificity of 100%. Lara-Oya *et al*., (2013) has also reported 99% specificity. Double hybridization attributes for the higher specificity of the assay which avoids the detection of unspecific amplified fragments providing excellent test specificity.

The overall specificity level of SO-DMT assay found in the present study is within the range of other similar commercial molecular diagnostics reported by different studies for the diagnosis of MTBC in respiratory specimens. Amplicor MTB Test has shown to have a specificity of ranging from 91.3% to 100% (Piersimoni and Scarparo, 2003; Palomino, 2009), Amplified MTD Test has reported to have specificity of 92.1% to 100% and BD ProbeTec MTB test specificity of 98.9% to 100% (Piersimoni and Scarparo, 2003; Parsons *et al.*, 2011).

The SO-DMT assay detected all 63 smear positive specimens. Hence, sensitivity of SO-DMT assay for detection of MTBC considering smear-positive specimens was 100% (Table 4). This is similar with sensitivity of 100% observed by Lara-Oya *et al*., (2013) for detection of MTBC by SO-DMT assay. However, sensitivity of SO-DMT in AFB smear-

negative samples for the detection of MTBC was 89.5% which is much higher than previously reported for smear-negative samples by Lara-Oya *et al*., (2013) who has reported a sensitivity level of 68%. Antonenka *et al*., (2013) even reported very much lower sensitivity level of 42.1% for smear-negative specimens. The higher sensitivity observed in smear-negative samples compared to Antonenka *et al*., (2013) might be due to not the inclusion of MOTT specimens in this study. Lara-Oya *et al*., (2013) previously has reported that sensitivity of 50% in smear-positive and 16% in smear-negative specimens for MOTT. The other reason could be, as mentioned earlier, the higher sensitivity for smear-negative specimens in the present study might be for the reason that two major improvements had done in this new SO-DMT assay to improve sensitivity of the assay.

Sensitivity levels reported for smear positive and negative specimens by SO-DMT assay are within the range of sensitivity levels reported by other studies on various commercially available NAATs for the diagnosis of MTBC in respiratory specimens. Sensitivity of Amplicor MTB Test has shown to have from 90% to 100% in smear positive samples and from 50% to 95.9% in smear-negative samples. Sensitivity of Amplified MTD Test ranges from 91.7% to 100% in smear-positive and 65.5% to 92.9% in smear-negative respiratory samples (Piersimoni and Scarparo, 2003). A literature report for BD ProbeTec MTB test has shown that a sensitivity of 98.5% to 100% in smear-positive samples. However, a lower sensitivity than the present finding in smear-negative samples ranging from 33% to 85.7% (Piersimoni and Scarparo, 2003; Parsons *et al.*, 2011).

In general, there was excellent agreement between the new SO-DMT assay results and culture and clinical data results, as the kappa coefficient was calculated to be 0.936 which was higher than previously reported kappa coefficient of 0.85 (Lara-Oya *et al.*, 2013) and it was statistically significant ($P<0.05$).

In the present study, the SO-DMT assay was also compared with WHO endorsed Xpert MTB/RIF assay in detecting MTBC. Both assays have shown the same sensitivity of 94.8% (Table 5). Chang *et al*., (2012) has explained that the sensitivity of Xpert MTB/RIF assay ranges from 73% to 100% for pulmonary specimens, which is in agreement with the present finding. On the other hand, the SO-DMT assay was performed on decontaminated sputum pellets, whereas Xpert MTB/RIF assay was on raw sputum which could result in non-equivalent fraction when the raw sputum was split (Dorman *et al.*, 2012). However, the data showed that no differences observed in sensitivity of SO-DMT and Xpert MTB/RIF assays.

The SO-DMT assay (100%) yielded higher specificity than Xpert MTB/RIF assay (93.8%) (Table 5). This is because of unlike SO-DMT assay, Xpert MTB/RIF assay was positive for two samples which were both smear and culture negative specimens. Miller *et al*., (2011) explained that the diagnosis of infectious disease will lead to positive DNA results in the absence of viable organisms. These type of false-positive reactions on molecular diagnostic tools could be due to contaminating amplicons (Mazzarelli *et al.*, 2003).

Both the sensitivity and specificity of Xpert MTB/RIF and SO-DMT assays for smear-positive specimens were 100% with reference to culture results and clinical data. Previous literatures have demonstrated that the Xpert MTB/RIF assay displays high percentages of sensitivities ranging from 95% to 100% in smear-positive specimens for the detection of MTBC isolates (Chang *et al.*, 2012; Bateson *et al.*, 2013). The sensitivity of both SO-DMT and Xpert MTB/RIF assays for smear-negative specimens were 87.1%, whereas the specificity for SO-DMT assay and Xpert MTB/RIF assay were 100% and 93%, respectively. The difference in specificity of smear-negative specimens was due to the detection of two false positive results by Xpert MTB/RIF assay. Generally, the agreement between Xpert MTB/RIF and SO-DMT assays was very good with a kappa coefficient of 0.832 (SE, 0.057) which was statistically significant ($P<0.05$) when they compared directly.

7. CONCLUSIONS AND RECCOMENDATIONS

7.1. Conclusions

1. SO-DMT assay is more sensitive than ZN since it detected more MTBC in patients with ZN negative specimens.

2. The data demonstrated that the SO-DMT assay is a very sensitive and specific assay for detection of MTBC directly in smear positive specimens, allowing rapid confirmation of positive smear results as true tuberculosis.

3. The results appear to indicate that the sensitivity of the SO-DMT and Xpert MTB/RIF assays is similar. On the other hand, the specificity of SO-DMT is higher than Xpert MTB/RIF assay.

7.2. Limitation of the study

Culture identification was not confirmed either by biochemical, genotypic or immunochromatographic methods due to financial constraints.

7.3. Recommendations

1. In general, the new SO-DMT assay can be used for diagnosis of MTBC infection; specially, in smear positive specimens in addition to conventional methods. This assay might be a good alternative to real-time PCR assays for laboratories not equipped with real-time PCR instruments.

2. This study is restricted to sputum specimens. Therefore, the performance of new SO-DMT assay for early detection of MTBC and MOTT in other clinical specimens needs to be investigated in further studies under different settings.

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APPENDICES

Annex 1. Clinical information from TB patients

1.1. General Information

Patient name_____

Card number_____

Sex _____ (Female/Male)

Age _____ (Years)

Address: Region_____ Zone_____ Kebele_____

Date of sputum collection_____/___/_____ G.C. (dd/mm/yyyy)

Patient type (New/Previously treated)

1.2. Current clinical presentations

TB symptoms and manifestations_____(Yes/No)

If yes duration in weeks_____

X-ray_____(Normal/Abnormal)

HIV status_____(Positive/Negative/Not known)

1.3. Treatment

Treatment regimen_____

Time of initiation of treatment____/____/____ G.C. (dd/mm/yyyy)

Annex 2. Ziehl-Nelseen reagent preparation

1. Fuchsin- 0.3 g of basic fuchsin was dissolved in 10 ml of 95% ethanol.

2. Phenol- 5 g of phenol crystals was dissolved in 100 ml of water and gently heated.

3. Carbol fuchsin- Solution 1 was mixed with 90 ml of solution 2.

4. Acid alcohol- 3 ml of concentrated hydrochloric acid was carefully added to 97 ml of 95% ethanol and mixed gently.

5. Methylene blue- 0.3 g of methylene blue chloride was dissolved in 100 ml distilled water.

Annex 3. Löwenstein-Jensen medium preparation

The mineral salt solution was prepared by dissolving 2.4 g potassium dihydrogen phosphate (KH_2PO_4), 0.24 g magnesium sulphate ($MgSO_4.7H_2O$), 0.6 g magnesium citrate, 3.6 g asparagines and 12 ml glycerol in 600 ml distilled water. The solution was sterilized at 121°C for 30 min in autoclave and cooled to room temperature. Then in separated sterile flask 2.0 g malachite green dye was dissolved in 100 ml sterile distilled water by placing the solution in the incubator for 1hr.

Next to that, 24 fresh hens' eggs, not more than 1 week old, were cleaned by scrubbing thoroughly with a hand brush in soap solution. Then the eggs were soaked for 30 min in the soap solution and rinsed thoroughly in running water and soaked them in 70% ethanol for 15 min. After rinsed the eggs, hands washed and scrubbed well before breaking the eggs into a sterile flask. Then the eggs were homogenized with a sterile blender and filtered through four layers of sterile gauze into a sterile cylinder.

Finally, 600 ml of mineral salt solution, 20 ml malachite green solution and 1000 ml of homogenized eggs were aseptically pooled in a large sterile flask and mixed well and the

complete egg medium is distributed in 6-8 ml volumes in sterile 14 ml or 28 ml McCartney bottles and the tops are securely fastened. The medium was inspissated within 15 min of distribution. The inspissator was heated to 80°C before loading. The bottles were placed in a slanted position in the inspissator and the medium was coagulated for 45 min at 80°C-85°C. After inspissation, the whole media batch or a representative sample of culture bottles were incubated at 35°C-37°C for 24 hr as a check of sterility. For the cultivation of *M. bovis* glycerol is omitted and 8.0 g sodium pyruvate was added to the mineral solution.

Annex 4. Laboratory data

Patient Code	Ziehl-Neelsen results		LJ culture results		SO-DMT assay results		Xpert MTB/RIF assay results	
	+ve	-ve	+ve	-ve	+ve	-ve	+ve	-ve

Annex 5: Information sheet for study subject

1. Purpose of the study

This study is being conducted to evaluate a PCR-based assay for direct detection of *Mycobacterium tuberculosis* complex in sputum specimens.

2. Duration of sample collection

The sample will be collected for 6 months.

3. Sample taking procedures

One sputum sample per patient will be collected in 50ml falcon tube by appropriate health workers.

4. Risks and discomfort

There will be no discomfort or risk while collecting sputum samples.

5. Benefits of the study

By participating in this study, you will be providing information to the study of the performance of a molecular assay. The person with positive TB test will get free treatment. We hope the information learned from this study will benefit in controlling and preventing TB or what is being studied in the future.

6. Confidentiality of your personal information

Information gathered in this research study may be published or presented in public forums, however your name and other identifying information will not be used or revealed and your specimens are used only for this study purpose.

7. Voluntary Participation/Withdrawal from the Study

Your decision to take part in this study is voluntary. You may refuse to participate or you may withdraw from the study at any time. Your decision not to participate or to withdraw from the study will not affect your other medical care at this site.

8. Contact address

Name of researcher: Abiy Aklilu

Mobile: 0912104022

Institutional review board: Addis Ababa University, College of Natural Sciences

Phone №: +251-011-895-9216

P.O.Box: 1176

Addis Ababa

የጥናቱ ማብራሪያ ቅፅ ለጥናቱ ተሳታፊዎች

1. የጥናቱ ዓላማ

የጥናቱ ዓላማ የቲቢ ባክቴሪያ መመርመሪያነት በሚዉል የጤና መመርመሪያ መሳሪያ ላይ ሲሆን ጥናቱም የሚካሄደዉ ከህሙማኑ የአክታ ናሙናዎች በመዉሰድ ነዉ፡፡

2. ናሙናዉ የሚወሰድበት ጊዜ

ናሙናዉ ለስድስት ወራት ይወሰዳል፡፡

3. የተሳትፎ ሁኔታ

ለአንድ ጊዜ የአክታ ናሙና በ50ሚሊ ብልቃጥ በተገቢዉ የጤና ባለሞያ መዉሰድ፡፡

4. ሊከሰቱ ስለሚችሉ ስጋቶችና የምቾት መጋደሎች

የአክታ ናሙና በሚወሰድበት ወቅት ምንም ዓይነት ችግር ወይም የምቾት መጋደል አይከሰትም፡፡

5. ጥቅሞች

ቲቢ ፖዝቲቭ ሆኖ ከተገኘ የነጻ ህክምና ማግኘት፡፡ ከዚህ ጥናት የሚገኘዉ መረጃ ቲቢን ለመቆጣጠርና ለመከላከል ይረዳል፡፡

6. ሚስጥር ስለ መጠበቅ

ከዚህ ጥናት የሚገኘዉ መረጃ ለህትመት ሊበቃና በተለያዩ መድረኮች ላይ ሊቀርብ ይችላል፡፡ ነገር ግን የርስዎ ማንነት በስም አይገለጽም፡፡ የተወሰዱት ናሙናዎች ለዚህ ጥናት ብቻ የሚዉሉ ናቸዉ፡፡

7. በጥናቱ ያለመሳተፍ ወይም ራስን የማግለል መብት

የእርስዎ ተሳትፎ በፈቃደኝነት ላይ የተመሰረተ ነዉ፡፡ እርስዎ በጥናቱ ላይ መሳተፍዎን ለመሰረዝ ከፈለጉ በማንኛዉም ሰዓት መሰረዝ ይችላሉ፡፡ በጥናት ዉስጥ መግባት አለመፈለግዎ በጤና እንክብካቤዎ ላይ ምንም ዓይነት ተጽእኖ አይኖረዉም፡፡

8. መረጃ ስለ ማግኘት

የተመራማሪዉ ስም፡- አብይ አክሊሉ

ሞባይል፡- 0912104022

የኢንስቲትዩሽናል ሪቪዉ ቦርድ፡- አዲስ አበባ ዩኒቨርሲቲ፣ የተፈጥሮ ሳይንስ ኮሌጅ

ስልክ፡- 251-011-895-9216

የመ.ሳ.ቁ፡- 1176

አዲስ አበባ

Annex 6: Informed Consent Form

Name of study participant: _____

I have read the information sheet, or it has been read to me. I have understood that it involves the study about evaluation of a PCR-based assay for direct detection of *Mycobacterium tuberculosis* complex in sputum specimens in southern Ethiopia. I will be asked to provide information and sputum samples for laboratory examination. I have had the chance to ask questions about it and I am satisfied with the answers I was given. I consent voluntarily to participate in the study and understand that I have the right to withdraw my consent and this will not affect my medical care.

I _____ hereby give my consent for giving of the requested information and specimens as the medical doctor and the researcher find best for me.

Participants signature_____Date_____

Investigators signature _____Date_____

Witness signature 1._____Date_____

2._____Date_____

የፈቃደኝነት መጠየቂያ ቅጽ

እኔ አቶ/ወ/ሮ/ወሪ----------ስለ ጥናቱ አስፈላጊ የሆኑትን መረጃዎች አንብቤያለሁ ወይም ተነቦልኛ። ጥናቱም የሚካሄደዉ በደቡብ ክልል ሲሆን ዓላማዉም የቲቢን ባክቴሪያ መመርመሪያነት በሚዉል የጤና መመርመሪያ መሳሪያ ሆኖ ጥናቱም የሚካሄደዉ ከህሙማኑ የአክታ ናሙናዎችን በመዉሰድ ነዉ። ለጥናቱ አስፈላጊ የሆኑትን መረጃዎችና የአክታ ናሙናዎች እንደምሰጥ ተረድቻለሁ። ከጥናቱ ጋር የተያያዙ ጥያቄዎችንም ጠይቄ ማብራሪያዎችም ተሰጥተዉኛል። በጥናቱ ላይ መሳተፌን ለመሰረዝ ከፈለኩ በማንኛዉም ሰዓት መሰረዝ እንደምችልና ይህም በጤና እንክብካቤዬ ላይ ምንም ዓይነት ተጽእኖ እንደማይኖረዉ ተረድቻለሁ።

እኔ ስሜ----------ለጥናቱ አስፈላጊ የሆኑ መረጃዎችንና ለምርመራ የሚያገለግሉ ናሙናዎችን ለመርማሪዉ ሀኪምና ለተመራማሪዉ ለመስጠት ፈቃደኛ ነኝ።

የተሳታፊዉ ፊርማ----------ቀን----------

የተመራማሪዉ ፊረማ----------ቀን----------

የምስክር ፊረማ 1. **----------ቀን----------**

2. **----------ቀን----------**