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Line Probe Assay for improved Tuberculosis Diagnosis and Detection of Isoniazid and Rifamicin Resistance

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Abstract

Introduction: *A sensitive, rapid and accurate laboratory diagnostic method is essential for detection of tuberculosis and its drug resistance. However, using conventional techniques for detection of acid-fast bacilli and mutations conferring resistance to anti-TB drugs have a number of draw backs.*
Objective: *To evaluate the diagnostic ability of Genotype MTBDRplus version 2.0 line probe assay for diagnosis of Mycobacterium tuberculosis complex (MTBC) and detection of drug resistant tuberculosis from direct sputum specimens.*

Methods: *A cross-sectional study was conducted in Addis Ababa regional laboratory from December 2013 to September 2014. A total of 96 drug resistant TB suspected patients were recruited from four hospitals and twenty eight health centers found in Addis Ababa. The specimens were analyzed in Addis Ababa regional laboratory and Armauer Hansen Research Institute. Culture by Lowenstein–Jensen medium, Genotype MTBDRplus version 2.0 line probe assay, pyrosequencing, spoligo-rifampicin-isoniazid-typing and Gene Xpert MTB/RIF assay were performed.*

Results: *The sensitivity and specificity of Genotype MTBDRplus version 2.0 line probe assay for detection of MTBC were 89.3% and 90.5%, respectively using culture method as a gold standard. The sensitivity and specificity of MTBDRplus were equivalent to that of Gene Xpert MTB/RIF assay in detecting MTBC in sputum samples. However, the performance of MTBDRplus in detecting resistance to rifampicin (47/66) was much higher than that of Gene Xpert MTB/RIF assay (30/66). The majority of mutations conferring resistance to rifampicin and isoniazid were found in codon S531L and S315T, respectively. The performances of MTBDRplus and spoligo-RIF-INH-typing were 96.2% concordant in detecting resistance to RIF, INH and multi-drug resistant tuberculosis.*

Conclusion and Recommendation: *Genotype MTBDRplus version 2.0 line probe assay improves diagnosis of tuberculosis and detection of drug resistance TB in sputum specimen regardless of its smear status. The method is effective, time saving and relatively simple method for detection of drug resistance to INH and RIF provided that the weak or faint appearance of wild type 8 band without the presence of related mutation band is considered as normal during interpretation of the results for rifampicin resistance. Further studies should be conducted to assess factors affecting wild type 8. The manufacturer should evaluate and review the manual for interpretation of Genotype MTBDRplus version 2.0 line probe assay results.*

Key words/ phrases: *Drug resistance, Genotype MTBDRplus version 2.0 line probe assay, M. tuberculosis complex, Tuberculosis*

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Acronyms and abbreviations

AAU	Addis Ababa University
AFB	Acid Fast Bacilli
AHRI	Armauer Hansen Research Institute
ALRT	All African Leprosy and Tuberculosis Rehabilitation and Training
AM-A	Amplification Mix-A
AM-B	Amplification Mix-B
AMK	Amikacin
A-NB	Neutralization buffer
BSC	Biosafety cabinet
BSL	Biosafety level
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleoside triphospate
DOTS	Directly observed treatment short course
DR	Drug resistance
DST	Drug susceptibility testing
EMB	Ethambutol
EPTB	Extrapulmonary tuberculosis
FLD	First line ant-tuberculosis drugs
FMOH	Federal Ministry of Health
INH	Isoniazid

IUATLD	International Union Against Tuberculosis and Lung Disease
LED	Light emitting diodes
LJ	Lowenstein -Jensen
LPA	Line probe assay
MDR-TB	Multi-drug resistant tuberculosis
MDR	Multi-drug resistance
MGIT	Mycobacteria Growth Indicator Tube
MTBC	Mycobacterium tuberculosis complex
NALC	N-acetyl-L-cysteine
NTM	Non-tuberculosis mycobacteria
PBS	Phosphate Buffered Saline
PCR	Polymerase chain reaction
PTB	Pulmonary tuberculosis
RIF	Rifampicin
RNA	Ribonucleic Acid
PCR	Polymerase Chain Reaction
SOPs	Standard operating procedures
SS	Sensitivity
SP	Specificity
STAG	Strategic and Technical Advisory Group
STR	Streptomycin
TB	Tuberculosis

TBLN	Tuberculous lymphadenitis
TDR	Totally drug resistant
TMAC	Tetramethyl ammonium chloride
WHO	World Health organization
WT	Wild type
XDR-TB	Extensively drug resistant tuberculosis
XXDR-TB	Extremely drug resistant tuberculosis
ZN	Ziehl-Neelsen

1. Introduction

1.1 Background Information

Tuberculosis (TB) is a bacterial infectious disease that affects mainly the lungs. It can also attack other parts of the body of infected humans and animals. The disease remains a major global health problem eventhough different diagnostic technology and control measures have been developed. It causes ill-health among millions of people each year and ranks as the second leading cause of death from an infectious disease worldwide after the human immunodeficiency virus (HIV) (WHO, 2013). It will still be among the 10 leading causes of global disease burden in the year 2020 (Corbett *et al.*, 2003). According to WHO 2012 report, there were almost 9 million new TB cases in 2011 and 1.4 million TB deaths which include 990, 000 among HIV negative people and 430, 000 HIV-associated TB deaths (WHO, 2012).

According to World Health Organization (WHO, 2009) report, one-third of the global community is infected with *M. tuberculosis* complex and also estimated 0.7 million cases and 0.2 million deaths in HIV positive people. An increased incidence of tuberculosis occurs mostly in Africa and Asia where the highest prevalence of co-infection with HIV and *M. tuberculosis* occur (WHO, 2012).

However, in 2012 the incidence of tuberculosis is decreased. There were 8.6 million new TB cases including 1.1million cases among people with HIV and 1.3 million TB deaths which include 320, 000 HIV-associated TB deaths (WHO, 2013). The majority of TB cases worldwide were in the South-East Asia (29%), African (27%) and Western Pacific (19%) regions. India and China alone accounted for 26% and 12% of total cases,

respectively. The TB incidence rate at country level ranges substantially, with around 1000 or more cases per 100, 000 people in South Africa and Swaziland, and fewer than 10 per 100, 000 population in parts of the Americas, several countries in western Europe, Japan, Australia and New Zealand (WHO, 2013).

1.2. Situation of Tuberculosis in Ethiopia

Tuberculosis continues to be a major public health problem in Ethiopia. It ranks seventh among the world's 22 countries with high tuberculosis burden (WHO, 2011). The Federal Ministry of Health (FMOH) hospital statistics data showed that tuberculosis is the leading cause of morbidity, the third cause of admission and the second cause of death in the country (MOH, 2008). According to WHO 2007 report, the prevalence and mortality rate of all forms of TB in Ethiopia was estimated to be 546 and 73 per 100,000 populations respectively but based on WHO 2011 report, the estimated prevalence and mortality rate was 394 and 35 per 100,000 respectively (WHO, 2011).

According to the WHO global TB report 2012, there were an estimated 220,000 (258 per 100,000 populations) incident cases of TB in Ethiopia in 2011. The same report also showed that the prevalence of TB was estimated to be 200,000 (237 per 100,000 populations) and 15,000 deaths (18 per 100,000 populations) due to TB, excluding HIV related deaths, in the country during the same period (WHO, 2012). In 2012, the mortality, incidence and prevalence rates of tuberculosis were 18, 247 and 224 per 100,000 populations respectively in the country (WHO, 2013). But the mortality, incidence and prevalence rates are 32, 224 and 211 per 100,000 population respectively in 2013 (WHO, 2014).This shows that the problem of tuberculosis is still high in the country.

1.3. Etiology and Its Characteristics

Tuberculosis is caused by the genus of mycobacterium mainly by the member of *Mycobacterium tuberculosis* complex (MTBC) (Singh *et al.*, 2000). Non *Mycobacterium tuberculosis* complex strains can also cause the disease. The MTC comprises closely related species responsible for strictly human and zoonotic tuberculosis. The species of MTC include *M. tuberculosis*, *M. bovis*, *M. africanum*, *M. microti, M. canetti and M. caprae.* Despite the different species tropisms, the species of mycobacteria have similarity of 99.9% or greater at nucleotide level and possess identical 16S rRNA sequence (Dye *et al.*, 2005; Smith *et al.*, 2006).

The mycobacteria are classified under the class Actinomycetes, order Actinomycetales, family Mycobacteriaceae and genus Mycobacterium (Shinnick and Good, 1994). Most of them grow slowly on solid Lowenstein - Jensen (LJ) medium and are resistant to drying, disinfectants and remain viable in clinical samples for a long period of time (Evans, 1998).

The cell wall of mycobacteria is thick consisting of a plasma membrane surrounded by a complex wall structure harboring virulence factor such as peptidoglycan, arabinogalactans, mycolic acids (long chain fatty acids), glycolipids, and lipoarabinomanans (Alamelu, 2004). The bacteria are rod shaped, acid fast because of their lipid-rich cell walls, aerobic or micro-aerophilic, non-spore forming, non-motile and non-capsulated bacteria (Frieden *et al.*, 2003). They are 2-4µm in length and 0.2-0.6µm in width. It is facultative intracellular parasite mostly of macrophage and has a slow generation time about 15-20 hours. Its lipid rich cell wall provides the bacteria with thick waxy coat which is responsible for acid fastness, hydrophobicity and other unfavorable

environmental conditions. The structure also provides resistance against different disinfectants, common laboratory reagents, antibiotics and physical injuries. Since it restricts the uptake of nutrients that are important for metabolic pathways, it probably contributes to the slow growth rate of some species (Palomino *et al.*, 2007).

The mycobacterial cell wall glycolipids interact with polymorphonuclear leukocytes and macrophage lysosomal membranes to prevent their fusion with phagosomes. The cell wall glycolipids, lipoproteins, and/or lipid may also play a role in the stimulation of delayed type of hypersensitivity and/or immunity (Flynn and Chan, 2001).

1.4. Disease Transmission and Risk Factors

Tuberculosis is most commonly transmitted by inhalation of infected droplet nuclei which are discharged in the air when somebody with untreated sputum-positive pulmonary TB is coughing, sneezing, talking and singing. These produce droplets that may contain tubercle bacilli (Nachega and Chaisson, 2003). The droplet nuclei may contain from one to ten bacilli and a diameter close to 10μm are expelled and suspended in the air and transported by air currents. The infected droplet nuclei can be kept airborne for prolonged periods of time and spread throughout rooms or buildings. Some of these droplet nuclei, usually larger than 10μm are inhaled and anchored in the upper respiratory tract (Wells, 1995).

The effective infective droplet nucleus that measures 5μm or less is able to avoid the mucus and ciliary system action and produce the anchorage in bronchioles and respiratory alveoli. The small size of the droplets allows them to remain suspended in the air. Although theoretically a single organism may cause the disease, it is generally

accepted that about 5 to 200 inhaled bacilli are necessary for a successful infection (Palomino *et al.*, 2007).

The presence of extensive pulmonary lesions such as cavities is the most important individual human factor in determining the infectious power. Since extensive pulmonary lesions are associated not only with an important concentration of oxygen that allows active bacillary multiplication but also with a rapid pathway to the external environment. The amount of bacilli released into the atmosphere under these conditions is enough to produce the transmission from person to person (Correa, 1997).The disease can also be transmitted to humans through ingestion of infected raw milk and close contact with infected animals (Skuce *et al.*, 2003).

Persons who have prolonged or intense contacts with infected individuals are at high risk of becoming infected. Residents and employees of high congregate settings, individuals living in TB endemic areas and health care workers who handle high risk TB patients are also at high risk to be infected by the disease (Kazwala, 2001). There are many factors that accelerate tuberculosis infection in the communities of developing countries. These are poverty, changing demographics with increasing crowding and changing age structure (children less than five years and the elderly greater than 65 years are more vulnerable), inadequate health coverage and lack of well organized laboratory capacity and well trained laboratory technicians. Other factors include: chronic infections, neglect and under-funding of TB control program, previous exposure to mycobacterium infections, migration, malnutrition, and cytotoxic therapy can also aggravate the disease progression (Yang *et al.*, 2004).

1.5 Clinical Presentation

There are two forms of tuberculosis based on the organs it affects. These are pulmonary tuberculosis (PTB) and extra pulmonary tuberculosis (EPTB). Although pulmonary tuberculosis is the most common presentation of the diseases, extra pulmonary tuberculosis is also an important clinical problem (WHO, 2011). Extra pulmonary tuberculosis can occur in isolation or along with a pulmonary focus as in the case of patients with a disseminated form of tuberculosis. Tuberculous lymphadenitis (TBLN) is a common form of extra pulmonary tuberculosis. Lymph nodes (cervical, axillary and inguinal) are the most common sites of involvement.

Pulmonary TB patients usually have weight loss and productive cough for more than three weeks. Symptoms such as chest pain, dyspnea, fever, weight loss, fatigue, cough and night sweating, anorexia and haemoptysis are common among TB patients (Nataraj *et al.*, 2002). Patients usually present with gradual enlarging of lymph nodes and may otherwise be asymptomatic. Isolated cervical lymphadenopathy is most commonly seen in about 67 % HIV negative patients (Sharma and Mohan, 2004). Among HIV negative as well as HIV positive patients, cervical lymph nodes were mostly affected as compared to axillary and inguinal lymph nodes (Bem, 1997). The enlarged lymph nodes may be of varying size, are usually firm, painless and may be discrete or matted. The lymph nodes are not tender unless secondary bacterial infection has occurred (Alamelu, 2004). Diagnosis of TBLN is a formidable challenge in developing countries where there is high rate of human immunodeficiency virus infection (Frieden *et al.*, 2003).

1.6 Drug Resistant Tuberculosis

This is a disease caused by *Mycobacterium tuberculosis* organisms that are resistant to at least one first line anti-tuberculosis drug. Multidrug resistance TB (MDR-TB) is defined as disease caused by an organism that is resistant to at least rifampin (RIF) and isoniazid (INH), the two most important first-line anti-TB drugs (FLD) with or without resistance to other anti-TB drugs (Jain and Dixit, 2008). MDR-TB strains could arise as a consequence of sequential accumulation of mutations conferring resistance to single therapeutic agents, or by a single-step process such as acquisition of an MDR element, or mutation that alters cell wall structure (Sharma and Mohan, 2004).

MDR-TB represents a serious problem for clinical management, and has become an important public health issue. Lack of access to quality laboratory diagnostics continues to jeopardize efforts to control the worldwide transmission of TB. This situation has been further complicated by the emergence of extensively drug resistant TB (XDR-TB). XDR-TB is a form of drug resistance TB caused by MDR strains of *M. tuberculosis* that have developed resistance to any fluoroquinolone and at least one of the following three second line injectable drugs: capreomycin, kanamycin and amikacin (Jassal and Bishai, 2009). It is the most problematic forms of resistance because treatment options are limited and the second-line drugs used for therapy are more toxic, less effective, more expensive, and must be administered for a longer period of time than standard first-line drug therapy (Migliori *et al.*, 2008).

Recently, a new dangerous form of resistant tuberculosis bacilli was also identified. This group of strains showed *in-vitro* resistance to the entire first and second–line drugs tested. They were named as totally drug resistant (TDR) or extremely drug resistant (XXDR-TB)

strains (Velayati *et al.*, 2009).The patients infected with XDR and XXDR-TB are difficult to treat and may increase the risk of disease transmission among the communities.

1.6.1 Burden of Drug Resistant Tuberculosis

Drug-resistant tuberculosis has emerged as an important global public health threat. The World Health Organization estimates that 489,000 cases of multi-drug resistant TB (MDR-TB) occur annually, predominantly in Eastern Europe and Asia (WHO, 2008). Countries such as South Africa, China, India and Russia have almost 60% of the world's MDR-TB burden (WHO, 2012). The prevalence of MDR-TB is rising in a number of geographic regions. According to WHO report, the median prevalence of MDR-TB is 1% (range 0%–14.1%) among new cases and 9.3% (range 0%–48%) among previously treated cases (WHO/IUATLD, 2004). The prevalence of MDR-TB in new TB cases is 0.8%, 0.9-2.6% and 1.8% in Botswana, South Africa and Zambia, respectively. However, among previously treated cases in high-HIV-burden African countries, MDR prevalence is estimated at 6.3% (Zignol *et al.*, 2006). Relatively, a lower frequency of MDR-TB has been reported in Bangladesh (0.23% for new cases and 5.56% for previously treated patients) (van Deun *et al.*, 1999). Alarming increases in MDR-TB, the emergence of extensively drug-resistant TB (XDR-TB), potential institutional transmission, and rapid mortality of MDR-TB and XDR-TB patient with HIV co-infection, have highlighted the urgency for rapid and effective screening methods (WHO, 2005).

1.6.2 Situation of Drug Resistant TB in Ethiopia

Drug resistance tuberculosis threatens the National Tuberculosis Control Programme in several countries including Ethiopia, and the major problem is multidrug resistance TB (MDR-TB) (WHO/IUATLD, 2000). The problem of drug resistant TB exists in different parts of Ethiopia, and data on patterns of resistance among Ethiopian isolates is ranging from 2%-21% for isoniazid, 2%-20% for streptomycin and 14%-15% for any of the drugs tested(Abate, 2002; Bruchfeld, 2002). According to WHO Global TB report, Ethiopia is ranked as 15th among 27 high burden M(X) DR-TB countries. In 2008, the prevalence of MDR-TB was reported as (0.9%–2.8%) for new cases and (5.6%–21%) for retreatment cases in the country (WHO, 2011). The study done in Bahir Dar (north west Ethiopia) indicated that 11.8% MDR-TB and 1% XDR-TB cases (Hussein *et al.*, 2013). According to Abate *et al.*, 2012, MDR-TB was found in 46.3% of retreatment cases and 9.7% by Ali *et al*., 2012 among all cases. This shows that the prevalence of MDR-TB is high in Ethiopia and suggests establishing diagnostic facilities for early case detection and treatment of MDR-TB in the country is important.

1.7 Statements of Problem

The diagnosis of tuberculosis remains a challenge in resource-limited countries including Ethiopia. This is particularly due to limitations in sputum smear microscopy, the cheapest and widely used test in such settings. Due to limited diagnostic facilities at hand in Ethiopia, much of the diagnosis depends on clinical expertise that may result in misdiagnosis and mistreatment of the disease which in turn facilitates the development of drug resistant TB. The clinical management of TB cases in the country is also hampered by the lack of rapid and effective diagnostic tests.

Although conventional methods for mycobacteriological culture and drug susceptibility testing (DST) are considered as gold standard, the processes are slow and cumbersome, requiring sequential procedures for isolation of mycobacteria from clinical specimens, identification of *Mycobacterium tuberculosis* complex, and *in vitro* testing of strain susceptibility to anti-TB drugs. During this time, patients may be inappropriately treated, drug-resistant strains may continue to spread, and resistance may become amplified (WHO, 2012). The utility of currently available novel molecular diagnostic technologies such as line probe assay for diagnosis of TB cases and detection of drug resistance in the context of Ethiopia remains poorly understood.

Because of the above diagnostic difficulties (need of too long time (8-12) weeks for culture and drug sensitivity test results, low sensitivity of smear microscopy and poor understanding of the utility of new molecular techniques), the initiation of appropriate therapy is often delayed in patients with TB and drug resistant TB, resulting in increased transmission, morbidity and mortality of the disease in the community. Patients without TB may receive unnecessary treatment for several weeks and even for months. During this time, the patients will suffer from the adverse effects (vomiting, nausea, loss of appetite, liver toxicity) of the treatment. This unnecessary treatment causes significant economic loss (Lee *et al.*, 2003). Late treatment of truly positive TB patients on the other hand can also jeopardize recovery and cure of the patient (Ismail, 2002). The limitations of the conventional methods for diagnosis of TB and drug resistant TB have spurred multi-faceted research activities in this field throughout the world. Therefore, a sensitive, rapid and accurate test would be of tremendous benefit in the diagnosis of TB and

detection of drug resistant TB. Thus, testing the feasibility of line probe assay is important.

1.8 Significance of the Study

It is well understood that rapid diagnosis of TB infection and drug resistance would facilitate rapid initiation of an effective treatment, leading to improved clinical management and reduce the transmission of the disease in the communities. However, the performance of Genotype MTBDR*plus* version 2.0 line probe assay to diagnose tuberculosis and detect drug resistance as an alternative to conventional methods is not well studied in Ethiopia. Hence, this study would provide the baseline information of the diagnostic accuracy of the assay on direct specimens of MDR-TB patients. As a result, the resources and time wasted by the TB patients, the laboratory workers and clinicians will be saved. Most importantly, the lives of many TB patients will be saved.

Study Hypothesis: Using Genotype MTBDR*plus* version 2.0 line probe assay as a diagnostic tool improves diagnosis of tuberculosis and detection of associated drug resistance compared to the gold standard method.

2. Literature Review

2.1 Laboratory Diagnosis of Tuberculosis and Detection of Drug Resistance

2.1.1. Microscopy

Direct smear examination with Ziehl-Neelsen (ZN) staining for the diagnosis of tuberculosis as employed in most low-income countries is cheap and easy to use, but its low sensitivity is a major drawback (Kivihya-Ndugga *et al.*, 2004). Moreover, it requires sputum samples collected on consecutive days, making the procedure slow and making patient compliance with the diagnostic process difficult. It is inexpensive and is relatively specific in settings where tuberculosis is endemic. However, direct smear microscopy can produce false-negative results, which have been observed in high HIV-prevalent settings and children (Frieden *et al.*, 2003; Newton *et al.*, 2008).The test may also identify certain types of bacteria that are not *M. tuberculosis*, thus yielding a false-positive result for tuberculosis. It is estimated that sputum smear microscopy only identifies 35 percent of patients with tuberculosis (Harris, 2004). Furthermore, the 2010 WHO report indicated that in 2009, 43 percent of the 4.6 million reported new cases of pulmonary TB were diagnosed without microbiological confirmation (WHO, 2010). The failure to confirm TB infection can delay initiation of the appropriate therapy to adequately treat cases, which could prevent further spreading of the disease(Cambanis *et al.*, 2007).

Several attempts have been made to improve and optimize the performance of smear microscopy, including with new technologies (Ramsay *et al.*, 2009), such as fluorescence microscopy, which uses inexpensive light-emitting diodes (LED) as an alternative for conventional ZN microscopy. This substitution increases the sensitivity of the test and is easier to use, even in peripheral laboratories where culture facilities are not available

(Hooja *et al.*, 2011). The World Health Organization Strategic and Technical Advisory Group (STAG) for TB recommended that fluorescence microscopy be phased in as an alternative for ZN (WHO, 2009), because it can be used even in low-income and high TB burden settings. It has been reported that LED fluorescence microscopy, either alone or in combination with single-specimen tests, could increase considerably the identification of smear-positive cases (Cattamanchi *et al.*, 2011).

2.1.2 Mycobacterial Culture Method

Identification of tubercle bacilli by culture is required for the ultimate proof of mycobacterial infection. However, due to lack of laboratory equipments and appropriate safety procedures, the method is not well practiced in resource-limited countries (Hung *et al.*, 2000).

2.1.2.1 Solid Culture

The ideal medium for isolation of tubercle bacilli by culture should be economical, simple to prepare from readily available ingredients, inhibit the growth of contaminants, support abundant growth of small numbers of bacilli in the specimen and permit preliminary differentiation of isolates on the basis of colony morphology, growth rate and pigment production (Ang *et al*., 2001, Kent and Kubica, 1985). Most of mycobacterial culture media fall into egg-potato-base media and agar-base media (American Thoracic Society, 2000). The most popular egg-based media are the Lowenstein-Jensen buffered egg-potato medium and the American Trudeau Society egg yolk-potato flour medium. Among the agar based media, Middlebrook 7H-10, Middlebrook 7H-11, and Dubose oleic-albumin agar are recommended. Bacterial culture on egg - based Lowenstein Jensen media are the commonly used media for mycobacterial culture in most laboratories since

they meet most of the aforementioned requirements (Singh *et al.,* 2000). LJ medium enriched with sodium glycerol favors the growth of *M. tuberculosis* but glycerol is inhibitory for most strains of *M. bovis*. LJ medium containing pyruvate improves the growth of *M. bovis* over that on glycerol medium. Both of them are recommended to be used in countries where patients may be infected with either organism (Small *et al.*, 1994).

Culture is still seen as the gold standard for TB detection because it is sensitive to live *M. tuberculosis* in the sputum sample; it can also provide data on the likely effectiveness of certain chemotherapeutic agents against TB. However, there are serious drawbacks to this method such as the time needed to obtain the result which is about 3–8 weeks (Szewzyk *et al.*, 1995) clinical and therapeutic decisions are often made before the culture results are available.

2.1.2.2 Liquid Culture

There are several manual and automated liquid culture systems that have been developed to reduce mycobacterium detection time in clinical specimens. Biphasic Septi-chek acid-fast bacilli (Becton Dickinson, Sparks, MD) and MB-Redox (BiotestAG, Dreieich, Germany) are examples of the manual systems. Radiometric BACTEC 460TB (Becton Dickinson), the fluorometric BACTEC MB9000 and BACTEC MGIT (Mycobacteria Growth Indicator Tube), 960 systems (Becton Dickinson), the carbon dioxide–sensing MB/BacT ALERT 3D System (Organon Teknika, Durham, NC), and the pressure-sensing ESP Culture System II (Trek Diagnostic Systems, Westlake, OH) are automated systems that developed as a result of advancement in technology. These liquid culture systems have their own advantage and limitation (Guillerm *et al.*, 2006).

Detection time and isolation of MTBC are considerably improved (7–21 days) by using radiometric BACTEC 460 TB broth-based system. However, this procedure still requires well trained laboratory technicians, well established laboratory and needs special attention to safety issues regarding radioisotopes (Salfinger and Pfyffer, 1994). Another limitation of this method is increased cost of radioactive waste disposal.

The fully automated BACTEC MGIT liquid medium system with early growth indicators (the BACTEC MGIT 960 system), is faster and more sensitive than both LJ and BACTEC 460 TB for testing the susceptibility of anti-tuberculosis agents, and it is more effective in diagnosing the disease in smear-negative samples; this feature shows great potential to reduce the mortality rate from TB (Morcillo *et al.*, 2010). The system is a high-capacity, fully automated continuous-monitoring system, which can test up to 960 samples for the rapid detection of mycobacteria, making it suitable for those laboratories dealing with a large number of specimens (Lee *et al.*, 2003). In the determination of the early bactericidal activity in the clinical studies of new anti-tuberculosis agents, it has been found that the time of detection of MGIT 960 is better than colony-forming units of MTBC on solid media (Diacon *et al.*, 2010).

WHO recommended the expanded use of liquid culture systems, such as MGIT, in resource-poor settings but the systems have not been used because of the high cost of the tests and contamination rates of the culture (5.5-15% and 29.3-33%) in high-income and resource constrained settings respectively (Chihota *et al* ., 2010).

Although automated systems such as BACTEC 460 TB, BACTEC 9000, and MGIT can be used to accelerate the growth of the bacteria, they can also produce inaccurate results (Daniel, 1987). In addition, it is not always possible to obtain bacteria in the sputum sample. False positives, which range from 0.1-65% because of laboratory contamination is another concern with the culture technique (Ruddy *et al.*, 2002). Therefore, even though culture has thus far been considered the gold standard for TB diagnosis, it still lacks the desired accuracy. It has also been estimated that no more than 81 percent of the confirmed TB cases can be detected by culture (API, 2006).

2.1.3 Molecular Methods

2.1.3.1 Polymerase Chain Reaction (PCR)

DNA amplification using PCR is considered as one of the most sensitive approaches for detection of mycobacterial DNA, which involves genomic DNA extraction, amplification and identification (Kurabachew *et al.*, 2004). It has allowed great progress to be made in the rapid and accurate diagnosis of infections due to organisms that are not cultivable by *in vitro* means, that require complex media, or cell cultures and prolonged incubation times (Bouakline *et al.*, 2003).

In contrast to pulmonary samples, PCR has less sensitivity in detection of mycobacterial DNA from extra pulmonary samples (Delportillo *et al.*, 1996). This might result from the use of very small sample volumes and the presence of inhibitors (both DNA extraction & PCR), which interfere with amplification based techniques. Inhibitory activity was often checked for discrepant results between culture and PCR results (Shah *et al.*, 2002). It is difficult to identify the substance(s) responsible for this inhibition, which might be blood,

detergents or heparin. In order to minimize the inhibitory substance (s), simple dilution of the specimen is of paramount importance (Rimek *et al.*, 2002).

The test has a specificity of 100% and sensitivities ranged from 20 to 94% for extra pulmonary specimens. It is important that any PCR - based assay includes positive and negative internal control to allow proper evaluation of DNA preparation and amplification (Bouakline *et al.*, 2003).

2.1.3.2 Line Probe Assay (LPA)

Line probe assay is one of the new molecular diagnostic tests used for TB diagnosis and detection of drug resistant TB. The World Health Organization recommended the use of the assay for rapid screening of MDR-TB in low and middle income settings from smear positive pulmonary specimens (WHO, 2008). There are two types of line probe assay. These are the INNO-LIPA Rif TB (Innogenetics, Ghent, Belgium) and the GenoType MTBDR (Hain Life-Science, Nehren, Germany). Both tests are based on multiplex polymerase chain reaction (PCR) amplification (more than one target sequence amplified by using multiple primer pairs in a reaction mixture) and reverse hybridization of amplicons (*rpoB* in the INNO-LIPA Rif TB and *rpoB* plus *katG* in the GenoType MTBDR) to immobilized membrane-bound probes, allowing the detection of mutations at the level of the most frequently mutated codons to identify MTBC and mutations to genes associated with RIF and INH resistance. The genes (*katG, inhA* and *rpoB)* coding for Catalase peroxidase, NADH enoyl Acp redactase and β-subunit of the RNA polymerase, respectively. The presence of a mutation in the genes is revealed by the absence of hybridization at the level of the wild-type probes (*rpoB* WT1 to WT5 and *katG* WT), with a possible positive hybridization signal at the level of the mutant probes

(*rpoB* MUT D516V, MUT H526Y, MUT H526D, MUT S531L, and *katG* MUT S315T) (Brossier, 2006). These probes are found in 81 base pair region or rifamicin resistance determining region which is part of rpoB gene encoding beta subunit of the DNA dependent RNA polymerase.

2.1.3.2.1 GenoType MTBDR*plus* Assay

Recently, the GenoType MTBDRplus was further developed from the original GenoType MTBDR version. In addition to the detection of mutations in the genes conferring resistance to RIF (*rpo*B) and high-level INH (*kat*G), as applied in the original version, probes to detect mutations within the *inh*A gene causing low-level INH resistance have been included. In the new version of the DNA strip, the *inh*A gene is covered by two *inh*A WT (−15, −16 and −8 nucleic acid positions) and four *inh*A mutation probes (C15T, A16G, T8C and T8A). In addition, three more *rpo*B WT probes targeting codons 505–533 were included (Vijdea *et al.*, 2008).

Currently, the GenoType MTBDR*plus* has two versions (GenoType MTBDR*plus* version 1 and version 2). The sensitivities of both versions are directly related to the specimen's bacillary burden (Dorman *et al.*, 2012). GenoType MTBDR*plus* version 1 has been limited for use on smear-positive patient material. It is also relatively time consuming. But the GenoType MTBDR*plus* version 2 is used for testing on both smear-positive and smear-negative patient material (Barnard *et al.*, 2012). The same study showed that the sensitivity and specificity of GenoType MTBDR*plus* (v2.0) assay for the detection of rifampin resistance detection is 100%. Barnard and his friends recommended that the GenoType MTBDR*plus* v2.0 assay can complement the Xpert MTB/RIF screening assay by validating rifampin susceptibility and providing information on isoniazid

susceptibility. In addition, they concluded that the assay can provide pharmacogenetic information that may be critical in guiding appropriate treatment.

The study done by Crudu and his friends indicated that as the assay has the ability to detect RIF and INH resistance with sensitivity and specificity of 94.3 and 96.0%, respectively. The authors also concluded that the MTBDR*plus* version 2.0 assay is rapid and highly sensitive test for the detection of *M. tuberculosis* strains from smear-positive and smear-negative clinical specimens and provides additional information on RIF and INH resistance status, which can easily be included in routine laboratory work flow (Crudu *et al.*, 2012).

The study done by Anek-vorapong and his colleagues showed the sensitivity of the assay is 95.3%, 100%, and 94.4% for INH resistance, RIF resistance, and MDR-TB, respectively, and the specificity is 100% for all resistance patterns and for both specimens and isolates (Anek-vorapong *et al.*, 2010). Moreover, the sensitivity and specificity for detection of resistance to rifampicin is 100% and 97.3%, and to INH is 91.9% and 98.4%, respectively (Raveendran *et al.*, 2013). Furthermore, the study done in Russia also showed that the sensitivity and specificity of the assay for the detection of rifampicin and isoniazid resistance and MDR are 96.2%, 97.4%, 97.1% and 90.7%, 83.3%, 88.9% respectively (Nikolayevsky *et al.*, 2009). In general, the authors concluded that MTBDR*plus* assay has good sensitivity and specificity with turnaround time of less than two days and it is also a useful tool for rapid detection of multidrug resistant tuberculosis.

2.1.3.2.2 INNO-LiPA Rif.TB Assay

This is one of the line probe assay used for the identification of *Mycobacterium tuberculosis* complex strains and the detection of rifampin (RIF) resistance. The result of the study done in Portugal showed that the specificity and sensitivity of the INNO-LiPA Rif.TB assay for the detection of the RIF resistance profile are 100.0% and 96.9%, respectively (Amaral *et al.*, 2005). Similarly, the data obtained from systematic reviews and meta-analyses that were used to evaluate assay performance against conventional DST methods showed the sensitivity greater than 95% and specificity 100% of the assay. According to the meta –analysis, the assay is highly sensitive and specific for detecting rifampicin-resistant TB both in culture isolates and, to a slightly lesser degree, in clinical specimens (Morgan *et al.*, 2005).The expert group concluded that INNO-LiPA Rif.TB is a highly sensitive and specific test for the detection of rifampicin resistance in isolates of *M. tuberculosis* complex (WHO, 2008).

2.1.3.2.3 GenoType MTBDRsl Test

This is a new molecular kit designed for rapid identification of the resistance to the second-line anti-tuberculosis drugs with a single strip (Feng *et al.*, 2013). It is a rapid and reliable drug susceptibility test that can be easily incorporated into the diagnostic algorithm. It significantly improves diagnostic yield while simultaneously decreasing diagnostic delay for reporting second-line drug susceptibility test (Barnard *et al.*, 2012).

The test is based on the DNA-strip technology and permits the molecular genetic identification of the *M.tuberculosis* complex and its resistance to fluoroquinolones, aminoglycosides and ethambutol from cultivated samples or pulmonary smear positive clinical specimens. The identification of resistance to fluoroquinolones is enabled by the

detection of the most significant mutations of the *gyrA* gene (coding for DNA gyrase). For detection of resistance to aminoglycosides the 16S rRNA gene *(rrs*) and for detection of resistance to ethambutol the *embB* gene (which together with the genes *embA* and *embC* codes for arabinosyl transferase) are examined.

One study showed that the sensitivity and specificity of the assay to detect resistant to different first line drugs are 100% and 100% for injectable second line drugs, 91% and 98% for fluoroquinolones, 56.2% and 81% for ethambutol, respectively (Ajbani *et al.*, 2012).

Another study result showed that the sensitivity of the test for detecting ofloxacin, amikacin, and extensive drug resistance is 90.7%, 100% and 92.3%, respectively, and the specificity for detection is 98.1%, 99.4% and 99.6,respectively (Barnard *et al.*, 2012). The study done in France also showed that the sensitivity and specificity of the Genotype MTBDR*sl* test are 87% and 96%, respectively, for fluoroquinolones; 100% for both for amikacin; 77% and 100%, respectively, for kanamycin; 80% and 98%, respectively, for capromycin; and 57% and 92%, respectively, for ethambutol (Brossier *et al.*, 2010).

2.1.3.3 Gene Xpert MTB/RIF Assay

Gene Xpert MTB/RIF assay is a novel integrated diagnostic device that performs sample processing and hemi nested real-time PCR analysis in a single hands-free step for the diagnosis of tuberculosis and rapid detection of RIF resistance in clinical specimens. The MTB/RIF assay detects *M. tuberculosis* and RIF resistance by PCR amplification of the 81-bp fragment of the *M. tuberculosis rpoB* gene and subsequent probing of this region for mutations that are associated with RIF resistance. The assay can generally be

completed in less than 2hrs (Blakemore *et al.*, 2010).The machine is robust under varying temperature and humidity conditions. It requires minimal training of the personnel. This molecular assay has also some limitations: it needs uninterrupted and stable electrical power supply and annual calibration of the modules, which may pose a problem in rural/remote settings (WHO, 2011).

The assay has shown 100% sensitivity for detecting smear-positive isolates but only 71.7% sensitivity for detecting smear-negative culture-positive isolates (Helb *et al.*, 2010). In a larger field trial, the assay was shown to be 98.2% sensitive for the identification of culture-positive isolates but only 72.5% sensitive for the identification of smear-negative culture positive isolates; the test had a reported specificity of 99.2% (Boehme *et al.*, 2010). In the same study, the assay was shown to be highly sensitive for detecting rifampin resistance, correctly identifying 97.6% of rifampin-resistant isolates and 98.1% of rifampin susceptible isolates.

Another study result showed that the assay has specificity and sensitivity of 90.6% and 94.3% respectively for pulmonary samples, and for the extrapulmonary samples, it has a specificity and sensitivity of 100% and 91.6% respectively. For microscopically negative specimens, the respective values were 86.3% and 93% (Loannidis *et al.*, 2011). According to the study done by Zeka and his colleagues, in pulmonary specimens, the sensitivities are 100% and 68.6% for smear-positive and smear-negative specimens, respectively. The test appeared to be as sensitive as culture with smear-positive specimens but less sensitive with smear-negative pulmonary and extrapulmonary specimens that include low numbers of bacilli (Zeka *et al*., 2011).

Even though the assay has high sensitivity and specificity for detection of drug resistance against rifampicin, it cannot detect isoniazid resistance. Other potential disadvantages include cost and, although to a lesser extent than line-probe assays, a continued need for adequate laboratory infrastructure and training of personnel (Boehme *et al.*, 2010).

2.1.3.4 DNA Sequencing

DNA sequencing methods determine the order of the nucleotide bases such as adenine, guanine, cytosine, and thymine in a molecule of DNA. This technique enables us to perform a thorough analysis of DNA because it provides us with the most basic information of all: the sequence of nucleotides. DNA sequencing is the most accurate and reliable method for mutations (point, insertion, deletion and substitution) detection and it is used as the gold standard technique for diagnosis of mutation. It allows detecting both previously recognized and unrecognized mutations (Kourout *et al.*, 2009). It has been performed by manual and automated procedures although the latter is now the most commonly used. It has been widely used for characterizing mutations in the *rpoB* gene in rifampicin-resistant strains and to detect mutations responsible for resistance to other antituberculosis drugs. The evaluation done on molecular methods showed that DNA sequencing is the most sensitive and specific assay for detecting rifampin resistance with the 100% sensitivity and 100% specificity. This indicates a 100% correlation with conventional susceptibility test results (Nachamkin *et al.*, 1997). However, except for RIF, DNA sequencing is unlikely to be used in routine detection of drug resistance mutations because it requires several sequencing reactions per isolate becoming labor-intensive and costly (Therese *et al.*, 2012).

2.1.3.5 Pyrosequencing

Pyrosequencing is a sequencing method which is rapid and accurate for detecting *M. tuberculosis* resistance to rifampin, isoniazid, ethambutol and fluoroquinolones. It determines the exact sequence, thereby providing the same accuracy as the conventional sequencing method (Zhao *et al.*, 2005).

The sensitivity and specificity of pyrosequencing for detecting rifampicin resistance are 97.2% and 97.9%, respectively for clinical strains (García-Sierra *et al.*, 2011). According to the study result reported from Philippines in 2009,the sensitivity and specificity of pyrosequencing are 96.7% and 97.3%, for the detection of resistance to rifampin (Bravo *et al.*, 2009). Another study result showed that the sensitivity and specificity estimates for detection of rifampicin resistance from clinical specimen are 89% and 99% respectively (Guo *et al.*, 2013). In general, the authors concluded that pyrosequencing is a highly sensitive and specific tool for the detection of rifampicin resistance in *M. tuberculosis* and can be directly applied on clinical specimen.

3. Objectives

3.1 General Objective

The general objective of this study was to evaluate the performance of line probe assay for diagnosis of tuberculosis and detection of associated drug resistance.

3.2 Specific Objectives

The specific objectives of the study were to:

- determine the sensitivity and specificity of Genotype MTBDR*plus* version 2.0 line probe assay for detection of *M. tuberculosis* complex from sputum specimens using LJ culture as a gold standard method.
- compare the performance of Genotype MTBDR*plus* version 2.0 line probe assay with that of Gene Xpert MTB/RIF assay for detection of MTBC and drug resistance to rifampicin.
- compare the performance of Genotype MTBDR*plus* version 2.0 line probe assay with Spoligo-rifampicin-isoniazid typing for detection of isoniazid and rifampicin resistance.
- Identify the resistant strain types circulating in Addis Ababa.

4. Materials and Methods

4.1 Study Site and Study Period

The study was conducted at Addis Ababa regional laboratory. However the study participants were selected from four hospitals and twenty eight health centers (Table 1) found in Addis Ababa, Ethiopia from December 2013 to September 2014.

Table 1 List of names of health centers and hospitals

Health Centers and hospitals			
Addis Ketema	Wereda 9 (Lafto)	Entoto	Wereda 11 (Kolfe)
Wereda 7 (Lafto)	Betesaida	Senay	Goro
Wereda12(Gulale)	Meshualeka	Brihane Selam	Bole 17/20
Beletshachew	Philipos	Yeka	Kirkose
Arada	Shegole	Kotebe	Hayat hospital
Kolfe	Wereda 23(Lideta)	Semen	Zewditu hospital
Lideta	Alem Bank	Bole	ALRT hospital
Amoraw	Merey	Hidase	Betezatha hospital

4.2 Study Design

A cross-sectional study design was employed to evaluate the diagnostic ability of Genotype MTBDR*plus* version 2.0 line probe assay for the diagnosis of TB and detection of drug resistance against rifampicin and isoniazid. All TB patients fulfilling the selection criterion and visited the selected hospitals and health centers in Addis Ababa during the study period were sampled by using non probability or convenient sampling methods.

4.3. Source of Study population

The source population was drug resistant TB suspected patients who had visited the selected hospitals and health centers (Table 1) for medical care during the study period, and their sputum samples were referred to Addis Ababa regional laboratory for further diagnosis.

4.4 Study Participants

All drug resistant TB suspected patients whose sputum samples had been referred to Addis Ababa regional laboratory and who had enough leftover sputum samples during the study period, were the study participants. The participants were selected as drug resistant TB suspected patients if they were previously treated with first line anti-TB drugs but failed to respond to the drugs, come after interruption of taking the drugs, remain smear positive at the end of intensive phase and previously treated with unknown treatment outcome.

4.5 Eligibility Criteria

4.5.1 Inclusion Criteria

- Drug resistant TB suspected patients.
- Volunteer to participate in the study.
- Eighteen years or above 18 years old.
- Agreed and signed in consent form.

4.5.2 Exclusion Criteria

- Drug resistant TB suspected patients whose leftover sputum samples were not enough for the study,
- Withdrawal of consent at any time during the study period and
- Younger than 18 years.

4.6 Measurement Variables

Table 2 Dependent and independent variable

Dependent variable	Independent variable
❖ LPA result ❖ Culture result	❖ Previous treatment ❖ Age ❖ HIV status ❖ Sex

4.7 Sample Size Determination

All TB patients fulfilling the selection criterion and visited the selected hospitals and health centers during the study period were sampled. Sputum samples were collected from 96 informed and consented patients with AFB smear positive and smear negative drug resistant TB suspected patients.

4.8 Data Collection and Laboratory Analysis

4.8.1 Sample Collection

Sputum samples were collected from patients fulfilling the inclusion criteria and visited the study areas. All samples were collected from the study subjects under the recommendation of health professionals working in the selected hospitals and health centers. The samples were collected by trained health professionals using sterile and tightly closed two 50ml falcon tubes with the maximum care and safety. This was done as part of the clinical management of the patients and no additional samples were collected for the purpose of this study, i.e. left over samples from consenting patients were used. In patients fulfilling the inclusion criteria and who agreed to participate in the study, patient's age, sex, clinical and medication taking history specifically, anti-TB treatment taking history was collected using a data collection sheet.

4.8.2 Sample Transportation and Storage

After sample collection was made, the tubes containing sputum specimen were covered with biohazard bag and packed in ice box. Then, it was sent to the Addis Ababa regional laboratory for both mycobacteriological and molecular analysis and stored at +4^{o}C until processed. After decontamination was performed, some portion (about 1ml) was transported to Armauer Hansen Research Institute (AHRI) TB laboratory in ice box for

culture purpose. The remaining portion of the sediments was stored in refrigerator at +4^{o}C in Addis Ababa regional laboratory until it was analyzed.

4.8.3 Sample Processing

Sample processing was done within the maximum of three days of sample collection. The digestion-decontamination procedure of all collected samples was based on the guideline of WHO (Kent and Kubica, 1985; Kantor *et al.*, 1998). The specimen in one falcon tube was analyzed by using Gene Xpert MTB/RIF assay. The sputum in another tube (about 5ml) was decontaminated from non-mycobacterium organisms by using N-acetyl-L-cysteine NaOH (NALC-NaOH) methods for 15 minutes. The decontamination process was strictly followed for the exact time. Then, the solution was neutralized with phosphate buffer saline (pH = 6.8) and centrifuged at 3000xg for 15 minutes. The supernatant was discarded and the sediment was mixed with 1ml phosphate buffer saline (pH = 6.8). Half of the sediment was used for mycobacterial culture on LJ media and the remaining sediment was used for DNA extraction to be analyzed by Genotype MTBDR*plus* version 2.0 line probe assay, spoligotyping and pyrosequencing methods (Fig. 1). All the clinical specimens were processed in biosafety level 3 (BSL-3) facilities.

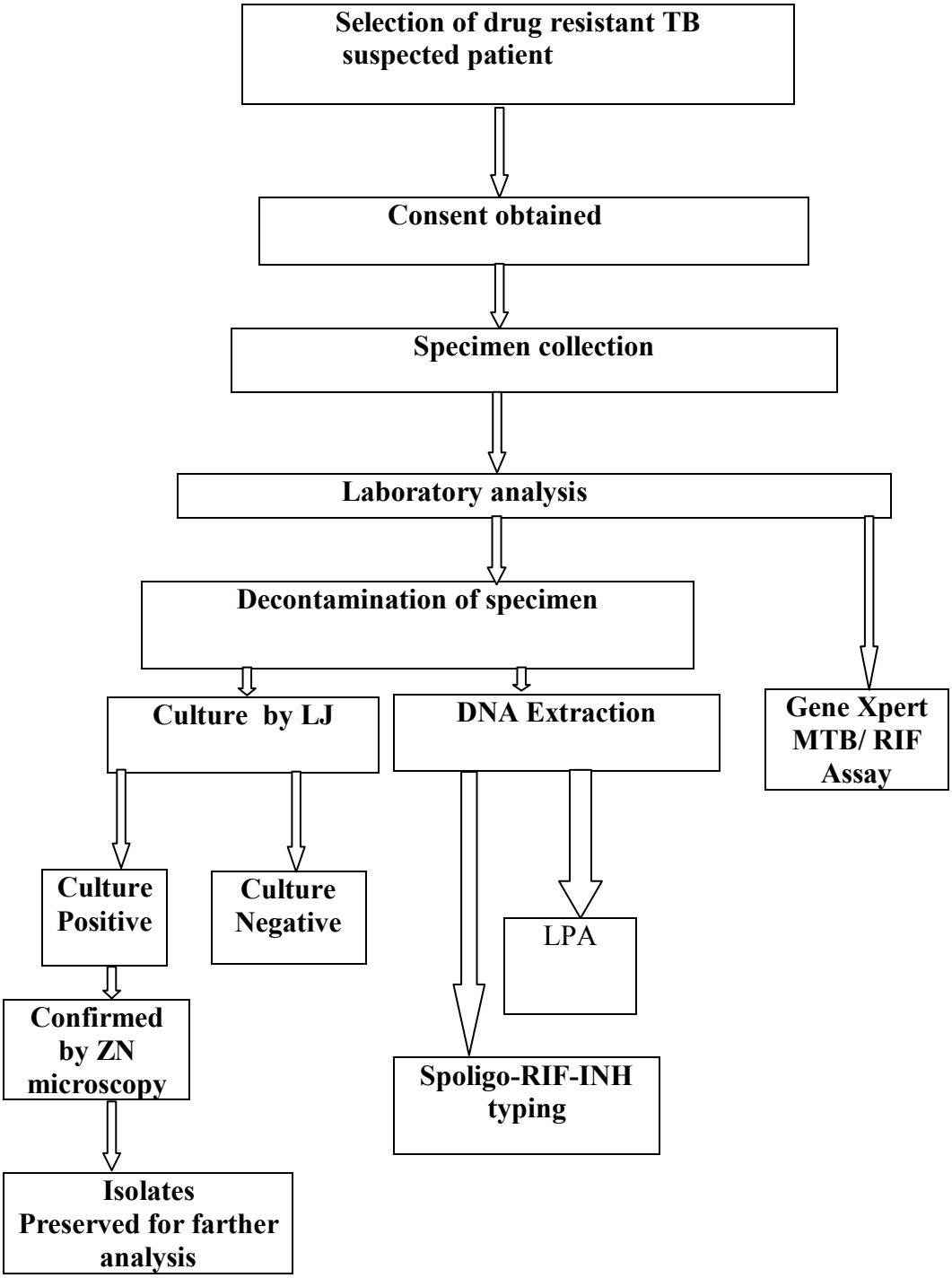

Figure 1 Shows the flow of the work

4.8.3.1 Mycobacterial Culture

Before culturing, Lowenstein-Jensen media were prepared for mycobacterium culture. Briefly, the mineral solution was prepared by suspending 37.2g of powder from TB medium base according to Lowenstein-Jensen in 600ml of purified water. The solution was heated with frequent agitation and boiled for one minute. Then, it was sterilized at 121^{o}C for 15 minutes. Fresh hens' eggs were cleaned by scrubbing thoroughly with hand brush in warm water and soap. The eggs were soaked for 30 minutes in the soap solution. After rinsing the eggs thoroughly in running water, they were soaked in 70% ethanol for 15 minutes. The eggs were washed and cracked with a sterile knife into a sterile flask and beaten with sterile blender. The sterilized mineral solution was cooled to 45-50^{o}C and mixed with 1litre of homogenized sterile eggs aseptically. For LJ media containing glycerol, 12mL of glycerol was added in 1600mL of media while for media containing sodium pyruvate, 6.65g was added into 1600mL media. Then, 6-8ml was dispensed into sterile culture tubes and coagulated at 85^{o}C for 45 minutes in slant position. Finally the LJ medium was labeled with date of preparation and stored in refrigerator.

The re-suspended sediment (about 3-4 drops) of the sputum was inoculated on three tubes of Lewenstein Jenson media of which two tubes containing glycerol and one tube containing pyruvate. The caps of the tubes were loosely applied to allow the entrance of oxygen for 48 hours. Each slant was properly labeled with the sample identification number and date of inoculation. The cultures were incubated in slanted position at 37°C until the inoculum was absorbed and were examined 48 hours after inoculation to check the liquid has completely evaporated, to tighten the caps and to detect contaminants. Then, they were examined weekly for eight consecutive weeks, during which time the

presence or absence of growth was noted. Cultures were considered as negative and discarded if no growth was detected by 8 weeks. In the presence of visible growth of colonies on LJ medium, visual detection of colonial morphology was performed. Suspicious colonies were confirmed by Ziehl Neelsen staining of cultures. Features like rough, crumbly, waxy, non-pigmented (cream, buff-coloured) were used as indicator for the presence of *M. tuberculosis*. White, small and round with a wrinkled surface and irregular thin margin were used for identification of colonies of *M. bovis* (Kantor *et al.*, 1998; Cheesbrough, 2006). From positive cultures, subcultures were also performed using 7H9 freezing media to keep the isolates for farther analysis.

4.8.3.2 Ziehl-Neelsen Stain

Ziehl-Neelsen staining procedure was applied to stain the smear. Briefly, the smear was made on properly labled microscopic slides. The slides were flamed to fix the samples. Carbol fuchsin solution was applied to cover the entire slide. The slides were slowly heated 2-3 times until steam rises using a Bunsen burner. The steaming was maintained for 5 minutes by using low or intermittent heat (i.e. by occasionally passing the flame from the Bunsen burner over the slides). After this, the slides were rinsed with water. Then, they were flooded with 3% acid alcohol. After rinsing the slides thoroughly with water, they were counter stained with 0.1% methylene blue approximately for 1minute. After rinsing with water and allowing to air dry, the slides were examined uhder the microscope using oil immersion lens.

4.8.3.3 GenoType MTBDRplus Version 2.0 Line probe Assay

The GenoType MTBDR*plus* line probe assay was carried out according to the manufacturer's (Hain Life Science, Nehren, Germany) instructions. The following steps were used: DNA extraction, multiplex polymerase chain reaction (m-PCR) amplification, hybridization and detection. These steps were performed in three separate rooms with restricted access and unidirectional workflow (WHO, 2008).

4.8.3.3.1 DNA Extraction

In order to extract the DNA of mycobacterium, 500µl of the decontaminated sputum was transferred into1.5ml screw capped tube and centrifuged at 10,000 x g for 15 minutes in an aerosol-tight rotor. The supernatant was discarded and the pellet was re-suspended in 100µl of lysis buffer (A-lys) and re-suspended. The bacteria were inactivated in heat block by incubating for 5 minutes at 95^0c. Then, the solution was neutralized with 100µl neutralization buffer (A-NB) and vortexed for about 5 seconds, and centrifuged at 15,000 x g for 5 minutes. Finally, the supernatant portion was transferred to a new 1.5ml tube and refrigerated at $+4^o$c until the DNA was amplified to be analyzed by Genotype MTBDR*plus* version 2.0 and processed by spoligotyping and pyrosequancing methods. The process was performed in DNA extraction room under biosafety level 3 cabinet.

4.8.3.3.2 DNA Amplification

Before amplification was performed, master mix had been prepared by combining 10µl of amplification mix A(AM-A) and 35µl of amplification mix B(AM-B).Then, 5µl of the extracted DNA was mixed with 45µl of amplification mix and the DNA was amplified using PCR machine according to the manufacturer's instructions. Amplification was done in a thermal cycler using the amplification profile: denaturation of 15 min at 95^oC,

followed by 1cycles of 30sec at 95^{o}C and 2 min at 65^{o}C, and 20 cycles of 25 sec at 95^{o}C, 40 sec at 50^{o}C and 40 sec at 70^{o}C and the extension step of 8 min at 70^{o}C. Finally, the PCR products (amplicons) were taken from the thermal cycler and kept in refrigerator at -20^{o}C until hybridization process was performed.

4.8.3.3.3 Hybridization and Detection (Visualization)

Following amplification, labeled PCR products were hybridized with specific oligonucleotide probes immobilized on a strip and then color was developed by enzyme mediated reaction. The process was performed by the following steps. From denaturation solution, 20µl was put in each well of the working tray and mixed with 20µl of DNA PCR product by pipetting up and down. Then, the mixture was incubated at room temperature for 5 minutes on the bench. This time was given to unzip the DNA strands. One milliliter of the hybridization solution was added to the mixture. The solution was mixed by tilting the mixture up and down. The strip labeled using DNA strip marker was placed in the solution and incubated for 30 minutes at 45^{o}C in the Twincubator. The whole quantity of hybridization solution was completely removed. One milliliter of stringent solution was added and incubated for 15 minutes in the Twincubator. Stringent solution was completely removed and 1ml of rinsing solution was added and incubated for a minute at room temperature in the Twincubator. The whole quantity of rinsing solution was removed.

In order to visualize the result, 1ml of diluted conjugate was added and incubated for 30 minutes at room temperature in the Twincubator. The conjugate was aspirated completely. One milliliter of rinsing solution was added and incubated for one minute. The quantity of rinsing solution was removed and rinsed once again with water for a

minute. One milliliter of the substrate solution was added and incubated for 5-8 minutes at room temperature. The substrate was aspirated and the reaction was stopped by rinsing twice with distill water for one minute in each rinse. The DNA strip was removed from the tray and air dried on absorbent paper. The dried strip was taped to the MTBDR*plus* 2.0 assay worksheet for interpretation. The captured labeled hybrids were detected by colorimetric development. This was used to detect the presence of *M. tuberculosis* complex as well as the presence of wild-type and mutation probes for resistance. The bands were visualized by naked eye. Finally, the Genotype MTBDR*plus* 2.0 results were interpreted as described in the manufacturer's instructions.

4.8.3.4 Gene Xpert MTB/RIF Assay

The assay was conducted following the manufacturer's (Cepheid, Sunnyvale, CA) instruction. From the collected sputum sample about 2ml was mixed with 4ml of buffer to obtain 2:1 buffer to sample solution. The purpose of the sample buffer is to homogenize the sputum sample and make it amenable for DNA extraction and to render any potentially infectious TB bacilli non-viable. The mixture was shaken and allowed to stand for 10 minutes. The mixture was also shaken and allowed to stand for further 5minutes. Then, 2ml of the mixture was transferred into the cartridge. The cartridge was put in the Gene Xpert and the test was undergone. Finally, the result was read and interpreted.

4.8.3.5 Pyrosequencing

Pyrosequencing is a sequencing method based on real-time monitoring of DNA synthesis, optimized to analyze single-nucleotide polymorphisms and short DNA sequences. The method is used for detection of resistance to rifampicin and isoniazid in *M. tuberculosis*

complex. Seventeen (17) discordant isolates were analyzed in Microbiology department, Hospital Universiteri Germans Trias i Pujol. Barcelona. The method was performed using the following steps: DNA extraction, DNA amplification, pyrosequencing reactions and result interpretation.

DNA extraction: The DNA was extracted using the same procedures as for Genotype MTBDR*plus* version 2.0 line probe assay.

DNA Amplification: Before the DNA was amplified, Master Mix was prepared. The Master Mix (Sigma ReadyMix™ Taq PCR Reaction Mix with MgCl2) was made by mixing primer forward (5' CGATCACACCGCAGACGTTGAT 3') (0.1µl), primer reverse (Biotin-5'_GGCACGCTCACGTGACAGACC_3') (0.1µl), water (7.8 µl) and MM (15µl). From the extracted DNA; 2µl was added to the master mix solution. Then, the solution was put in the PCR machine and amplified according to the PCR program. Briefly, denaturation and enzyme activation at 95 ^{o}C for 12 minutes and 40 cycles of 94 ^{o}C for 30 seconds,60 ^{o}C for 1 minutes, and 72 ^{o}C for 2 minutes, followed by extension at 72 ^{o}C for 7 minutes. The PCR product size (172bp) was checked by using gel electrophoresis.

Pyrosequencing reactions: The following steps were involved to perform the reaction. First the streptavidin mix was prepared by mixing streptavidin-coated Sepharose beads (12µl), binding buffer (108µl) and H_2O MQ (160µl). From the streptavidin mix, 70µl was put in the 96 wells plate and 10µl of the DNA was added. Then the mixture was incubated 1400rpm for 10 minutes at room temperature (24^{o}C).

The reagents to clean the DNA were prepared. The pyrosequencing primers (5' (ACCAG)CCAGCTGAGCCAATTC3',5'CCAGAACAACCCGCTGTCGGG3',5'CCG CTGTCGGGGTTGACC3')(dilution 1:250) 2.4µl primer in 600µl annealing buffer were prepared. In each well 40µl was added. After 10 minutes of incubation, the DNA was cleaned before the pyrosequencing reaction. The pyrosequencing plate was incubated with the primers and the DNA at 80^{o}C for 2 minutes. Then, added to the cartridge nucleotides, enzyme and substrate. The cartridge and the plate with the samples were put into the pyrosequencing instrument, and the program was undergone. After the reaction was completed, the results were saved and interpreted.

4.8.3.6 Spoligo-Rifampicin-Isoniazid Typing

Spoligo-Rifampicin-Isoniazid Typing is a method that based on a simultaneous analysis of the polymorphism in the clustered regularly interspersed short palindromic region and those in the *rpoB ,* katG and inhA genes hot spot regions conferring resistance to rifampicin and isoniazid. Twenty six (26) isolates were analyzed in University of Paris-Sud, Bat. 300, F-91405 Orsay-cedex, France. The method was performed as follows: In a total volume of 25 µl, using biot-Ra (5'GGTTTTGGGTCTGACGAC3',DRb (5'CCGAGAGGGGACGGAAAC3'),*rpoB*fw(5'CGGTGGTCGCCGCGATCAAGGAIII IITCGGCA3'), and *rpoB*-Drv (5' CCGTAGTGCGACGGGTGCACGTIIIIIACCTCC 3') dual-priming oligonucleotide primers, the reaction mixture contained 2µl of a DNA sample (20 to 40ng), 0.2mM each deoxynucleoside triphosphate (dNTP), 1mM each primer, PCR buffer (10mM Tris-HCl, pH 8.3, 50mM KCl), and 1.0Uof *Taq* polymerase. Then, the following PCR program was used: 3 min at 95°C, followed by 25 cycles of 30 s at 95°C, 30 s at 65°C, and 30 s at 72°C, with a final elongation step at 72°C for 5 min.

Hybridization of 2µl of the PCR products in 50µl of tetra methyl ammonium chloride (TMAC) buffer (1xTMAC) was performed after denaturation for 10 min at 95°C and then 20 min at 50°C. After centrifugation at 4,000 rpm and replacement of 35µl of supernatant by 1µl TMAC, streptavidin-phycoerythrin solution (Interchim SA, Montluçon, France) prepared in 1µl TMAC was added to a final concentration of 2µg/ml to reach a final volume of 75µl. Before reading the samples, it was allowed 5min of incubation in the system at 50°C. Then, the interpretation of the result was made based on the mean fluorescence intensity in the Luminex.

4.8.4 Quality assurance

Throughout this study, the protocol mentioned in the 2008 WHO report for sample collection, transportation and processing was strictly followed. In the preparation and storage of culture media, reagents and staining solutions that were used in this study, the manufacturers' instruction and standard operating procedures (SOPs) were followed. All tests were conducted after adequate training and optimizations were performed. The following quality control procedures were done for the tests that were used.

Quality Control for LJ Culture: Sterility testing was carried out by taking all batches of the media and incubated at 37^{o}C for 72 hours. Then, all media were refrigerated until the specimen was inoculated on them. Those media on which colonies are not seen were used for inoculation.

Quality Control for Line probe assay: The line probe assay has two internal controls on the strip. These are the conjugate control and the amplification control. When positive, these controls indicate that the test has been carried out correctly. To prevent DNA contamination, strict room separation was used including the work flow from DNA amplification to hybridization. In addition, a negative control was included in every run and was always negative. This gives strong indication of no laboratory contamination during the DNA was under the processes.

4.8.5 Laboratory Safety

M. tuberculosis is an important airborne laboratory hazard. Biosafety level 2 practices are adequate for processing specimens as long as the laboratory workers wear gowns and gloves/ protective clothing and equipment when splashes or aerosols may be produced and perform all manipulations in a certified biological safety cabinet. Biosafety level 3 practices should be used when working with cultured organisms. Specimens should be double sealed during centrifugation i.e. a specimen should be placed in a sealable centrifuge tube that is then put in a sealable safety carrier (Bartelt, 2000). All safety precautions were strictly followed throughout this study.

4.9 Data Management and Analysis

The data collected from the study area were entered into Microsoft Excel and analysis was done using SPSS 16th version statistical software (SPSS Inc. Chicago, 2007). Descriptive statistics was employed to quantify the results. Pearson chi-square test was used to compare differences of categorical variables (culture results, Genotype MTBDR*plus* version 2.0 line probe assay results). It was also used to evaluate the association of growth of MTBC, drug resistance, and socio-demographic status, gender,

HIV status and history of previous TB treatment. A p-value less than 0.05 was considered to be statistically significant. The performance indices (the sensitivity, specificity, positive and negative predictive value) of MTBDR*plus* version 2.0 were calculated using culture method as a reference test. Sensitivity and specificity were calculated using the following general formula (Macmillan, 2010):

Sensitivity= True positive / (True positive + False negative)

Specificity= True negative / (True negative + False positive)

Kappa was used to indicate the measure of agreement or concordance of MTBDR*plus* version 2.0 and Xpert MTB/RIF assay, and MTBDR*plus* version 2.0 and spoligo-RIF-INH typing.

4.10 Ethical Considerations

Participation in this study involves recruitment of patients suspected with drug resistant TB. But, the decision to collect sputum sample was made on clinical demand and not for the sake of participation in the study. The protocol of this study described in this application was reviewed and approved by College of Natural Sciences research ethics review committee, Addis Ababa University, to ensure complete fulfillment of both the protection of the study participants and the scientific validity of the proposed study before commencement of the actual activities. A formal letter of permission was obtained from Addis Ababa regional laboratory and Health Bureau in order to conduct the research in the study sites.

Patients were informed about the background and procedures of the study in order to use the leftover sample for research purpose only. All patients who had agreed to participate in the study read and signed a written informed consent before collection of the

specimens and medical history from their medical record. Study participants who are unwilling were not eligible for the study. For potential participant that were contacted, who cannot read, the information sheet and the consent form was read and his/her signature was collected. Codes were used and confidentiality was maintained throughout the study. After the specimen had been processed, the result was sent to the health center. The study participants whose specimen was positive either for tuberculosis or drug resistant TB has got treatment free of charge against the disease through his/her respective health center immediately.

4.11 Operational Definitions

- **Sensitivity:** is the probability of positive patients by the test under evaluation are positive by culture method (gold standard).
- **Specificity:** is the probability of negative patients by the test under evaluation are negative by culture method.
- **True positive:** when infected people are correctly diagnosed as having the disease.
- **Positive predictive value:** is the probability that a patient with a positive test result really does have the condition for which the test was conducted.
- **Negative predictive value**: the probability that a patient with a negative test result really is free of the condition for which the test was conducted.
- **False positive:** when uninfected or healthy people are incorrectly identified as diseased.
- **True negative:** when healthy people are correctly identified as non diseased.
- **False negative:** when diseased people are incorrectly identified as non diseased.

5. Results

5.1 Socio-demographic and clinical data of the study participants

A total of 96 sputum samples were collected from drug resistant TB suspected patients attending four hospitals and twenty eight health centers in Addis Ababa. Among these patients, 63 (65.6%) were males and 33 (34.4%) were females with minimum and maximum age of 18 and 75 years. Sixty nine subjects (71.9%) were between 18-35 years old. Nineteen (19.8%) of the study participants were HIV sero-positive. Sixty two (64.6%) of the total samples were smear positive. Seventy (72.9%) of the total participants had the history of previous tuberculosis infection and got treatment for the disease. Regarding their treatment status, 54.2 %, 32.3% and 13.5% account for relapse, new and treatment failure, respectively (Table 3).

Table 3 Socio-demographic data and clinical information of the study participant (N=96)

Variable	Study participants	Number	Percentage
Sex	Male	63	65.6%
	Female	33	34.4%
Age categories in years	18-25	34	35.4%
	26-35	35	36.5%
	36-45	14	14.6%
	46-55	8	8.3%
	>55	5	5.2%
HIV Status	Positive	19	19.8%
	Negative	77	80.2%
Category of cases	New	31	32.3%
	Relapse	52	54.2%
	Treatment failure	13	13.5%
Previous treatments against TB	Yes	70	72.9%
	No	26	27.1%
Smear status	Positive	62	64.6%
	Negative	30	31.2%
	Uncertain	4	4.2%

5.2 Association of risk factors with culture and MTBDR*plus* results

In this study, previous history of tuberculosis and age of the study participants had significant effect on the growth of *Mycobacterium tuberculosis* complex on LJ media (p=0.001 and p=0.005) and MTBDR*plus* version 2.0 results (p=0.027, p=0.002) respectively. History of past treatment with anti-TB drugs was significantly associated with the culture results (p=0.003), but it is not statistically significant for the detection of MTBC by GenoType MTBDR*plus* version 2.0 line probe assay (p=0.06). The age risk factors were not significantly associated with the detection of MTBC using culture method and MTBDR*plus* test (Table 4).

Detection of drug resistance to rifampicin and isoniazid using MTBDR*plus* was significantly affected by previous history of tuberculosis and age of the study participants (p=0.011, 0.035 and p=0.013, p=0.017, respectively). History of past treatment with anti-TB drugs was significantly associated with the detection of drug resistance to rifampicin (p=0.037) but not statistically significant for isoniazid resistance detection (p=0.134).Other risk factors such as sex and HIV status had no statistically significant effect on detection of drug resistance to both drugs (p>0.05).

Table 4 Associated factors with the results of culture and Genotype MTBDR*plus* test

Factors		Culture results			MTBDR*plus* version 2.0 results		
		Positive	Negative	p-value	Positive	Negative	p-value
Sex	Male	50	13		43	20	
	Female	25	8	0.725	26	7	0.276
Age	18-25	31	3		30	4	
	26-35	30	5		27	8	
	36-45	8	6	0.001	7	7	0.002
	46-55	5	3		4	4	
	>55	1	4		1	4	
HIV status	+Ve 19	15	4		15	4	
	-Ve 77	60	17	0.923	54	23	0.444
Previous TB	Yes 70	60	10		54	16	
	No 26	15	11	0.005	15	11	0.027
History of Treatment	Yes 70	60	10		54	16	0.060
	No 26	15	11	0.003	15	11	

+Ve: positive, -Ve: negative

5.3 Culture and GenoType MTBDR*plus* version 2.0 line probe assay results

A total of 96 specimens were used for performance evaluation of Genotype MTBDR*plus* version 2.0 line probe assay. Culture method correctly identified 75 (78.1%) specimens as positive for *Mycobacterium tuberculosis* complex. Sixteen of them had been from smear negative patients.

Figure 2 depicts positive cultures for *Mycobacterium tuberculosis* complex. From 96 study participants, 69 (71.9%) were detected MTBC positive by using Genotype MTBDR*plus* version 2.0 line probe assay (Table 3). Of the 69 MTBC positive specimens, 13(18.8%) were smear negative and 12(17.4%) of which were also positive by culture. Ten of these smear negative specimens were collected from patients who had history of previous treatment with anti-tuberculosis. Of 52 relapse cases, 45 (86.5%) and 41 (78.8%) were positive by culture and MTBDR*plus* version 2.0 line probe assay respectively. The overall sensitivity, specificity, positive predictive value and negative predictive value of Genotype MTBDR*plus* version 2.0 line probe assay to detect MTBC is found to be 67/75 (89.3%), 19/21 (90.5%), 67/69(97.1%) and 19/27 (70.4%), respectively (Table 5).

Table 5 GenoType MTBDR*plus* version 2.0 line probe assay (LPA) results for TB detection in comparison to culture results.

LPA	Culture			Performance of LPA (95% CI)			
	Positive	**Negative**	**Total**	**Sensitivity (CI)**	**Specificity (CI)**	**PPV (CI)**	**NPV(CI)**
Positive	67	2	69	89.3% (80.1-95.3)	90.5% (69.6-98.6)	97.1% (89.9-99.6)	70.4% (49.8-86.2)
Negative	8	19	27				
Total	75	21	96				

PPV: positive predictive value, NPV: negative predictive value, CI: confidence interval

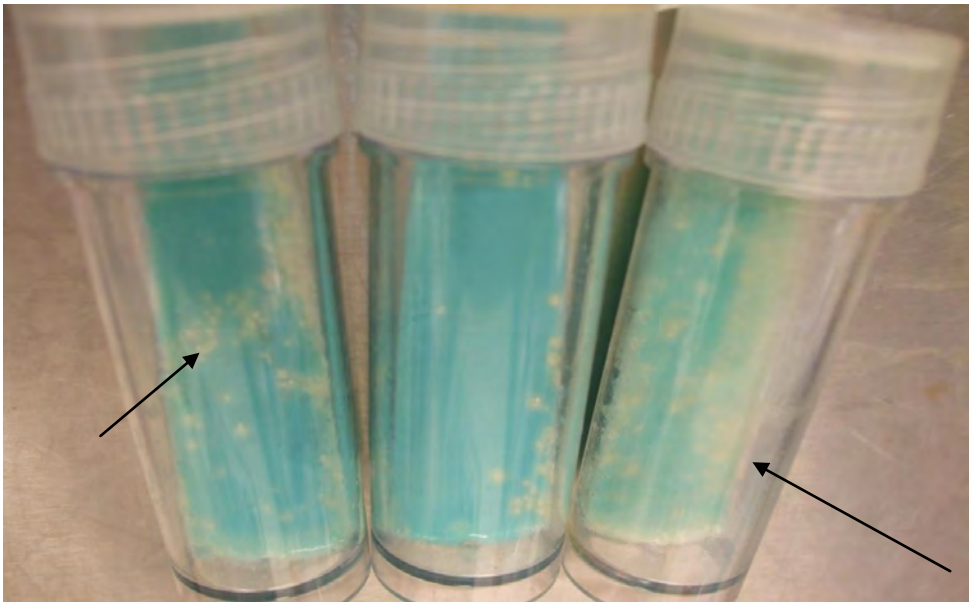

Figure 2 Positive culture for *Mycobacterium tuberculosis* complex. The arrows show the colonies of the bacteria on LJ medium enriched with glycerol.

5.4 Performance of MTBDR*plus* version 2.0 in detecting resistance to rifampicin and isoniazid

Of 69 isolates, 19(27.5%) and 50 (72.5%) were detected sensitive and resistant, respectively to rifampicin using MTBDR*plus* version 2.0 line probe assay. Similarly, 31(44.9%) and 38 (55.1%) isolates were detected sensitive and resistant, respectively to isoniazid by the same assay. Forty two (84%) and 8 (16%) of rifampicin resistant isolates had been from smear positive and smear negative samples respectively. Thirty two (84.2%) and 5(13.2%) of isoniazid resistant isolates were from smear positive and smear negative samples, respectively (Table 6).

Table 6 Frequencies of resistant and sensitive MTBC isolates detected in smear positive and negative samples using MTBDR*plus* (N=69)

	Rifampicin		Isoniazid	
Smear Status	**Resistant**	**Sensitive**	**Resistant**	**Sensitive**
Smear positive	42 (84%)	13 (68.4%)	32(84.2%)	23(74.2%)
Smear negative	8 (16%)	5(26.3%)	5(13.2%)	8(25.8%)
Uncertain	0 (0%)	1(5.3%)	1(2.6%)	0(0%)
Total	**50** (72.5%)	**19**(27.5%)	**38** (55.1%)	**31**(44.9%)

5.5 Pattern of mutations detected by MTBDRplus version 2.0 line probe assay associated with RIF and INH resistance

Most mutations conferring resistance to rifampicin were identified in wild type 8.These were detected in 46 (92%) of the rifampicin resistant isolates. Of the mutations, S531L mutation was detected in 30 (60%) of the isolates. The second most frequently occurred rpoB gene mutation was found in wild type7. Three (6%) of the rifampicin resistant MTBC isolates was due to the mutation in H526Y (Table 7).

Thirty five (92.1%) of isoniazid resistant isolates had mutations in katG gene region. Of the mutations, S315T (ACG→ACC) mutation was detected in 33(86.8%) of INH resistant MTBC isolates. One isolate had a mutation in S315T (ACG→AAC) of katG gene and 3(7.9%) of the INH resistant isolates showed loss of wild type1 and wild type 2 of inhA promoter region without the presence of mutation band at respective mutation sites (Table 7).

Table 7 Distribution of mutations detected using MTBDR*plus*

Probes	Resistance-associated mutation site	Number (%)	Specific mutation detected	Number (%)
rpoB WT8	531,533	46 (92)	***	16 (32)
rpoB MUT3			S531L	30 (60)
rpoB WT7	526	4(8)	***	1(2)
rpoB MUT2A			H526Y)	3 (6)
katG WT	315	35(92.1)	***	1(2.6)
KatG MUT1			S315T (ACG→ACC)	33 (86.8)
katG MUT2			S315T (ACG→AAC)	1 (3)
inhA WT1	-15,-16	3(7.9)	***	3(7.9)
inhA WT2	-8			

Note: WT: Wild type, MUT: Mutation, ***: The specific mutation is not identified.

Among all 50 RIF resistant isolates, 14 (28%) were rifampicin mono-resistant and 36 (72%) were multidrug resistant tuberculosis. Two (5.3%) of the 38 INH resistant isolates were isoniazid mono resistant and the rest were multidrug resistant.

Of rifampicin resistant isolates, 29 (80.6%) of MDR-TB isolates and 13(92.9%) of the rifampicin mono-resistant isolates were due to mutation in rpoB S531L (MUT3). Three (8.3%) of the MDR-TB isolates had a mutation in wild type 7 specifically in H526Y. The rest of MDR-TB isolates lost wild type7 and 8 without the appearance of respective mutation band (Table 8).

Among isoniazid resistant isolates, 33(91.7%) of MDR-TB isolates and all of INH mono-resistant isolates were due to mutation in katG S315T (MUT1).Two isolates (5.6%) of the MDR-TB isolates had mutation in inhA WT1 and WT2 without the development of known mutation band (Table 8). Nine (25%), 18 (50%) and 9(25%) of multidrug resistant isolates had been from new, relapse and treatment failure cases, respectively. Figure 3 shows sensitive, mono and multi-drug resistant strains of *Mycobacterium tuberculosis* complex.

Table 8. Frequency of *rpoB*, *KatG* and *inhA* mutation detected through LPA in mono and multidrug resistant isolates

Isolates	LPA Probes	Number	Specific mutation detected	Number (%)
RIF mono-resistant	rpoB WT8	14	***	13 (92.9)
	rpoB MUT3		S531L	1 (7.1)
INH mono-resistant	katG WT	2	S315T (ACG→ACC)	2 (100)
	katG MUT1			
Multi-drug resistant	rpoB WT7	4	***	1 (2.8)
	rpoB MUT2A		H526Y	3 (8.3)
	rpoB WT8	32	***	3 (8.3)
	rpoB MUT3		S531L	29 (80.6)
	katG WT	34	S315T (ACG→ACC)	33 (91.7)
	katG MUT1			
	katG MUT2		S315T (ACG→AAC)	1(2.8)
	inhAWT1	2	***	2(5.6)
	inhA WT2			

WT: Wild type, MUT: Mutation, ***: The specific mutation was not identified

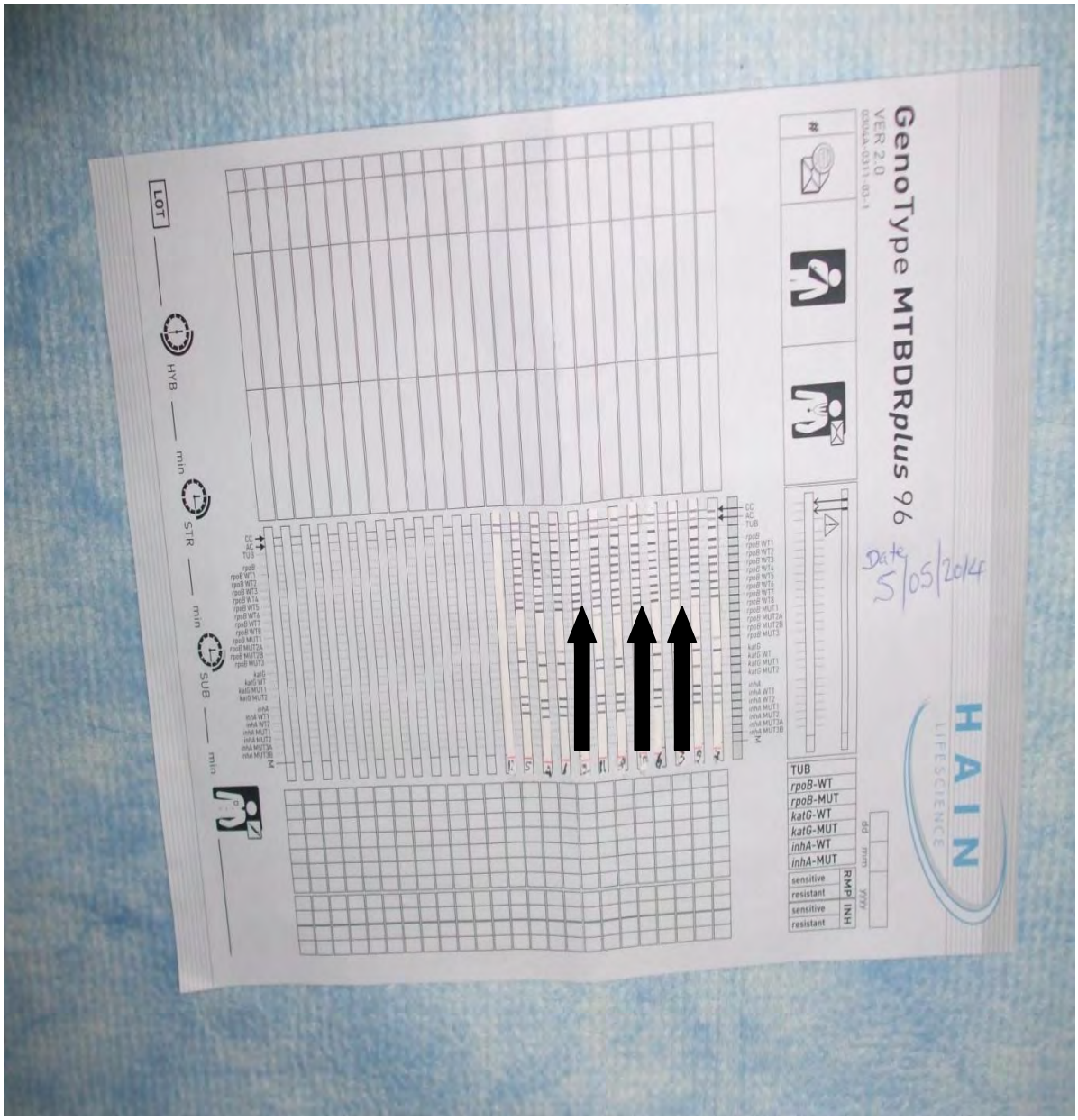

Figure 3 MTBC positive LPA results. Arrows indicate multidrug resistant, sensitive and mono resistant isolates of *Mycobacterium tuberculosis* complex, respectively from left to right

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5.6 Gene Xpert results

From 96 samples, 68 (70.8%) samples were detected positive for MTBC using Gene Xpert. These positive samples had been from smear positive 54 (56.3%), smear negative 13 (13.5%) and uncertain 1 (1%) samples. From 68 isolates, 32 (47.1%) and 36 (52.9%) were detected resistant and sensitive to rifampicin, respectively (Table 9).

Table 9 Performance of Gene Xpert in detection of MTBC and associated resistance to rifampicin

	Gene Xpert		
Smear status	**Positive**	**Resistant**	**Sensitive**
Smear positive	54 (56.3%)	28 (41.2%)	26 (38.2%)
Smear negative	13 (13.5%)	4 (5.9%)	9 (13.2%)
Uncertain	1 (1%)	0 (0%)	1 (1.5%)
Total	**68** (70.8%)	**32** (47.1%)	**36** (52.9%)

5.7 Comparison of MTBDRplus and GeneXpert in diagnosis of TB and its resistance to RIF

Of the total 96 samples, 69 and 68 were positive for MTBC using MTBDR*plus* and Gene Xpert MTB/RIF assay, respectively. Sixty six (68.8%) of the total samples were detected positive for tuberculosis by the two methods. Two specimens that had been from smear negative samples were positive for MTBC by Gene Xpert but negative by MTBDR*plus*. Similarly, 2 specimens that had been from smear negative were also negative for MTBC using Gene Xpert but positive by MTBDR*plus* and one smear positive specimen was also positive by MTBDR*plus* but negative by Gene Xpert. Considering the culture result as a gold standard, the sensitivity and specificity of the Gene Xpert in detecting the presence

of MTBC in sputum samples were 88.0% and 90.5, respectively and that of MTBDR*plus* were 89.3% and 90.5%, respectively.

From 66 isolates, 47 samples (71.2%) were found to be resistant to rifampicin by MTBDR*plus,* and 30 samples 45.5%) were resistant to rifampicin *using* the Gene Xpert (Table 10). Twenty eight and 8 resistant strains were detected from smear positive and smear negative samples, respectively using Gene Xpert. However, the number of resistant strains detected from smear positive (42) and smear negative (8) samples using MTBDR*plus* were higher than that of the Gene Xpert. The measure of agreement of the two methods in detecting resistance to rifampicin was found to be substantial agreement (k=0.724).

Table 10 Comparison of performance of MTBDR*plus* and Gene Xpert for detection of resistance to rifampicin from clinical specimens

	Gene Xpert			**kappa value**
MTBDR*plus*	**Resistant**	**Sensitive**	**Total**	
Resistant	30	17	47	**0.724**
Sensitive	0	19	19	
Total	**30**	**36**	**66**	

5.8 Analysis of discordants

Of the total 69 isolates, 17 were detected sensitive to rifampicin using Gene Xpert MTB/RIF assay but resistant to rifampicin by MTBDR*plus* version 2.0 line probe assay. Based on the manufacturer's instruction for interpretation of the results of MTBDR*plus* version 2.0, the assay was 100% concordant with that of pyrosequencing in detecting

resistance to rifampicin. However, 11 isolates with weak wild type 8 without presence of respective mutation band, and an isolate with no wild type 8 without appearance of mutation band were detected sensitive and resistant to rifampicin using pyrosequencing and MTBDR*plus* version 2.0, respectively (Table 11).

Table 11 Comparison of discordant results with that of pyrosequencing

Patterns of WT/MUT in MTBDR*plus* results	**rpoB Pyrosequencing results**	**Gene Xpert result**	**Number of isolates**
No wild type8 and no mutation band **(R)**	Wild type (**S**)	Sensitive	1
No wild type8 but MUT3**(R)**	Mutation at ntd 19-20 (**R**)	Sensitive	1
No wild type8 and No mutation band **(R)**	Mutation at ntd 17 (**R**)	Sensitive	1
Wild type 8 weak and no mutation band **(R)**	Wild type (**S**)	Sensitive	11
MUT3 band and No wild Type8 **(R)**	Mutation at ntds 19-21 **(R)**	Sensitive	1
No Wild type8 and no mutation band **(R)**	Mutation at ntds 19-20 (**R**)	Sensitive	2

ntd: nucleotide, S: Sensitive, R: Resistant, WT: Wild type, MUT: Mutation type **Total=17**

5.9 Spoligo-RIF-INH Typing Results

Among 26 isolates, 12(46.2%) were identified as T3-ETH and resistant to both rifampicin and isonizid. Seven (26.9%) and 2(7.7%) were found as CAS1-KILI and CAS1-DELHI, respectively. These strains were also found resistant to RIF and INH. One (3.8%) T3_ETH new SIT was also detected by the method. All except one isolate were found resistant to both RIF and INH (Table 12).

Table 12 Performance of Spoligo-RIF- INH typing in detection of resistance to RIF & INH, and identification of circulating strain type.

Rifampicin resistance	Isoniazidr esistance	SIT	Octal code	Clades/ Lineage	N (%)	MDR
R	R	149	777000377760771	T3_ETH	12(46.2%)	Yes
R	R	21	703377400001771	CAS1_KILI	7(26.9%)	Yes
R	R	25	703777740003171	CAS1_DELHI	2(7.7%)	Yes
R	R	New	777000317760771	T3_ETH	1(3.8%)	Yes
R	R	53	777777777760771	T1	1(3.8%)	Yes
R	R	2277	703377600001771	CAS	1(3.8%)	Yes
S	R	149	777000377760771	T3_ETH	1(3.8%)	Mono resistant
R	R	156	776177777760771	T1	1(3.8%)	Yes

R: Resistance, S: Sensitive, MDR multidrug resistance, SIT: Spoligo international type, New: not present in the international data base.T1: Euro-American, T3-ETH: Euro-American, CAS: East African-Indian, CAS1_DELHI: East African-Indian, CAS1_KILI: East African-Indian

5.10 Comparison of MTBDR*plus* with spoligo-rifampicin-isoniazid typing for detection of RIF and INH resistance

Thirty one isolates were included to be analyzed by MTBDR*plus* and spoligo-rifampicin-isoniazid typing method. But 5 specimens were excluded due to insufficient amount of DNA left for spoligo-rifampicin-isoniazid typing. Therefore, 26 isolates were used to compare the performance of MTBDR*plus* version 2.0 with spoligo-rifampicin-isoniazid typing in detecting resistance to isoniazid and rifampicin. The spoligo-rifampicin-isoniazid typing method identified 26(100%) and 25(96.2%) of the total isolates as resistant to isoniazid and rifampicin, respectively. Using the MTBDR*plus* version 2.0, the corresponding rates of detected resistance were 25(96.2%) and 26(100%). Twenty five (96.2%) isolates were detected multi-drug resistant using spoligo-rifampicin-isoniazid typing method. Similarly, 25 isolates were identified as multi-drug resistant by MTBDR*plus* version 2.0 (Table 13). The concordance between GenoType MTBDR*plus* and spoligo-rifampicin-isoniazid typing was 96.2% in detecting resistance to RIF, INH and MDR-TB. Figure 3 shows the mutation conferring resistance to RIF, INH and multi-drug resistance. One isolate was found to be discordant in the status of its resistance to rifampicin by the two methods. The methods did also not agree on the status of resistance to isoniazid of another one isolate. The overall agreement of the two methods was almost perfect agreement (k=0.92) in detecting resistance to rifampicin and isoniazid.

Table 13 Detection of mono and multi-drug resistance using MTBDR*plus* and Spoligo-rifampicin-isoniazid typing (N=26)

	MTBDRplus	Spoligo-RIF-INH typing
Resistance	N (%)	N (%)
INH resistance	25 (96.2)	26 (100)
RIF resistance	26(100)	25 (96.2)
MDR-TB	25 (96.2)	25 (96.2)

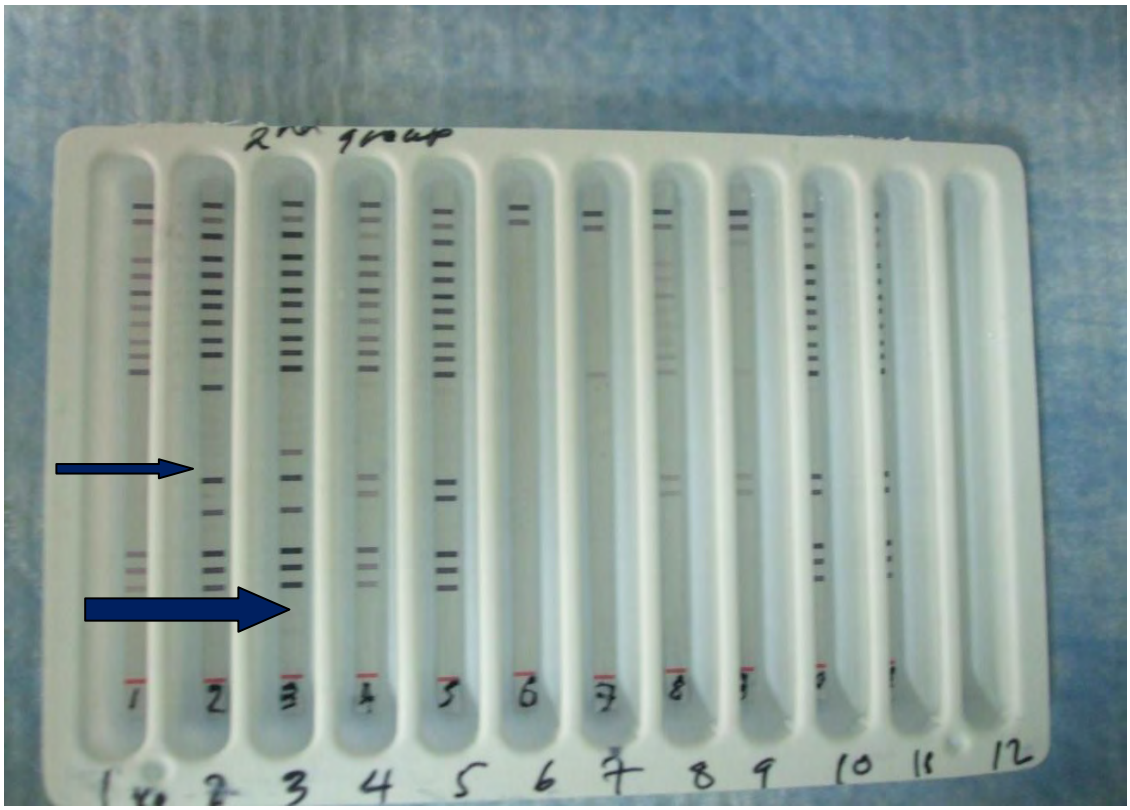

Figure 4 *Lane* 2 indicates multi-drug resistant tuberculosis (MDR-TB) due to absence of rpoB WT7, and absence of katG WT and presence of katG MUT1. *Lane* 3 also shows MDR-TB isolate as a result of absence of WT 8 and presence of MUT3 conferring resistance to RIF, and absence of katGWT and appearance of katG MUT1 (high level resistance) which causes resistance to INH.

6. Discussion

In the present study, the evaluation of association between different risk factors and the diagnosis of tuberculosis and drug resistance detection showed that the growth of MTBC and the detection of drug resistance by MTBDR*plus* were not affected by sex and HIV status of the study participants. Similar observations were made in previous studies reported from Ethiopia and UK (Melzer *et al*., 2010; Tessema *et al*., 2012).

Previous history of tuberculosis and age of the participants had significant effect on the culture results and detection of resistance to RIF and INH using MTBDR*plus*. These might be associated to the concentration of the bacilli in the sputum and the time for the development of resistance. The concentration of the bacilli might be increased if the sample had been taken from patients with previous tuberculosis and older age groups (since the immune system of the individual is weak in this groups), and the long stay of the bacteria in the patient might give the chance for spontaneous development of drug resistance.

It was also found that previous treatment had a significant effect on culture results and detection of MTBC and resistance to rifampicin using MTBDR*plus.* This could be due to the fact that the sample collected from the patient under the treatment might contain dead bacilli so that the bacteria cannot grow on the medium and MTBDR*plus* detects the DNA of dead bacilli. A meta-analysis of drug resistant tuberculosis done in sub-Saharan Africa had also demonstrated that past exposure to anti-tuberculosis drugs has strong association with drug resistance (Asres *et al.*, 2013). However, our result showed that past treatment had no statistically significant effect on drug resistance to isoniazid which is consistent with the study done in UK by Melzer and his colleagues (Melzer *et al.*, 2010).

The result of our study showed that the sensitivity and specificity of MTBDR*plus* version 2.0 for detection of *Mycobacterium tuberculosis* complex were 89.3% and 90.5% respectively. When these findings are compared to the findings reported by Crudu *et al* in 2012, the sensitivity is slightly higher and the specificity is lower (87.6 and 99.2 % respectively) (Crudu *et al.*, 2012). This difference might be due to the two different methods for DNA extraction, larger sample size (348) and combined tests as a reference test used by Crudu and his colleagues.

The sensitivity of MTBDR*plus* version 2.0 for detection of MTBC in smear negative specimens (30) were 75% which is almost similar to the previous published report (76%) (Crudu *et al.*, 2012), but slightly higher when compared with the previously reported sensitivities (72.5% and 71.2%) of the Gene Xpert (Boehme *et al.*, 2010; Barnard *et al.*, 2012) respectively. This might be associated with the difference in the principles in the two methods, the status of treatment history of the patients and large sample size under the studies. Boehme and colleagues excluded patients that are under treatment from the final analysis but in the present study, the samples were collected and tested regardless of the status of their treatments.

The sensitivity of MTBDR*plus* for detection of MTBC in smear positive specimens was 87.1% which is lower than the previously reported result (94.6%) (Luetkemeyer *et al.*, 2014). This might be due to the large sample size and different methodologies used in previous study.

Two specimens were negative for MTBC by culture but positive by using MTBDR*plus* version 2.0. The reasons for this difference could be the specimens were collected from

patients who had a history of treatment with anti-tuberculosis drugs, and few bacteria and dead bacilli can be detected by MTBDR*plus* version 2.0. Another reason could be the portion of the specimens inoculated in culture tubes might not contain live bacteria.

From culture negative specimens, eight were detected negative for MTBC using MTBDR*plus* version 2.0. However, the specimens were positive for non-tuberculosis mycobacteria (NTM) by using MTBDR*plus* version 2.0. This difference might be attributed to the negative effect of the ingredients incorporated in the LJ medium on the growth of those NTM species and the absence of live bacteria in the portion of specimen used for culture.

Studies showed that the majority of mutations conferring resistance to rifampicin are found in the rpoB gene (Ali *et al.*, 2009; Riccardi *et al.*, 2009). In the present study, 60 % of rifampicin resistant isolates showed mutation at codon S531L which is in agreement with previous studies done in South Africa (Barnard *et al.*, 2008) and Pakistan(Ali *et al.*, 2009). However, it is higher than the reported rates of mutation (52%) from Pakistan in 2013 (Khan *et al.*, 2013). This difference might be attributed to the smaller sample size used in the previous study, the methodologies used for DNA extraction and strain difference.

The next frequently showed mutation in this study was found at codon H526Y of rpoB gene of 6% of the rifampicin resistant isolates which is higher when compared to the previous reports from Georgia (2.1%) (Shubladze *et al.*, 2013) but much lower than that of reported from China (40%) and Iran (45.6%) (Yue *et al.*, 2003; Bahrmand *et al.*,

2009).This might be due to strain differences in Ethiopia and the two countries and the sample size included in these studies.

In this study, 86.8% of INH resistant MTBC strains showed mutation detected by LPA in catalase peroxidase gene (katG) at codon S315T (ACG→ACC). This finding is in agreement with the range (75-90%) recognized as the mutations in the 315th amino acid mainly in S315T1 and S315T2 (Hazbo´n *et al.*, 2006; Riccardi *et al.*, 2009; Vilchèze and Jacobs, 2007). Two of INH resistant strains showed mutation at inhA promoter region in our study. However, the study done in Ethiopia to determine the magnitude of gene mutations conferring drug resistance in *Mycobacterium tuberculosis* showed that there is no mutation in the region and all the mutation conferring resistance to INH had been found in katG gene (Biadglegne *et al.*, 2013).This might be attributed to the source of samples which were collected from lymph node aspirates in case of the previous report and from pulmonary in our cases. The different methodologies used in the studies might also be the reason for the difference.

The present study showed that the sensitivity (89.3%) of MTBDR*plus* version 2.0 were slightly higher than that of Gene Xpert MTB/RIF assay (sensitivity (88%)) in detecting *Mycobacterium tuberculosis* complex in direct sputum specimens. This finding is consistent with the results reported previously (Barnard *et al.*, 2012). However, the sensitivities of both assays in our study were higher than the previous sensitivities (71.2% for Gene Xpert and 73.1% for MTBDR*plus*) reported before. The specificities of both assays were similar (90.5) and much lower than the previous result (100%) (Barnard *et al.*, 2012). This might be due to the large sample size in previous study and the difference in the prevalence rate of tuberculosis in the study areas.

In this study, three specimens were found to be positive for MTBC using MTBDR*plus* but negative by Gene Xpert and 2 samples were positive by Gene Xpert but negative by MTBDR*plus*. The disagreement between the two methods might be attributed to the presence or absence of the bacilli in the portion of the specimens used by the methods.

The performance of MTBDR*plus* in detecting resistance to rifampicin (47 (71.2%)) was much higher than that of Gene Xpert (30(45.5%)).This finding is inconsistent with the results reported previously from South Africa which reflected as the two methods are equivalent in detecting resistance to rifampicin (Barnard *et al.*, 2012). This difference could be due to the presence of hetro-resistance of strains in the specimens and the difference in principles of the two methods. The limitation of the manufacturer's instruction for the interpretation of the MTBDR*plus* results might also be the reason.

Seventeen discordant isolates were encountered using Gene Xpert MTB/RIF assay and MTBDR*plus* version 2.0 based on the manufacturers' instructions for interpretation of the results. The problem of discordance was also observed in the study results reported from Cote d'ivoire (N'guessan *et al.*, 2014). For confirmation, all the specimens were further analyzed using pyrosequencing methods. The results showed that for isolates with no wild type 8 with or without appearance of respective mutation band, MTBDR*plus* version 2.0 were 100% concordant with pyrosequencing in detecting resistance to rifampicin. But in 11 isolates with weak wild type 8 band and 1 isolates with no wild type 8 band without the presence of respective mutation bands, the two methods were inconsistent. The observed discordance between the two tests might not be originated from the test performance but it might be from the limitation of manufacturer's instruction in the manual for interpretation of MTBDR*plus* version 2.0 results. According to the manual,

the weak or faint band at the wild type and absence of wild type band with presence or absence of band at respective mutation sites were considered as an indicator of mutation conferring resistance against rifampicin. But the result of pyrosequencing indicated that weak or faint band of wild type 8 without the presence of mutation band at respective sites, does not mean that the isolate developed resistance against the drug. If there is no band of wild type 8 with or without presence of mutation band, the isolate is resistant to the drug. Accordingly, weak or faint appearance of band at wild type 8 without the presence of related mutation band is not an indicator of resistance to rifampicin. These findings were also supported by the positive response to the first line anti-tuberculous drugs in these patients.

In the present study, T3-ETH (Euro-American lineage) and CAS1-Kili (East African-Indian lineage) were identified as the first and second most frequently circulating strains, respectively, among the multi-drug resistant isolates. Similar finding was also found in previous study done in Ethiopia (Agonafir *et al*., 2010). However, the spoligo international typing (SIT) number 21 (CAS1-Kili) was found as the most predominant strain in the study done by Diriba and his colleges in Addis Ababa (Diriba *et al*., 2013). This difference might be attributed to the larger sample size in the previous study. The frequencies of SIT25 (CAS1-DELHI) and SIT53 (T1) strains were very low (only in 2 and 1 isolates, respectively) in our study. But, in the previous study done at Bahir Dar, the two spoligotypes were found as the most predominant strains consisting of 22 and 14 isolates, respectively (Debebe *et al*., 2013). This might be due to the difference in geographical location of the study areas in the country and the sample size difference used for the studies.

One isolate to rifampicin and another one isolate to isoniazid were found to be discordant in the status of their resistance using MTBDR*plus* version 2.0 and spoligo-rifapmicin-isoniazid typing. This difference might be attributed to the simultaneous presence of resistant and sensitive isolates (heteroresistance) in the specimen.

Our work result also showed that the performances of MTBDR*plus* version 2.0 and spoligo-rifapmicin-isoniazid typing were 96.2% concordant for detection of resistance to isoniazid and rifampicin in sputum specimens. The finding is in agreement with the range of concordance (84.4-98.1%) reported by previous study for RIF-resistance detection but higher than the range (87.3-90.14%) for detection of INH-resistance (Balabanova *et al.*, 2009; Ma¨kinen *et al.*, 2006). The difference might be due to the methodologies used for analysis of samples and the reference tests used in the studies.

7. Conclusion

Genotype MTBDR*plus* version 2.0 line probe assay has a very good sensitivity and specificity for detection of *Mycobacterium tuberculosis* complex (MTBC) from sputum specimens using culture as a reference test.

The sensitivities and specificities of MTBDR*plus* and Gene Xpert MTB/RIF assay in detecting MTBC in sputum samples were equivalent. However, the performance of MTBDR*plus* in detecting resistance to rifampicin was found to be much higher than that of Gene Xpert MTB/RIF assay.

The majority of mutations conferring resistance to rifampicin and isoniazid were found in codon S531L and S315T, respectively. Weak or faint band at wild type 8 without appearance of respective mutation band doesn't indicate resistance to rifampicin. The T3-ETH (Euro-American lineage) is the most predominant strain circulating in multidrug resistant isolates in Addis Ababa. Genotype MTBDR*plus* version 2.0 and spoligo-rifampicin-isoniazid-typing have a very good concordance in detecting resistance to RIF, INH and MDR-TB. In conclusion, Genotype MTBDR*plus* version 2.0 line probe assay was found to be a sensitive and effective method for diagnosis of tuberculosis in clinical specimens. The assay was also found to be rapid and effective method for detection of drug resistance to RIF and INH from both smear negative and smear positive sputum specimens provided that the weak or faint appearance of wild type 8 band without presence of related mutation band is considered as normal during interpretation of the results for rifampicin resistance.

8. Recommendation

Based on the above conclusion, it is possible to forward the following ideas:

- To use the Genotype MTBDR*plus* version 2.0 line probe assay, it should be interpreted based on the total absence of wild type 8 band with or without appearance of band at respective mutation site for resistant strains to rifampicin to avoid treatment of false positive cases.
- It will be good to perform further studies on factors affecting the appearance of wild type 8 band.
- Large scale study should be done on performance characteristics of MTBDR*plus* version 2.0 line probe assay.
- The instructions for interpretation of MTBDR*plus* version 2.0 line probe assay results for rifampicin resistance should be revised by the manufacturer.

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10. Appendices

Annex 1. Patient Information Sheet and Consent Form

Annex 1.1. Patient Information Sheet (English Version)

Please read this information sheet and consent form entirely and ask the principal investigator and/or study co-investigator any question you may have about the study before deciding to participate and signing. If you prefer, I can read this information sheet and consent form to you knowing that you can stop me at any time in order to ask about any aspect you may not understand or may require clarification.

Principal Investigator: Chala Chaburte, **Name of the institute:** AAU, College of natural sciences.

1. **Research Title:** Evaluation of Line Probe Assay as a diagnostic tool for improvement of tuberculosis detection and identification of INH and RIF drug resistance in Addis Ababa region, Ethiopia

2. **Research Objective:** The aim of the study is to evaluate the diagnostic ability of line probe assay for improvement of tuberculosis detection and identification INH and RIF drug resistance identification in Addis Ababa region.

3. **Study Procedure:** To achieve the planned objective of this study, TB suspected patients whose will be contacted in order to get permission to accesses their clinical history and get their consent to utilize the leftover clinical specimens (about 6ml sputum), which will be collected by health professionals as part of their clinical diagnosis not for the sake of participation in the study. Following consent to use the leftover specimens, it will be analyzed by using different laboratory methods for the confirmation of tuberculosis infection and detection of drug resistant TB. Your attending Physician/Nurse will tell you the laboratory test result and you will get appropriate treatment as part of your routine medical care.

4. **Risks:** There will not be harm to your health during sputum collection and your time will not be wasted due to the participation in this study since you come for the sake of your health services.

5. **Participants' Role:** If you agree to participate in this study, you will be requested to permit accessing of your clinical history and the utilization of the leftover samples that will be collected

as part of the routine clinical practice. Your full commitment to participate in this study is helpful for the prevention, diagnosis and control of tuberculosis

6. **Participants' Right:** Your participation in this research is completely voluntary. You have the right to withdraw your consent and discontinue participation at any time. This decision will not have any impact on your usual care and treatment services at this hospital/health center. You also have the right to get information regarding the procedure of the study as well as questions related to the study with your preferred language.

7. Benefit to the Participants: The laboratory result of the tests that we will perform will be given to your health care provider as early as possible. If the result of the test indicates positive for tuberculosis as well as for drug resistant TB, you will get anti-TB treatment immediately. As a result, the transmission of the disease will be reduced in your family and the community. This research could also help in the development of improved diagnostic tests which will have a great role in early diagnosis, treatment and control of TB in the future.

8. **Confidentiality:** The information concerning your participation in this study will be kept confidential and used only for scientific purposes. No one except members of the research team will have access to the information. Your name and/or personal information will not be notified in any report. Your sample will not be labeled with your name but instead with a unique number assigned to you. All paper and computer records of the study will be kept in a secured place under lock when not in use. The results of this research may be published for scientific purposes. However, your identity will not be given out.

9. **Agreement:** If you agree to participate in this study, you will be asked for signature of concurrence. This is to make sure that your agreement to participate in the mentioned study is on volunteer and informed basis. Otherwise, there is no other reason for signing. The study is approved by ethical committee of researcher's institutes (department of microbial, cellular and molecular biology, Addis Ababa University). Getting signatures of agreement from study participant is one of the criteria of committees for the indication of no one did not participate in the study without participant consent and agreement.

10. **Communication:** You can ask the following researchers mentioned below any question you may have about this study. You may ask questions in the future if you do not understand any aspect of your voluntary participation or about the study itself. If you need further information

and have any doubt, feel free to contact any one of the following researchers in person or by phone.

Annex 1.2. Patient Information Sheet (Amharic Version)

ለጥናቱ ተሳታፊዎች የሚሰጥ መረጃ

ዋናው ተመራማሪ፡ ጫላ ጫቡርቴ የተቋሙ ስም፡ አዲስ አበባ ዩኒቨርስቲ

1. የጥናቱ ርዕስ: የሳንባ ነቀርሳ በሽታን(ቲቢን) እንዲሁም አይሶኒያዚድ እና ሪፋፕሲን የተለማመደ የቲቢ በሽታ አምጪ ህዋስን ለማወቅ የሚያገለግለውን ላይን ፕሮብ አሴይ የመመርመር ብቃት ግምገማ

2. የምርምሩ ዓላማ: የዚህ ጥናት ዓላማ የቲቢንና የቲቢ በሽታን መድኃኒት የተለማመደውን የበሽታው አምጪ ህዋስን ለመመርመር የሚያገለግለውን ላይን ፕሮብ አሴይ የሚባል አዲስ ዘዴን የመመርመር ችሎታን ለመገምገም ነው::

3. የጥናቱ ቅደም ተከተል: ከላይ የተጠቀሰውን የጥናቱን ዓላማ ለማሳካት፤ናሙና የተወሰደላቸውንና ሐኪም በወሰደው የህመም ታሪክ ቲቢ ይኖርባቸዋል ተብሎ የተጠበቁ ህምምተኞችን ካገኘን በኋላ ከህክምና ቻርታቸው ላይ አጠቃላይ የምርመራ መረጃ ቻርቱ ላይ መጠቀም እንድንችልና ከላቦራቶሪ ምርመራ የሚተርፈውን ናሙና ለጥናቱ እንዲንጠቀምበት ዘንድ ፈቃደኝነታቸውን እንጠይቃልን::ለህመምዎ መመረመሪያ ተብሎ የተወሰደውን ናሙና ምርመራው ከተደረገ በኋላ የሚተርፈውን እንድንጠቀም ከፈቀዱልን፣ ከተወሰደው ናሙና ላይ የቲቢ አምጪ ተህዋስ መኖሩንና መድሐኒት መለማመዱን ለማረጋገጥ የሚረዱ የተለያዩ የላቦራቶሪ ምርመራዎች ይሰራሉ:: የጥናቱ ውጤትም በተቋሙ ሐኪም ወይም ነርስ በኩል ይነገሮታል::

4. ሊደርስ የሚችል ጉዳትና ህመም: በጥናቱ ተሳታፊዎች ፈቃድ የሚወሰደው ከላቦራቶሪ ምርመራው በኋላ ለጥናቱ የምንጠቀምበት ናሙና በዚህ ጥናት በመሳተፈዎ ምንም አይነት ጉዳትና ህመም አያደርስቦትም:: ለራሶት ጤና አገለግሎት ስለሚመጡ የርሶ ጊዜም አይባክንም::

5. የጥናት ተሳታፊ ሚና : በዚህ ጥናት ላይ ለመሳተፍ ከተስማሙ ፤ ለህክምናዎ ምርመራ ይረዳ ዘንድ የሰጡትን የህምም ታሪክ እና ከላቦራቶሪ ምርመራ የሚተርፈውን ናሙና በመጠቀም እንችል ዘንድ ፈቃደኝነትዎትን እንጠይቃለን:: በዚህ ጥናት የእርስዎ ተሳትፎ የቲቢ በሽታን ለመከላከል፣ ለምርመራና ለመቆጣጠር ትልቅ ቦታ ይኖረዋል፡

6. የጥናቱ ተሳታፊ መብት: እርስዎ በዚህ ጥናት የመሳተፍና ያለመሳተፍ ሙሉ መብት አለዎት፡፡ ተሳትፎዎ በፍቃደኝነት ላይ የተመሰረተ ስለሆነ በጥናቱ የመቀጠል ወይም በማንኛውም ሁኔታና በፈለጉበት ጊዜ ከጥናቱ የመውጣት መብት አለዎት፡፡ ይህ በመሆኑ ግን በዚህ ሆስታል ወይም የህክምና ማእከል የሚያገኙት የሕክምና አገልግሎት ላይ ተፅእኖ አይኖርም፡፡ ስለ ጥናቱ ቅደም ተከተልና ተያያዥ መረጃዎችን በፈለጉት ቋንቋ የማግኘት ሙሉ መብት አለዎት፡፡

7. ተሳታፊ በመሆን የሚገኝ ጥቅም : በናሙናው ላይ የምናደርገውን የላቦራቶሪ ምርመራ ውጤት ምርመራው ተስርቶ ከተጠናቀቀ በኋላ ለሐኪምዎ የምንሰጥ ይሆናል፡፡ ይህም በመሆኑ ለሚደረግልዎ የህክምና ክትትል ትልቅ ድርሻ ይኖረዋል፡፡የበሽታው ስርጭትም በቤተሰብዎ እና በጎብረተሰቡ ዘንድ ይቀንሳል፡፡ይህ ጥናት የሳንባ በሽታን በተሻለ ሁኔታ ለማወቅ የሚረዳ በመሆኑ በሽታውን ለመከላከል፣ ለመመርመር ብሎም ለመቆጣጠር የሚረዳ ይሆናል፡፡

8. ሚስጥር አጠባባቅ: በዚህ ጥናት ላይ የሚሰጡት መረጃ በሙሉ ምስጥራዊ በሆነ መንገድ የሚቀምጡ ሲሆን የተገኙት መረጃዎች ለጥናቱ ዓላማ ብቻ የሚውል ይሆናል፡፡ የእርስዎ ስም ወይም የግል መረጃ በማንኛውም ሪፖርት ላይ አይጠቀስም፡፡ ከጥናቱ ተመራማሪዎች ውጭ ማንም ሰው የእርስዎን የህክምናና የላቦራቶሪ ውጤት በማንኛውም መንገድ በቀጥታ ሊያገኝ አይችልም፡፡ ለጥናቱ የምንጠቀምበት ናሙና በእርስዎ ስም ሳይሆን በተለየ መለያ ቁጥር የሚሰየም ይሆናል፡፡ እርስዎ ላይ የምንሰበስባቸውን በወረቀት ወይንም በኮምፒዩተር ላይ የሚሰፍሩ መረጃዎች በማንጠቀምባቸው ጊዜ ተቆልፈው ይቀመጣሉ፡፡ የዚህ ምርምር ውጤት ለህትመት ሊውል ይችላል፡፡ ነገር ግን የእርስዎ ማንነት በሕትመት ውጤቱ ላይ አይገለፅም፡፡

9. ስምምነት: በጥናቱ ላይ ለመሳተፍ ከተስማሙ የስምምነት ፊርማ እንዲፈርሙ ይጠየቃሉ፡፡ ይህም የሚደረገው ከተጠቀሰው ጥናቱ ላይ የተሳታፉት በእርስዎ ፈቃደኝነት መሆኑን ለማረጋገጥ ነው፡፡ ከዚህ ምክንያት ውጪ የሚፈርሙበት ሌላ ምክንያት ምንም የለም፡፡ ይህ ጥናት የጥናት ተቋማት ስነ-ምግባር ኮሚቴ (አዲስ አበባ ዩኒቨርስቲ፣ሳይንስ ፋኩልቲ ማይክሮቢያል ፣ሰሉላር እና ሞልኩላር ባዮሎጂ ዲፓርትመንት) የተረጋገጠ ነው፡፡ እርስዎ በስምምነት ቅፁ ላይ መፈረምዎ ሁሉም ተሳታፊ በፈቃደኝነት እና በስምምነት መሳተፍን የሚያመለክትበት አንዱ መለኪያ ነው፡፡

Annex 1.3. Informed Consent for Participants ≥ 18 Years (English Version)

Principal Investigator: Chala Chaburte, AAU Name of the institute: AAU, Faculty of Science

Research Title: Evaluation of Line Probe Assay as a diagnostic tool for improvement of tuberculosis detection, INH and RIF drug resistance in Addis Ababa region, Ethiopia

Study Code number: _____ Hospital case number: _____

I have read the patient information sheet above & been told the detail explanation about the aim of the study and have been given the opportunity to discuss and to ask questions by the language of my preference. By agreeing to participate in this study, I give permission for the researchers to have an access of the clinical data from my medical card and to utilize the leftover sample which was collected for the laboratory diagnosis of my disease as part of the routine clinical practice after laboratory analysis. I have been told that all the laboratory information will be registered and kept confidential in a locked cabinet. I have been also given a brief explanation that my participation is on volunteer basis. My refusal to participate has no consequence in the health care service and treatment that I will get from this hospital/health centre and I can withdraw from participation without any prerequisite at any time.

I have agreed to be part of this study and I would like to confirm my agreement by signing.

_____ _____ _____

Name of study participant Participant's Signature Date

_____ _____ _____

Name of researcher/health professional Participant's Signature Date

Thank You for Your Participation

Annex 1.4. Informed Consent for Participants ≥18 Years (Amharic Version)

የጥናቱ ተሳታፊ የስምምነት ቅጽ

ዋናው ተመራማሪ፡ ጫላ ጫቡርቴ የተቋሙ ስም፡ አዲስ አበባ ዩኒቨርስቲ ሳይንስ ፋኩልቲ

የጥናቱ ርእስ: የሳንባ ነቀርሳ በሽታን(ቲቢን) እንዲሁም ኤይሶኒያዚድ እና ሪፋምፕሲን የተለማመደ የቲቢ በሽታ አምጪ ህዋስን ለማወቅ የሚያገለግለውን ላይን ፕሮብ አሴይ የመመርመር ብቃት ግምገማ

የጥናቱ መለያ ኮድ፡ _____ የሆስፒታል መለያ ቁጥር፡ _____

ከላይ የተጠቀሰው የጥናቱ ተሳታፊ መረጃ ያነበብኩና ማብራሪያ የተሰጠኝ ሲሆን ስለጥናቱ ዓላማና የጥናቱ ምንነት ተነግሮኛል፡፡ ግልፅ ላልሆነልኝም ጥይቄ በምረዳው ቋንቋ መረጃ እንዳገኝ እድሉ ተሰጥቶኛል፡፡ በዚህ ጥናት ለመሳተፍ ፈቃደኛ የሆንኩ ሲሆን የህክምና መረጃዬ ላይ ለጥናቱ የሚያስፈልገውን መረጃ እንዲወስዱና ለህመሜ ምርመራ የተወሰደውን የላቦራቶሪ ናሙና ምርመራው ከተካሄደ በኃላ የሚተርፈውን ለጥናቱ ዓላማ እንዲጠቀሙበት ፈቃደኛ ነኝ፡፡

በጥናቱ ውስጥ የሚሰበሰቡት የላቦራቶሪ ሆነ የህክምና ሁኔታን የሚገልፁ መረጃዎች ምስጥራዊነታቸው የሚጠበቅና ተቆልፈው እንደሚቀመጡም ተነግሮኛል፡፡ በዚህ ጥናት መሳተፍ በእኔ ሙሉ ፈቃደኝነት ላይ የተመሰረተ መሆኑ ተገልፆልኛል፡፡ የተሳትፎ ሁኔታዬ ከዚህ ሆስፒታል/ጤና ጣቢያ በማገኘው የሕክምና አገልግሎት ላይ ምንም አይነት ተፅእኖ እንደሌለው ተነግሮኛል፡፡ ከዚህም በተጨማሪም እዚህ ጥናት ላይ በፈለግኩት ጊዜ ያለምንም ቅድመ ሁኔታ ማቋረጥ እንደምችልም ተነግሮኛል፡፡ የዚህ ጥናት አካል ለመሆን የተስማማሁ ሲሆን ስምምነቴንም በፊርማዬ አረጋግጣለሁ፡፡

_____ _____ _____

የጥናቱ ተሳታፊ ስም ፊርማ ቀን

_____ _____ _____

የተመራማሪው ወይም የጤና ባለሙያ ስም ፊርማ

እናመሰግናለን!

Annex 2. Data Collection Sheet

Please circle on the letter of your choice and fill the space provided with the requested information when applicable.

A.To be filled by requesting Physician

Name of the Hospital/Health center_____Hospital/Health center Case number_____ Code No._____

Patient name_____Phone number_____Age (in years)_____Sex_____ Kebele_____ Wereda_____

1. Type of TB case A, New B, Relapse C, Treatment failure

2. Past TB treatment A, Yes, (Duration) _____ B, No

3. Anti-TB drugs taken A, INH B, RIF C, Ethambutol D, Strepomycin

E, Pyrazinamide F, Others_____

4. Chest X-ray result: A, Normal B, Abnormal

5. Sputum Direct Microscopy A, AFB present B, AFB absent C, Uncertain

6. HIV serostatus A, Reactive B, Non reactive

7. Type of specimen A, Sputum B, Urine C, pleural fluid D, Pus

8. Date of collection _____

9. Types of test requested A, Diagnosis B, Drug sensitivity test

B. Laboratory analysis Result

10. Culture result A, Positive B, Negative C, Colony count_____

12. LPA result A, Positive B, Negative if positive: Resistance or Sensitive

14. Gene Xpert result A, Positive B, Negative if positive Resistance or Sensitive

Number_____ Date reported_____Name and signature_____

Declaration

I hereby declare that this thesis is the result of my original research work carried out at Addis Ababa University, Ethiopia, Natural sciences, department of microbial, cellular and molecular biology.

I hereby admit that this thesis has never been presented for award of a degree in any university and all the resources of materials used for this thesis have been duly acknowledged.

Chala Chaburte _____ _____

Signature Date

This thesis has been submitted for examination with my approval as university main advisor.

Dr Silvia Blanco  23/2/2015

Addis Ababa University

Department of Microbial, Cellular and Molecular Biology