ADDIS ABABA UNIVERSITY
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Performance Evaluation of MDR-TB Color Plate Test for Rapid Detection of *Mycobacterium tuberculosis* and Multidrug-resistant Tuberculosis in Resource-limited Setting, Addis Ababa, Ethiopia

MSc Thesis

By:
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A Thesis Submitted to Department of Microbiology, Immunology and Parasitology, Collage of Health Sciences, Addis Ababa University, In Partial Fulfillment of the Requirement for the Degree of Masters of Science in Medical Microbiology

June, 2017
Addis Ababa, Ethiopia

Addis Ababa University
College of Health Sciences, School of Medicine,
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M.Sc. Thesis

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ADDIS ABABA UNIVERSITY
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DEDICATION

This thesis is dedicated to those who lost their lives, from infant to elderly, due to rubbish dump landslide in "**Koshe**", Addis Ababa, Ethiopia on Saturday March 11, 2017.

ACKNOWLEDGMENTS

"Praise to **God** according to his excellent greatness" Psalm 150.

This study was done as a collaborative research between Department of Microbiology, Immunology and Parasitology, Collage of Health Sciences, School of Medicine, Addis Ababa University and Ohio State University, USA that aimed to improve the diagnosis of Tuberculosis, and I am thankful for providing logistic and technical support during this study. I would like to extend my immense acknowledgement to the Armauer Hansen Research Institute (AHRI) for its significant and core logistic supplies, and allowing me to access its conventional TB laboratory.

I gratefully acknowledge Dr.Tamrat Abebe, Dr. Adane Mihret and Dr. Shu-Wang my scientific advisors, for their unreserved help started from giving me to work on this 'golden chance' and continued with endurance and patience to rectify my work and providing me relevant materials, constructive suggestions, encouragement and intellectual criticism to shape and build my work at its present level. My sincerely gratitude also extended to my co-advisor, W/t Muluwork Getahun for her provision of essential test drugs (first line anti-TB drugs) and technical support particularly during conventional culturing and drug susceptibility test.

I would like to acknowledge and thank profoundly Dr. Abrham Assefa (MD, PhD, AHRI director) for his willingness and generous permission to use AHRI TB laboratory setup and facilities. Your scientific instruction and encouragement has contributed a lot for this research work.

I am grateful to Ethiopian Public Health Institute (EPHI) for its valuable supports of TB laboratory materials particularly personal protective equipment kits. Moreover, my deep appreciation and acknowledgment go to All African Leprosy, Tuberculosis and Rehabilitation Training Center (ALERT) hospital and St. Peter's TB referral hospitals for their consent and facilitation during sputa specimen collection.

It is also my pleasure to acknowledge EPHI TB department staffs particularly Dr. Desta and Mr. Zelalem; AHRI TB laboratory particularly W/t Marechigne Yimer, and AHRI cleaning

and sterilization section staff members; St. Peter's hospital officer, TB laboratory technicians and MDR-TB care center workers particularly Dr. Addisu and W/t Kidist Tsigemaryam; ALERT TB laboratory and MDR-TB care center particularly W/ro Asnakech for their unreserved all rounded support and kind hospitalities in moral and other social values while my stay in the study area.

I owe special thanks to all study participants for giving precious information during sample taking and interview. Really i am sorely thankful and may God give them his mercy in their recovery process.

I am also grateful to Mekelle University, College of Veterinary Medicine, and Addis Ababa University, School of Medicine, College of Health Science, Department of Medical Microbiology, Immunology and Parasitology, for giving the chance and admitting me to continue post graduate study, advice, encouragement and all round support during my MSc. program.

Finally, I am indebted to my beloved family and friends here and abroad particularly Mr. Abrham Wondmu (PhD candidate) Dr. Solomon Abebe, Dr. Sindu, Z, Dr. Desalegn, Mr. Tadesse, T., W/t Tiruwork, A., W/t Haymanot A., W/t Nebiyat, T., W/t Tigist T. and Lt. Col. Sissay G. who encouraged, assisted me and unreserved prayerful support in the completion of this study.

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ABBERVIATIONS

AFB	Acid Fast Bacilli
AIDS	Acquired Immune Deficiency Syndrome
ALERT	All African Leprosy, Tuberculosis and Rehabilitation Training Center
BCG	Bacillus Calmette–Guérin
BSC	Bio Safety Cabinet
CFP-10	Cultures Filtered Protein-10
CFX	Ciprofloxacin
CI	Confidences Interval
DMF	Di-Methyl-Formamide
DNA	DeoxyriboNucleic Acid
DST	Drug Susceptibility Test
DVM	Degree of Doctor of Veterinary Medicine
ELISA	Enzyme Linked Immunosorabent Assay
EMB	Ethambutol
EHNRI	Ethiopian Health and Nutrition Research Institute
EPHI	Ethiopian Public Health Institute
ESAT-6	Secretary Antigenic Target-6
FDA	Food and Drug Administration
FIND	Foundation for Innovative New Diagnostics
FMOH	Federal Ministry of Health of Ethiopia
HIV	Human Immunodeficiency Virus
IFN-γ	Interferon-gamma
IGRA	Interferon Gamma Release Assay
INH	Isoniazid
KatG	Gene encoding catalase-peroxidase
LED	Light-Emitting Diode
LJ IMP	Löwenstein-Jensen Indirect proportional Method
LJ	Löwenstein-Jensen
LPA	Line-Probe Assay
LTBI	Latent Tuberculosis Infection
MDG	Millennium Developmental Goal
MDR	Multi-Drug Resistant
MDR-TB	Multi-Drug Resistant Tuberculosis
MGIT	Mycobacteria Growth Indicator Tube
MIC	Minimum Inhibitory Concentration
MODS	Microscopic Observation broth-Drug Susceptibility Assay
MTB	*Mycobacterium tuberculosis*
MTBC	*Mycobacterium tuberculosis* complex

NAATs	Nucleic Acid Amplification Tests
NALC	N-Acetyl-L-Cysteine
NPV	Negative Predictive value
NTM	Non-Tuberculosis Mycobacterium
OSU	Ohio State University
PBS	Phosphate Buffer Solution
PCR	Polymerase Chain Reaction
PNB	Paranitrobenzoic Acid
rpoA	Gene encoding pyrazinamidase
PPD	Purified Protein Derivatives
PPV	Positive Predictive Value
PTB	Pulmonary Tuberculosis
PZA	Pyrazinamide
RDs	Regions of differences
RFLP	Restriction Fragment Length Polymorphism
RMP/RIF	Rifampicin
rpoB	Gene encoding β-subunit of RNA
rpm	Revolution per minute
rRNA	ribosomal Ribonucleic Acid
SDA	Strand Displacement Amplification
SE	Standard error
SM	Streptomycin
SOP	Standard Operating Procedures
SPSS	Statistical Package Software for Social Sciences
STC	2, 3 diphenyl-5-(2-thienyl) Tetrazolium Chloride
TAT	Turnaround Time
TB	Tuberculosis
TCH	Thiophene-2-Carboxylic acid Hydrazide
TDR	Total Dug Resistant Tuberculosis
TLA	Thin-Layer Agar
TST	Tuberculin Skin Test
USA	United States of America
WHO	World Health Organization
XDR	Extensively Drug Resistant
ZN	Ziehl-Neelsen
Mm	Micrometer
$\chi 2$	Chi-square

ABSTRACT

Background: Tuberculosis (TB) is a top global public health concern and its controlling program is chiefly hampered by diagnostic difficulties. Timely TB diagnosis and detection of drug-resistant TB has a key significance for both patient management and the disease control. However, the commercially existing diagnostic tools are slow and unaffordable for resource-limited settings.

Objective: The current study was aimed to evaluate the performance of MDR-TB color plate test (color plate test) for rapid concurrent detection of *Mycobacterium tuberculosis* (MTB) and multidrug-resistant TB (MDR-TB) directly from sputum specimens.

Materials and Methods: A cross-sectional, hospital-based diagnostic test evaluation study was conducted from March 2016 to February 2017 at All Africa Leprosy, Tuberculosis and Rehabilitation Training Center (ALERT) hospital and St. Peter's TB referral hospital in Addis Ababa, Ethiopia. The performance of the color plate test was prospectively compared to the conventional Löwenstein-Jensen (LJ) culture and LJ indirect proportional drug susceptibility test (DST), and the molecular GeneXpert MTB/RIF® assay. A paired morning expectorated sputum samples were collected from 147 MDR-TB suspected patients using a convenience sampling technique in both hospitals. One of the two sputum samples is used for diagnosis purpose was tested directly by GeneXpert assay at each respective hospital's laboratory and internally validated. But, the other sputa were refrigerated (4-8^{0}C) and transported to the Armauer Hansen Research Institute (AHRI) TB laboratory where the color plate test and the conventional LJ culture and DST were conducted. Data were analyzed using SPSS version 20, and kappa statistic was applied to test agreement between results of the three assays.

Result: Of the 147 sputa, 137 (93.2%) sputa produced interpretable MTB detection results for all the three assays and were included in the analysis. Compared to the conventional LJ culture, color plate test was 93.6% sensitive and 98.3% specific while GeneXpert assay was 96.2% sensitive and 79.7% specific. Moreover, the color plate test had showed a strong agreement with both the conventional LJ culture (95.62%, kappa 0.9114) and GeneXpert assay (89.05%, kappa 0.7756) for the detection of MTB. Moreover, all conventional LJ

culture positives, 78 isolates were further subjected to conventional DST, and valid results obtained by color plate test and GeneXpert assay were 70 and 72 isolates respectively. The color plate test demonstrated sensitivity of 91.3% and 93.6% specific for the detection of isoniazid resistant MTB isolates with strong agreements (kappa 0.8399). However, the accuracy for detection of rifampicin resistance was suboptimal with low agreement (kappa value of 0.08 and concordance 39%). The color plate test was also in low agreement with the GeneXpert assay (38.57%, kappa 0.0773) for detection of RMP drug resistance. In the present study, the results of color plate test can be obtained in a median time of 13 days while the conventional indirect DST method requires an average of 50 days. Besides, the color plate test is simple to use and the cost per test result also minimal relatively.

Conclusion: In this study, the color plate test was found to be a good alternative method for screening of TB and selective drug resistant-TB in a timely and affordable way in resource-limited setting despite limitations of the test need to be addressed before test approvals.

Key Words: *Color plate test, Conventional LJ culture, Diagnostic performance, GeneXpert assay, indirect proportional DST, Multidrug-resistant tuberculosis, Tuberculosis*

1. INTRODUCTION

1.1. Background

Tuberculosis (TB) is an aerosol-transmitted disease caused by members of the species *Mycobacterium tuberculosis* complex (MTBC), primarily by *Mycobacterium tuberculosis* (MTB). It is one of the oldest well-known diseases since known as the “white plague” and “consumption. Besides to plaguing medieval and renaissance Europe, TB has been traced back and documented in ancient civilizations including Greek, Egyptian, and Peruvian (Daniel, 2006). In 2016, TB was still a major global health concern and presenting significant challenges in both diagnosis and treatment (Mario and Giorgia, 2016; WHO, 2016).

TB was declared as a global emergency in 1993 by the World Health Organization (WHO) and at present it is a leading cause of morbidity and mortality worldwide, accounting for about 9.6 million new cases and 1.5 million deaths estimated to have occurred in 2014 (WHO, 2015). In 2015, TB ranked above human immunodeficiency virus (HIV) infections and one of the top 10 causes of death worldwide (WHO, 2015). The recent report indicated that two-three billion people, roughly one third of the world's population are latently infected with *M. tuberculosis* (Juan, *et al*., 2008; WHO, 2016). The global burden of tuberculosis is not evenly distributed rather more than two thirds of the global TB burden is reported in Africa and Asia. Furthermore, the actual TB burden extends beyond the direct morbidity and mortality of human beings rather has an extensive social and economic impacts. The social impact may be described in the fact that 10 million children were orphaned because of TB in 2009 (WHO, 2011). Additionally, the economic burden due to the disease is very high, for example, US$100-500 and 5000-10000 US$ costs per patient were required for treatment of drug-susceptible TB and MDR-TB respectively in 2014, a gigantic financial burden on the struggling economies of developing nations (WHO, 2015).

Although TB is curable completely, in addition to acquired immunodeficiency syndrome (AIDS), the emergence of drug-resistant tuberculosis, particularly multidrug-resistant (MDR) and extensively drug-resistant (XDR) TB is a potential challenge for global TB

control program (Falzon *et al*., 2015; Mario and Giorgia, 2016). Despite extended treatment duration, MDR and XDR-TB have considerably worse outcomes (Orenstein, *et al*., 2009) and longer infectious periods than does drug-susceptible TB (Wells, *et al*., 2007).

Ethiopia is also highly troubled by the TB pandemic and is the most serious public health challenges. The incidence rates of TB in the country was 192/100,000 populations in 2015 (WHO, 2016). In addition, the incidence of MDR-TB is also a continuing challenge to the TB program, with almost 5000 MDR-TB patients emerging each year (FMOH, 2015). The up-to-date report of WHO indicated that an increment of the incidence rate of MDR-TB, from 1.6% to 2.7% in new TB cases and also rose from 12% to 14% in previously treated TB cases. This figure is getting closer to global average, where MDR-TB cases accounted for about 3.3% of all new TB cases and 20% of previously treated cases (WHO, 2016).

TB prevention and management efforts rely primarily on the vaccination of infants and the detection and appropriate treatment of active cases. Thus, new vaccines, diagnostics tools and drugs are the main focus and parts of the 'End TB Strategy' where 90% and 80% reduction in TB deaths and TB incidence rates respectively are expected by 2030, compared with 2015 (WHO, 2016).

There is only one available vaccine, the Bacillus Calmette–Guérin (BCG), a live attenuated vaccine, which has been in use since the 1920s, and whose actual benefits are questionable. It has a significantly variable efficacy (reportedly 0-80%) and does not prevent the occurrence of pulmonary TB infection (Checkley and McShane, 2011). However, it protects against severe forms of TB such as miliary TB, mainly in infants (Andersen and Doherty, 2005; Checkley and McShane, 2011). Recently, fifteen vaccine candidates are in clinical trials and their emphasis has shifted from children to youth and adults (WHO, 2015).

Timely MTB diagnosis, detection of drug resistance and treatment are pillars for the global control strategies of the disease, because optimized treatment is initiated when infection is detected and is based on rapid results of DST (Michael and Wilson, 2011; WHO 2015). World health organization (WHO) has achieved some success with improved diagnostic tool and effective treatment regimens which was credited for averting 49 million deaths and

significant reduction (22%) of TB incidence globally since the Millennium Development Goal (MDG, between 2000 and 2015) was set (WHO, 2016).

However, the diagnostic and therapeutic gaps still remain in resource-limited settings. Recent report indicated that about 3.6 million TB cases are estimated to be missing every year comparing with the estimated figure (Herbert, *et al*., 2014). In 2015, only 57% of TB cases were confirmed bacteriologically, merely about 26% of the estimated (half a million) MDR-TB patients were enrolled and confirmed with laboratory testing, and only 50% of the enrolled MDR-TB patients were successfully treated. All these large unknown cases are not diagnosed and/or notified to public health systems, are responsible for continuing challenge to the global TB control program (WHO, 2015; Mario and Giorgia, 2016, WHO, 2016).

These serious diagnostic gaps can be reduced significantly with optimal TB diagnostic assays. Accordingly, several new optimal TB diagnostic methods have been endorsed by the WHO since 2007 and many others are under investigation. Of these, the non-molecular methods are the modification of conventional culture methods with the prime aim of circumventing the shortcomings of high cost and long turnaround time conventional culture DST (Michael and Wilson, 2011, WHO, 2015; WHO, 2016). With this regard, thin-layer agar (TLA) assay is one of the emerging and promising diagnostic tests, waiting sufficient evidences for endorsement (Minion *et al*., 2010). The color plate test is based on TLA technology, with both culture and direct DST method on a single agar plate. Despite limited data, the color plate test have demonstrated a good performance for rapid diagnosis of MDR-TB and pre-XDR-TB (Herrera, *et al*., 2007; Minion, *et al*., 2010; Toit, *et al*., 2012).

1.2. Statement of the Problem

The current global diagnostic pipeline of TB, particularly in high TB burdened countries such as Ethiopia, was the main driver for studying an alternative TB diagnostic technique. Despite some advances with the adoption of rapid molecular tests in recent years, the existing commercially available assays for diagnosis of TB and MDR-TB are not in a timely and affordable ways in resource-limited settings. As a result, the majority of TB cases are treated without DST results and unable to routinely carry out surveillance for MDR-TB,

thus, continuing challenge to the global TB control program (Wright, *et al*., 2009; Michael and Wilson, 2011; FMOH, 2015; WHO, 2015; WHO, 2016).

Sputum smear microscopy is still the mainstay of diagnostic tools in high TB burdened countries like Ethiopia while several other countries are phasing out the use in favor of GeneXpert assay. The test is rapid and economical ($0.50 USD per test) but it has lower sensitivity, it misses more than 30% of positive cases, and unable to detect drug-resistant TB (FMOH 2012; Molicotti, *et al*., 2014). The GeneXpert assay represents a paradigm shift in the diagnosis of TB and MDR-TB by simultaneously detecting MTBC and rifampicin resistance (a surrogate marker for MDR-TB) directly from sputa in 2 hours. It has higher sensitivity than smear microscopy (about 40% more sensitive) and has comparable sensitivity to the conventional culture (Steingart, *et al*., 2014). Thus, it allows rapid initiation of MDR-TB treatment, however, it is very expensive and unaffordable ($25-30 per test) (Molicotti, *et al*., 2014). As a result, most developing nations including Ethiopia could not replace smear microscopy with GeneXpert assay. Culture remains the gold standard method for the detection of MTBC (WHO, 2016). The solid culture-based DST methods can provide definitive results, but it can take 3-4 months for results to initiate treatment and is laborious (Ani, 2008; Molicotti, *et al*., 2014). Commercial DST with liquid culture (MGIT) can shorten the detection time to few days but requires expensive laboratory facilities so that it is not implemented widely though recommends for rapid detection of MDR-TB (Raviglione, *et al*., 2012; WHO, 2015).

Consequently, there has been a marked increase in the development and testing of rapid and cost-effective TB diagnostic assays. In this thesis work, we present the diagnostic performance of color plate test and compared it to the conventional culture-based DST, and the molecular GeneXpert MTB/RIF assay.

1.3. Significance of the Study

Based on the above figure and facts, it is possible to hypothesize that TB and the growing drug-resistant TB, particularly in resource-limited countries, could not be controlled by only the existing commercially available diagnostic tests. Therefore, it is urgently essential to

have optimal methods for diagnosis of TB and identifying drug-resistant TB in a timely and affordable way. Color plate test is a reliable diagnostic test for simultaneous detection of TB and drug-resistant TB. Results are obtained significantly more quickly than with conventional DST as a result optimized treatment can be initiated. It is also affordable than GeneXpert assay, hence, it may satisfy the demand of developing nations. The color plate test may also be safer than with the conventional method because it is double-sealed from inoculation to discarding and is discarded much faster than conventional culture methods. Thus, it may be suitable to outside conventional laboratory settings. The current study findings (data) may be essential and offer evidences for TB diagnostic pipelines and for also endorsement of the test by world health organization.

2. LITERATURE REVIEW

2.1. Etiologic Agents of Tuberculosis

The genus *Mycobacterium* is classified under the order Actinomycetales and family Mycobacteriaceae. There are three species under this genus and these include *M. tuberculosis* complex, the non-tuberculosis mycobacteria and *M. leprae* (Shinnick and Good, 1994). The *M. tuberculosis* complex (MTBC) has a number of members, evolved in Africa and most probably in the horn of Africa (Blouin, *et al*., 2012; Comas, *et al*., 2013). They are a group of strongly genetically related *Mycobacteria* that causes TB in mammalian species. These includes *M. bovis* (causes TB mainly in cattle), *M. bovis* BCG (the attenuated strain used in vaccines), *M. africanum* (less pathogenic than *Mycobacterium tuberculosis* (MTB), causes TB-like symptoms, found mainly in West Africa), and *M. canettii* (found mainly in horn of Africa). Additionally, *M. microti*, *M. caprae* and *M. pinnipedi* are causative agents of TB in goats, deer, and seals, respectively, despite human infection also possible. Of the members of MTBC, *M. tuberculosis* and *M. bovis* are best known and share 99.9 % of the same genome (Brosch, *et al*., 2002) and *M. tuberculosis* is the primary TB-causing pathogen in human beings (Forbes, *et al*., 2007; Gordon, *et al*., 2009). *M. bovis* may be an issue in rural or pastoral areas where close association between human and cattle or where increase usage of unpasteurized milk products is consumed (Gumi, *et al*., 2011; Ameni, *et al*., 2013; Torres-Gonzalez, *et al*., 2013).

Mycobacterium tuberculosis (MTB) was first discovered in 1882 by Robert Koch (Ryan and Ray, 2004). It is a rod shaped acid fast bacilli, measuring 0.5 x 3 μm, non-motile and don't produce spores. MTB are intracellular an obligate aerobic, requires oxygen to grow and for this reason they are always found in the well-aerated upper lobes of the lungs. They are extremely slow-growing (18-24 hr generation time) compared with other bacteria that divides in minutes (*E. coli* can divide roughly every 20 minutes) (Forbes, *et al*., 2007).

Mycobacterium is distinguished by having peptidoglycan cell walls with exceptional high lipid content, mainly mycolic acid, and thus cannot absorb any conventional stains such as gram stain; as a result, they cannot be classified as either gram-positive or gram-negative. Instead, they can be stained with lipid-soluble acid-fast stains such as Ziehl-Neelsen stain

with a property known acid-fastness or fluorescent stains such as auramine are used (Daniel, 2006; Forbes, *et al*., 2007; Gordon, *et al*., 2009; Knechel, 2009; El Khechine and Drancourt, 2011; Cudahy and Shenoi, 2016). This exceptional cell wall structure of MTB is also likely responsible for its resistance to desiccation and is a key virulence factor, as this hydrophobic cell wall is impermeable to many antibiotics, and contains channels and porins that pump out drugs. Besides, this lipid-rich rigid cell wall containing the carbohydrate lipoarabinomannan allows MTB to survive within the patients' macrophages that engulf the bacilli (Knechel, 2009; Almeida, *et al*., 2011; El Khechine and Drancourt, 2011).

MTB can be grown in the microbiology laboratory, and the commonly used artificial media includes liquids media such as Middlebrook 7H9 or 7H12, and solid media namely Löwenstein-Jensen (egg-based), and agar-based Middlebrook 7H11 or 7H10 (Eugene, *et al*., 2015). They grow slowly; roughly doubling once per day, thus several weeks (4-6 weeks) required seeing visible colonies on solid media with small and buff/yellow colored. It is also distinguished from other *Mycobacterium* by its production of catalase and niacin (McMurray and David, 1996), other testes like molecular test confirm its identity (Bicmen, *et al.*, 2011*).* Moreover, under microscopy cells are curved rod-shaped and are often seen wrapped together, due to the presence of fatty acids in the cell wall that stick together (Kenneth Todar and Madison, 2016). This appearance is referred to as cording, like strands of cord that make up a rope (Eugene, *et al*., 2015).

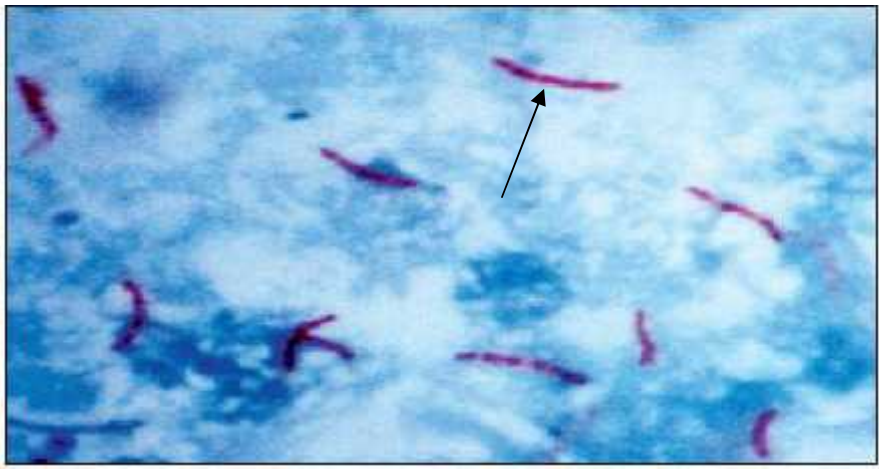

Figure 1: Acid-fast Staining of MTB Bacilli (arrow indicated) in Sputum (x1000 magnification) (Lawn and Zumla, 2011)

2.2. Genome of *Mycobacterium tuberculosis*

Genetic homogeneity is a feature of MTB and the complete genome of the reference virulent *M. tuberculosis* strain (H37Rv) was sequenced and published in 1998. This revealed a circular genome in the region of 4.4 million base pairs containing approximately 4000 genes with a high (65.6%) GC content (Cole, *et al*., 1998; Gordon, *et al*., 2009). The H37Rv strain is the presently available universal control for drug susceptibility test (DST), as a characterized pathogenic pan-susceptible strain (Ängeby, *et al*., 2012).

Genome of complex pathogenic organisms like *M. tuberculosis* can encode several thousand protein and most of them are shared with non-pathogenic mycobacteria and the vaccine strain of *M. bovis* BCG (Mustafa, 2001). However, comparative genomic studies have identified 11 regions of difference (RD1, RD4, RD5, RD6, RD7, RD9, RD10, RD11, RD12 RD13 and RD15) in *M. tuberculosis* genome which are absent or deleted in all vaccine strains of *M. bovis* BCG (Behr, *et al*., 1999). The proteins encoded by genes predicted in these RDs could be useful as protective vaccine and/or exacerbated the disease process by inducing cellular immune response involved in protection and pathogenesis of TB. In the recent years, because of specificity to MTB, the proteins encoded by various MTB RDs have been a major focus for diagnostic application (Mustafa and Rajaa, 2009).

The phenotypic and virulence variations observed between different MTB strains may be molecularly attributed to polymorphic genomic 'regions of difference' (RD), which measure about 0.5 kb (Cole, *et al*., 1998; Gordon, *et al*., 2009). Certain RD loci can also be of utility in strain lineage identification, such as MTB deleted region 1 (TbD1) whose presence or absence helps define MTB lineages (Gordon, *et al*., 2009).

2.3. Epidemiology of Tuberculosis

The magnitude of the global TB burden is expressed as the number of incident cases per 100,000 populations. Asia (South-East Asia and Western Pacific regions) accounted for 58% of the world's TB cases in 2014. The African Region had 28% of the world's cases, and the Americas, European and Eastern Mediterranean regions accounted for the remainder. Moreover, the WHO 2015 report has showed India, Indonesia and China had the largest

numbers of cases of the global total 23%, 10% and 10% respectively. Globally in 2014, there were an estimated 9.6 million incident cases of TB and of these noted figures, 5.4 million, 3.2 million, and 1.0 million were men, women, and children respectively (WHO, 2015). In Ethiopia, 192(142–250) incident cases per 100 000 population were reported in 2015. Among these, 106(77–136) and 85(52–117) cases were reported in male and females respectively. Besides, the estimated TB incidence by age showed 18(12–24) cases in children with age of 0-14 years, and 173(143–203) cases in those whose ages were >14 years (WHO, 2016).

2.4. Transmission and Pathogenesis of Tuberculosis

MTB is transmitted from person to person via inhalation of airborne particles called droplet nuclei. The air droplet nuclei are carried the bacilli, originated from a person who has the disease during coughing, sneezing, speaking, or singing. The larger droplets either settle to ground quickly or lodges in nose and throat if inhaled, while smaller droplets desiccate (via evaporation) to form 'droplet nuclei' of 1-5μm diameter, reach to pulmonary alveoli where infection is begin, and only 1-3 bacilli are sufficient for infection (Smith, *et al*., 2009; CDC, 2016). Once in the lung alveoli, there could be three possible outcomes. The first chance can be elimination of the bacilli and infection clearance, or failure to control the infection and development of active symptomatic tuberculosis, or the third fate can be control of infection by the immune system without clearance of tubercle bacilli. In the third option, the patient is non-infective and exhibits no symptoms, though they are carrier of dormant bacilli which may be reactivated when the person's immunity is sorely compromised with chronic conditions for instance HIV infection and diabetes mellitus (Knechel, 2009; Gengenbacher and Kaufmann, 2012).

About 90% of those infected with *M. tuberculosis* have asymptomatic, latent TB infections and with only less than 10% of individuals who become infected with TB bacilli will develop an active tuberculosis disease, regardless of the level immune system. Based on the level of immunity, this figure can reach 30%, and co-infection with HIV makes progression to active TB disease 20-30 times more likely than people without HIV (Gordon, *et al*., 2009; McNerney, *et al*., 2012; WHO, 2012).

When an infective droplet carrying bacilli are inhaled, a portion may be trapped and get rid of by innate immune system of the host, the mucociliary systems of the respiratory tract. The escaping bacilli that reach the lungs are engulfed by alveolar macrophages as an infection containment defense, but unable to kill and digest the mycobacterium due to MTB possesses a unique ability to persist within the macrophages and counterbalance their destruction mechanisms. Some of the mechanisms include the vigorous cell wall prevents the fusion of the phagosome with the lysosome, which contains a host of antibacterial factors; thereby, it could be the first-line of defense for *M. tuberculosis* against the harsh environment inside macrophages. On another front, MTB changes the pH within the macrophage from the lethal 4.5-5.0, to nearly a neutral 6.4 via neutralizing the acidification process initiated by the macrophages. Besides, the entire metabolic activity of MTB modifies so as to favor survival within the unsociable macrophage environment with inadequate nutrients (Forbes, *et al*., 2007; Knechel, 2009; Gengenbacher and Kaufmann, 2012).

Within 2-6 weeks, the accumulation of macrophages, T-lymphocytes, and other host cells (dendritic cells, fibroblasts, endothelial cells, and stromal cells) leads to the formation of granuloma at the site of infection around the tubercle bacilli. The protective granuloma also called a barrier shell that keeps the bacilli contained and under control. Then after, necrosis set in and slowly the bacilli are trapped within the granuloma lesions (caseous centers of the granuloma) in a microenvironment with low oxygen, pH, and nutrients. As a result, the metabolic function of MTB drops off and come into a non-replicative dormant state, and eventually latent TB infection (LTBI) is established. By the time the patient's immune system compromised where can't keep the tubercle bacilli under control, or if latent TB is reactivated, the bacilli cannot be contained within the granulomas, which burst open and the bacilli spread. They can spread to the alveoli and cause pulmonary TB, or the infected macrophages can take the bacilli to other organs causing extra-pulmonary TB (20% of all TB cases) e.g. lymphatic TB. The clinical symptoms of active TB are not specific but the general sign and symptom includes low-grade fever, night chill and sweats, fatigue, loss of appetite, progressive weight loss, coughing, and sputum with tainted blood in pulmonary TB (Forbes, *et al*., 2007; Knechel, 2009; Lawn and Zumla, 2001; Gengenbacher and Kaufmann, 2012).

2.5. Treatment of Tuberculosis

The principal goals for tuberculosis treatment are to cure the individual patient, and to reduce the transmission of *Mycobacterium tuberculosis* to the community population. Therefore, successful treatment has public health benefits through prescribing appropriate regimens and follows up completion of therapy. Even though TB has remained one of the most difficult infections to treat, it has become a curable disease since the discovery and availability of effective drugs. Treatment of TB is a prolonged and demanding process; the previous standard treatment regimen took 9-12 months. The purpose of these lengthy treatments is to completely eliminate *M. tuberculosis* and preventing the formation of drug resistant mutants. On the contrary, despite high cost, the prolonged therapies are highly toxic to patients and it contribute to poor patient adherence and leading to relapse and likely emergence of drug resistant strains (Heifets and Cangelosi, 2009; Pinto and Menzies, 2011; Tasha, *et al*., 2013). Thus, treatment challenges have exacerbated by the emergence of drug resistant TB. In spite of presenting a significant challenge, MDR-TB is treatable with second-line drugs (e.g. fluoroquinolones and injectable). However, early diagnosis is crucial for proper management of the patient (Ormerod, 2005; Yew and Leung, 2008). The treatment of MDR-TB is prolonged lasting 18 to 24 months therapy but less effective and more toxic (Nathanson, *et al*., 2010).

The first discovered anti-TB drug was streptomycin (SM) in 1944. However, SM resistance developed rapidly due to extensive SM mono-therapy (Daniel, 2006; Zhang and Yew, 2009; Almeida, 2011). The second drug, INH was first discovered in 1952 and, is the first oral anti-TB drug (Daniel, 2006; Inge and Wilson, 2008; Zhang and Yew, 2009). The discovery of rifamycin compounds in 1957 strengthened the anti-TB arsenal with another oral drug, rifampicin (RMP) (Daniel, 2006; Heifets and Cangelosi, 2009). The addition of pyrazinamide (PZA) with isoniazid and rifampicin forms the cornerstone of modern TB therapy, shortening the TB therapy from previously 9-12 months (1year) to current 6 months (Zhang, *et al*., 2003).

The currently recommended treatment for new cases of drug-susceptible TB is a 6-month regimen of four first-line drugs: isoniazid (INH), rifampicin (RMP), ethambutol (EMB) and

pyrazinamide (PZA) (WHO, 2016), where INH and RMP are the two most potent drugs. RMP must always be given in conjunction with another potent anti-mycobacterial as resistance readily emerges to it and resistance to rifampicin is sometimes considered a marker for MDR-TB (O'Grady, *et al*., 2011).

The need for using the drugs cocktail for TB treatment is to eliminate tubercle bacilli at different metabolic and growth phases; thereby each drug has a different role in treatment, to ensure killing of all mycobacterium in the body. INH is the most active drug against continuously multiplying and metabolically active bacilli, RMP and SM are also effective in killing these bacilli. In addition, RMP is also active against metabolically inactive bacilli, but exhibit irregular spurts of metabolism. Ethambutol has a bacteriostatic effect on metabolically active bacilli subpopulation of bacilli. The drug PZA is favored for treatment of a semi-dormant MTB bacilli population in acidic pH environments (caseous necrotic cavities and inside the phagolysosomes of macrophages), where other anti-TB drugs fail to act (Mitchison, 2005; Zhang and Yew, *et al*., 2009; Pinto and Menzies, 2011).

The global TB drug facility supplies a complete 6-month course for about US$ 40 per person, and treatment success rates of at least 85% for new cases of drug-susceptible TB. Treatment for rifampicin-resistant-TB (RR-TB) and multidrug-resistant TB (MDR-TB) is longer and more expensive. Until early 2016, the re-treatment regimens lasted for 20 months, and cost about US$ 2000–5000 per person. As a result of new evidence from several countries, as of May 2016, shorter MDR-TB regimen of 9–12 months is now recommended for all patients (excluding pregnant women) with pulmonary MDR/RR-TB that is not resistant to second-line drugs. The cost of a shortened drug regimen is about US$ 1000 per person (WHO, 2016). The shorter MDR-TB regimen consists of seven drugs (four to six months with kanamycin, moxifloxacin, prothionamide, clofazamine, pyrazinamide and high dose INH followed by five months of moxifloxacin, clofazamine, pyrazinamide, and ethambutol (WHO 2016). Moreover, the report indicated that treatment success rates of 52% for MDR-TB and 28% for extensively drug-resistant TB.

2.6. Drug-resistant Tuberculosis

Drug resistance is the reduction in effectiveness of a drug in curing a disease or condition, and it is the ability of bacteria to survive and multiply in the presence of a particular antimicrobial agent. Generally, bacteria demonstrate two types of resistance namely natural (intrinsic) and acquired resistances. Natural resistance is the innate ability to resist the activity of drugs through its inherent structural or functional characteristics of bacteria. Whereas, acquired resistance occur when a bacteria gain/acquire the ability to resist against the action of a particular antibiotics to which it was previously susceptible, and it might occur through either mutation (gene change) and/or horizontal gene transfer (exchange) (Tasha, *et al.*, 2013).

MTB and other members of *M. tuberculosis* complex use several strategies such as intrinsic and acquired to resist the action of anti-TB drugs. In addition to β-lactamase activity, an inherent resistance to a variety of antibiotics by virtue of its extraordinary cell wall structure which also include multiple efflux mechanisms to counteract drugs (Almeida and Palomino, 2011). In *M. tuberculosis*, horizontal transfer of resistance genes via plasmids or transposon elements has not been reported. By contrast, all currently known acquired resistances are mediated exclusively through chromosomal mutations that arise under selective pressure of antibiotic use (Gillespie, 2002; Huitric, *et al*., 2010).

Drug resistance of TB can either be primary TB resistance, which occurs in naive patients who have never been treated with any anti-TB drugs, might be due to contact with drug-resistant patients or acquired TB resistance, in patients who get inadequate treatment due to prescription error, inadequate absorption (sub-therapeutic drug dose) or non-adherence to the appropriate regimen (Heifets and Cangelosi, 2009; Almeida and Palomino, 2011), and TB drug resistance is significantly more likely in retreatment patients (Almeida and Palomino, 2011; WHO, 2015). Drug resistance to at least the two most powerful first-line anti-TB drugs, isoniazid (INH) and rifampicin (RMP) is known as multi-drug resistant (MDR)-TB while extended-drug resistant (XDR-TB) is MDR-TB plus resistance to at least one fluoroquinolone and second line injectable drugs such as amikacin, kanamycin,

and capreomycin (Almeida and Palomino, 2011). The MDR-TB inclusive of pyrazinamide (PZA) drug resistance definitely worsens the prognoses of the patients.

Isoniazid is a potent drug that inhibits the synthesis of mycolic acids in MTB. It is a pro-drug that becomes active and acts on its target when catalyzed by the mycobacterial enzyme catalase-peroxidase. At least three genes, *kat*G, *inh*A and *ahp*C are involved in resistance to isoniazid. Among these genes, *kat*G (mycobacterial catalase-peroxidase gene) that encodes catalase-peroxidase is mainly responsible for isoniazid resistance. A missense mutation of the *inh*A gene which encodes an enzyme involved in the mycolic acid biosynthetic pathway also causes resistance (Zhang, *et al*., 1992).

Resistance to rifampicin results from missense mutations in the *rpo*B gene, which encodes the β-subunit of RNA polymerase (Miller, *et al*., 1994). RNA polymerase is an essential enzyme with five subunits that catalyzes the process of transcription. Rifampicin specifically binds to the β-subunit and prevents early steps of transcription that leads to the bacterial death. However, mutation in *rpo*B gene results in resistance by decreasing rifampicin-binding affinity. However, it is rapidly results in the selection of mutants that are resistant to other anti-TB drugs (Goble, *et al*., 1993). Most commonly, rifampicin resistant TB exists in conjunction with isoniazid resistance, and thus defines a strain as being MDR-TB (Turett, *et al*., 1995). Spontaneous mutations resulting in resistance to both isoniazid and rifampicin is the results of the individual probabilities (Iseman and Madsen, 1989).

Pyrazinamide (PZA) is a pro-drug and converted into the active bactericidal form pyrazinoic acid (POA) by mycobacterial enzyme pyrazinamidase, POA accumulate that lowers intracellular pH to a suboptimal level that is likely to inactivate a vital target enzyme such as fatty acid synthase-I and eventually result in cell death (Yeager, *et al*., 1952; Zimhony, *et al*, 2000; Singh, *et al*., 2006). MTB develop resistance to PZA drug predominantly results from mutation in *pncA* gene. Pyrazinamidase (PZase) is encoded by *pncA* gene, and mutations in this gene have been demonstrated as the major mechanism of PZA resistance. Numerous mutations, including missense, insertion, deletion and nonsense mutations have been reported and located in both the putative promoter and coding regions of *pncA* (Scorpio and Zhang, 1996; Ando, *et al*., 2010). In spite of some PZA-resistant strains that contained

wild-type and retain PZase activity, vast majority of PZA-resistant *M. tuberculosis* strains are strongly associated with defective PZase activity. This suggest that the involvement of other resistance mechanisms namely pyrazinamide uptake, *pncA* regulation, or POA efflux (Scorpio and Zhang, 1996; Mestdagh, *et al*., 1999; Raynaud, *et al*., 1999; Singh, *et al*., 2006). Of the members of the *M. tuberculosis* complex, *M. bovis* and *M. bovis* BCG, are naturally/inherently resistant to PZA. In these organisms, PZA resistance is due to a unique C to G point mutation in codon 169 of *pncA*. In contrast, mutations in PZA-resistant *M. tuberculosis* have been found scattered throughout *pncA* (Scorpio and Zhang, 1996). Thus, detection of PZA drug resistance can be a surrogate marker for *M. bovis*.

The bacterial mutation rate is defined as the probability of a mutation occurring per cell division (Pope, *et al*., 2008). The rate of spontaneous drug resistance-conferring gene mutations in *M. tuberculosis* isolate can vary among anti-TB drugs. Resistance to a single drug can be the outcome of a spontaneous mutation in the particular resistance-conferring gene, an event which occurs in wild MTB strains every 10^{-6} to10^{-8} replications (McGrath, *et al*., 2014). For the two main anti-TB drugs isoniazid (INH) and rifampicin (RMP), the rate of spontaneous resistance-conferring mutations are 2.56x 10^{-8} and 2.25x10^{-10}, respectively (Pinto and Menzies, 2011). The use of drug combinations in TB therapy reduces the likelihood of emergence of resistance, as the risk of a strain harboring two resistance mutations is less than 10^{-18} (Almeida, *et al*., 2011). Naturally occurring two-drug resistance is very uncommon, therapy with two (or more) drugs prevents the emergence of progressive resistance. Moreover, this rate can be reduced due to human intervention. The frequency of acquired resistance to pyrazinamide is determined to be 1x10^{-5} bacilli in vitro (Stoffels, *et al*., 2012).

When mutation confers resistance to a certain antibiotic, all sensitive bacteria is killed and the resistant ones will grow and become the dominant variant in the population (Porter and Adam, 1992). Generally, when one percent or more (>1%) of organisms in an isolate are found resistant to an anti-TB drug, therapeutic success is less likely to occur. It is then that the strain is considered resistant to the drug (Garay, 1996). Normally any large population of mycobacterium regardless of their exposure to antibiotics will contain some organisms

resistant to one of the five first line drugs, isoniazid, rifampicin, streptomycin, ethambutol, and pyrazinamide (Selwyn, *et al*., 1992).

An issue with global ramification is the rise of the incidence of drug-resistance in TB, particularly MDR-and XDR-TB. MDR-TB strains currently infect more than 50 million patients worldwide, with almost 500,000 new cases emerges annually (Gengenbacher and Kaufmann, 2012, WHO, 2016). An estimated 3.3% of new TB cases and 20% of previously treated cases are MDR-TB and 9.5% of people with MDR-TB have XDR-TB, the serious form of drug-resistant TB (WHO, 2016). In Ethiopia, 2.7% of newly diagnosed MTB cases are classified as MDR-TB, however, this figure rises to 14% among retreatment cases (WHO, 2016). Moreover, some studies reported XDR-TB in the range of 1% - 4.4% in Ethiopia. The most powerful predictor of the emergence of MDR-TB reported in Ethiopia is previous exposure to anti-TB drug treatment (Fantahun, *et al*., 2014). These figures may be an under estimation since not all *M. tuberculosis* isolates are tested for drug resistance. For instance, in 2015, only 57% of new and relapse pulmonary TB cases are bacteriologically confirmed (WHO, 2016). Besides, from the estimated MDR-TB cases to have annually (nearly half a million cases), only about a quarter of these (26%) were enrolled and had laboratory testing and confirmation (WHO, 2015; WHO, 2016).

2.7. Diagnosis of Tuberculosis

Laboratory diagnosis of *M. tuberculosis* can be performed using direct and indirect methods. An isolation of *mycobacterium*, species-level identification, and the determination of the isolated strain's drug susceptibility are made by direct diagnostic method. It is used to diagnose active TB using sputum smear microscopy, isolating MTB by conventional culture method and identifying specific MTB DNA sequences using molecular diagnostic tools. Whereas, the indirect diagnostic tool is mainly used to detect latent TB infection indirectly, for example tuberculin skin test and interferon-gamma release assays (IGRAs) can identify the subjects infected with MTB (eg. detection of interferon-gamma in response to the certain MTB antigens) (Molicotti, *et al*., 2014). The main diagnostic approaches of *M. tuberculosis* detection have not been changed very much for decades in most high TB burden countries. Sputum smear microscopy remains the most common method for detection of active TB

infection though it is phasing out from some countries. TB is also diagnosed via culture methods, the current standard references, in countries with more advanced laboratory settings. In the last few years, the use of rapid molecular testes to diagnose TB and drug-resistant TB is increasing (WHO, 2015).

2.7.1. Microscopic Examination

Sputum smear microscopy was developed more than 100 years ago. Sputum samples are examined under a microscope to see if *mycobacterium* is present and it relies on finding of acid-fast bacilli (AFB) using conventional stain, acid-fast/Ziehl Neelsen (ZN) stain. For detection of positive samples, a minimum of 300 microscopic fields must be observed, and it requires the presence of at least 5,000 to 10,000 bacilli per mL of sputum. Thus, a lower bacillary load would be missed (Martin and Palomino, 2009; WHO, 2016). Currently, the number of sputa specimen is reduced from three to two, for screening of TB in well-established laboratory settings. Out of the two specimens, morning specimens are preferred, and one positive result is required for a diagnosis of smear-positive pulmonary TB (Parsons, *et al*., 2011; WHO, 2011a).

AFB smear microscopy is useful for detection of active TB infection as non-viable bacilli with damaged cell walls may lack the acid-fastness upon which the staining is based (Mitchison, 2005; Palomino, 2012). It is simple, inexpensive and rapid but it has several drawbacks such as significant performance variation with the operator (Molicotti, *et al*., 2014), low sensitivity, it misses about 30-35% of positive cases (Kashino, *et al*., 2008; WHO, 2011a). An estimated minimum 17% of new infections are acquired from others who tested smear-negative (Heifets and Cangelosi, 2009). Moreover, MDR strains cannot be detected by AFB smears and it cannot also differentiate MTB from non-tuberculous mycobacteria (NTM) as both of them have similar chording feature under microscopy, which can cause a misidentification problem (Saltini, 2006; Steingart, *et al*., 2007; Weyer, *et al*., 2011).

The performance of smear microscopy has been improved recently with different approaches. One of the suggested approache is concentration of sputum prior to examination

either by passive sedimentation or treatment with bleach or ammonium sulphate, followed by centrifugation (Parsons, *et al*., 2011), 11-26% improvement in sensitivity has been reported when the latter method was used (Bruchfeld, *et al*., 2000). The other performance enhancement approach is the use auramine/rhodamine fluorescent dyes for staining smears and the use of fluorescence microscopes for examination. In spite of the fact that a significant increase in cost due to expensive prices for the stain, microscopes and their bulbs, it providing considerable improvement in sensitivity, throughput, and speed of detection. Thus, the use of cheaper light-emitting diode (LED) microscopes can balance the cost and performance advantages of fluorescence microscopy. LED microscopy is more sensitive and equally specific, compared with either conventional light or fluorescent microscopy. The use of LED smear microscopy has been endorsed by the WHO, and several commercial products for TB diagnosis are now available (Parsons, *et al*., 2011; Drobniewski, *et al*., 2012; Palomino, 2012).

2.7.2. Culture Methods

Culture remains the gold standard method for the diagnosis of MTB infection in clinical specimens and it is the current gold standard for the isolation of bacillus. The test can detect as few as 10-100 viable bacilli/mL of specimen (Martin and Palomino, 2009). However, it demands considerable infrastructure and bio-safety requirements, and also it is extremely time consuming usually takes 3-4 weeks or longer (Mitchison, 2005; Forbes, *et al*., 2007; Palomino, 2012). Solid and broth media are used for the initial isolation of organisms from specimens.

The solid media includes the conventional egg-based Löwenstein-Jensen (LJ), the agar-based Middlebrook 7H10, and the TK medium, and Middlebrook 7H9 is the liquid culture media (Mitchison, 2005; Forbes, *et al*., 2007; Weyer, *et al*., 2011; Palomino, 2012; Molicotti, *et al*., 2014). In spite of limited works, the TK medium has comparable sensitivity to conventional LJ, and has the advantage of having a colorimetric indicator incorporated within it, which shows the mycobacterium growth even prior to colony visualization. Besides, the metabolic activity of the mycobacterium will produce a change in the original color of the medium (red) that allows differentiating mycobacterial growth (yellow) from

growth of contaminants (green) (Baylan *et al*., 2004; Forbes, *et al*., 2007; Migliori, *et al*., 2008; El Khechine and Drancourt, 2011; Parrish and Carroll, 2011).

The liquid culture media is Middlebrook 7H9 and requires an automatized incubator allowing incubation with an atmosphere containing 5% to 10% of CO_2 and a regular and automatized detection of bacterial growth (Molicotti, *et al*., 2014). The development of liquid media such as the Middlebrook 7H9 has offered speed (10-14 days) and higher mycobacterial recovery rates in cultures. According to 2011 world health organization report, superior sensitivity is achieved and case detection yield can be up to 10% higher than solid media. However, they are more liable to contamination and expensive, in addition to the need for stricter biosafety and staff training measures due to risk of aerosol formation (Forbes, *et al*., 2007; El Khechine and Drancourt, 2011; Parrish and Carroll, 2011; McNerney, *et al*., 2012; Palomino, 2012). It is known that a contamination rate under 10% in liquid culture is considered as acceptable. Hence, it is recommended to use in low and middle-income regions for the reason that the overall performance merit of culture using liquid media (Parsons, *et al*., 2011).

Before planting on/in culture medium (solid/liquid media), sputum sample is decontaminated by NALC-NaOH (2%) treatment. NaOH kills normal flora in the sputum and liquefied using mucolytic agent NALC (N-acetyl-L-cysteine). The treatment continues with centrifugation for concentration of bacilli. However, overtreatment of the sputum using this approach is lethal to *mycobacterium*. For instance, one third of the *mycobacterium* in the specimen would be killed if using excessive decontaminant reagent volume (>1:1 ratio) with respect to the sample volume (Forbes, *et al*., 2007; Parrish and Carroll, 2011; Parsons, *et al*., 2011). The bacilli can also be killed abundantly if time contact is beyond optimal (>15min).

Specific identification of *M. tuberculosis* is possible using conventional culture methods with subsequent biochemical properties. MTB has the capacity to reduce niacin and nitrate but yields a negative result with catalase, tellurite, and thiophene-2-carboxylic acid hydrazide (TCH) tests. Besides, MTB can be differentiated from NTM using media that contains para-nitrobenzoic acid (PNB); this inhibits MTB growth (Forbes, *et al*., 2007; Nepali, *et al*., 2010).

2.7.3. Molecular Diagnostic Methods

A molecular (genotypic) method is aimed at nucleic acid composition of the *M. tuberculosis* complex. One of the commonest targets for bacterial identification including *mycobacterium* is the highly conserved 16S ribosomal ribonucleic acid (rRNA) gene (stable RNA genes). The genes coding for it are referred to as 16S rDNA and is used in reconstructing phylogenies, because it often contains specific sequences that doesn't change overtime (slow rates of evolution of this region) and is present in the cells and media in high quantity/large enough (~1,500 bp). Sequencing of the 16S rDNA remains the reference method for *mycobacterium* identification, even though other options include restriction fragment length polymorphism (RFLP) analysis of amplified 16S rDNA regions or probe hybridization (Leao, *et al*., 2004; Chakravorty, *et al*., 2007; Neonakis, *et al*., 2008). A minimum of 94.3% similarity between mycobacterial species is found in the 16S rDNA (Smith, *et al*., 2009).

Molecular detection of MTB continues to change the landscape of TB diagnostics for shortening the time of detection to few hours as conventional culture takes several weeks to yield results due to the growth nature of *mycobacterium* (slow grower) though rapid detection of MTB is possible with the introduction of liquid culture-based techniques. With the aim of obtaining faster results and earlier diagnosis of tuberculosis, several molecular detection methods were introduced and have been evaluated in numerous studies. MTB-specific nucleic acid amplification tests (NAATs) are the most frequently used molecular tests for laboratory diagnosis of tuberculosis (Sperhacke, *et al*., 2004). The majority of molecular techniques are amplification-based e.g. conventional and real-time PCR, strand displacement amplification (SDA), followed by electrophoresis detection or probe hybridization. Isothermal amplification methods like loop-mediated isothermal amplification (LAMP) are also available (Parsons, *et al*., 2011). Despite molecular assays can shorten detection time to a few hours, their routine use is not always deem practical particularly in low-income regions. This is due to the high cost of equipment set up and maintenance and, requirement for highly trained personnel (Saltini, 2006; Palomino, 2009).

2.7.4. Others TB diagnostic tests

The tuberculin skin test (TST) and interferon gamma release assay (IGRA) are the major TB diagnostic tests for screening latent tuberculosis infection (LTBI). LTBI is a condition in which a person is infected with MTB, but does not have any signs or symptoms consistent with active TB disease. Tuberculin skin test (TST) also called as the Mantoux test is one of the oldest (over 100 years ago) and most commonly used test that involves intradermal injection of TB purified protein derivatives (PPD) and then examining the area of injection after 2-3 days for swelling. TST detects the classic delayed hypersensitivity reaction (cell-mediated immune reaction) to the infection, and the intradermal reaction may be indicative of a positive PPD reaction (5mm diameter in infants or immunocompromised subjects and a 10 mm diameter in adults) or a negative response. The test has a low sensitivity; false negatives are observed in case of immune-compromised patients and those with recent active infection. False positive results can be observed in previously vaccinated (antigenic cross-reactivity with BCG vaccine) or exposed individuals, and cross reaction with other mycobacteria, as some antigenic components of PPD are conserved among mycobacteria (Pai, *et al*., 2006; Knechel, 2009; Lawn and Zumla, 2011; McNerney, *et al*., 2012), all are responsible for its low specificity.

Because of the difficulties with the tuberculin test interpretation, the interferon-gamma assay test was developed. The IGRA is a serum diagnostic method for MTB infection based on detection of or measures the interferon-gamma (IFN-γ) release by T-cells in response to the certain MTB antigens. IGRAs have verified superior specificity to TST and similar sensitivity (Pai, *et al*., 2006; Gordon, *et al*., 2009; Fan, *et al*., 2012). However, clinical performance of IGRAs is inadequate for detection of extra pulmonary TB. Following demonstration of significantly superior performance to TST, IGRAs are the most chosen and currently available methodology for accurate detection of latent TB infection (Pai, *et al*., 2006; Knechel, 2009; Cattamanchi, *et al*., 2011; Lawn and Zumla, 2011; Palomino, 2012; Parrish and Carroll, 2011; Fan, *et al*., 2012).

Furthermore, several immunological assays have been conducted in attempt to provide fast, direct, non-invasive diagnosis of TB by detection of relative antigens in urine. The

advantage of detection of TB antigens is that it can distinguish between active and latent TB infections as antigens are generally linked with actively replicating pathogens. It helps also to avoid false results due to modulations of the host immune response as in the case with HIV/AIDS patients when testing for antibodies. Despite limited performance date, lipoarabinomannan urine testing is currently detected using enzyme-linked immunosorbent assay (ELISA) and is particularly sensitive in HIV-positive patients and in smear-positive patients (Daley, *et al*., 2009; Mutetwa, *et al*., 2009). In addition to lipoarabinomannan, several other urine antigens are being investigated as markers of active pulmonary TB infection. These consist of MTB ornithin carboamyltransferase, phosphoadenosin phosphosulphate reductase, homoserine O-acetyltransferase, and MoA-related protein (Kashino, *et al*., 2008; Napolitano, *et al*., 2008). Sero-diagnostic test are not recommended by WHO due to inconsistent and imprecise sensitivity and specificity (WHO 2011).

2.8. Drug Susceptibility Test (DST)

Drug resistant TB strains cannot be detected by the current most commonly used detection method of active TB, smear microscopy; instead antimicrobial susceptibility testing is required (WHO, 2011). Unfortunately, DST is not carried out as a routine step in most low resource high TB burden countries rather it is usually done when the patient fail to respond to standard therapy, this mean that DST would be performed anywhere from 2 to 8 months after start of treatment (Heifets and Cangelosi, 2009). If drug resistance is present but not known, the patient may be receiving inadequate therapy and continue to be infectious and transmit MTB. In addition, for patients with adverse drug reactions to first line TB drugs, DST to first and second line agents are needed to change to effective therapy.

2.8.1. Conventional DST Methods

Conventional methods for detecting drug-resistance in *M. tuberculosis* are slow and labor-intensive due to the lengthy time required for culturing MTB. Generally, primary isolation of MTB is the first step before performing drug susceptibility testing (DST) and take 3-4 weeks. The laboratory diagnosis is further complicated by the fastidious growth of the tubercle bacillus. The traditionally accepted DST methods include the proportion method,

the absolute concentration method, the resistance ratio method and the radiometric BACTEC TB-460 system (Becton Dickinson, Sparks, MD) (Canetti *et al*., 1969; Kent and Kubica, 1985; Heifets and Cangelosi,1999). The most commonly used and the current gold standard proportion method on Löwenstein–Jensen (LJ) medium or Middlebrook agar requires a minimum of 3-4 weeks (long turn-around time) to produce results. On the other hand, the radiometric BACTEC TB-460 system using an enriched liquid medium shorten the turnaround time (TAT), DST to 1-2 weeks, but the fact that it relies on radioactive materials, significantly more expensive and inconvenience for its wider application especially in low-resource countries (Ang, *et al*., 2010).

2.8.1.1. Phenotypic DST methods

The phenotypic methods are depends on detection of MTB growth in the presence or absence of anti-TB dugs. DST can be performed directly on clinical specimen, or indirectly by testing on pure isolates cultured from clinical specimens. Direct testing is produce rapid result via saving the time needed (3-4 weeks) to isolate a pure colony from sputum and several assays can be used to perform it. However, DST is generally carried out indirectly so as to avoid about 15% of failed testes attributed to problems and interference from contaminating bacteria or NTMs in sputum, despite it has long TAT. As compared to genotypic tools, phenotypic methods are receiving renewed attention because in general they are simple to perform, allow for several drugs being tested simultaneously, and in principle they are applicable to all drugs (Heifets and Cangelosi, 2011; Parrish and Carroll, 2011).

The proportion method (solid-culture based)

It is a popular one, about 50 years since its development, and remains a gold standard for DST. It can be performed using different solid media including the most popular LJ, and Middle brook 7H10 agar. It starts with taking representative colonies, and the bacterial suspension is adjusted with sterile distilled water to a turbidity matching a McFarland standard No1 (approximately equivalent to $3x10^8$ CFU/ml). Then, two serial dilutions (10^{-2} and 10^{-4}) made from the original suspension are used for inoculation. Finally, 100µL (0.1ml) of the bacterial suspension are inoculated in both drug-containing media and drug-free media, test media containing the drug INH, RMP, SM, EMB are inoculated with dilutions

10^{-2} while two growth control (drug-free) media are inoculated with 10^{-2} and 10^{-4} (to get countable colony) and incubated at 37 ºC. The first reading of drug susceptibility test results is done at 4 weeks (28 days) of incubation, and the number of colonies in each tube is counted. Resistance is expressed as the percentage of colonies on drug-containing media with recommended critical concentration in comparison to the growth on drug-free media. If the proportion exceeds 1% for all first line drugs, the isolate is designated resistant. In order to designate an isolate as susceptible, incubation must be extended and on the 42^{nd} day (6weeks) if the proportion does not exceed 1%, the isolate is considered susceptible (Hans, *et al*., 1998; O'Grady, *et al*., 2011). The test is repeated instead of "borderline resistance" (about 1% growth on drug containing medium).

Liquid culture-based methods

Several liquid culture-based methods for cultivation and DST of *M. tuberculosis* have been introduced during the last years. Some of them are simple manual methods while others rely on more sophisticated automated systems. The BACTEC MGIT 960 system (Becton Dickinson, Sparks, MD) is the only one recommended by the WHO for surveillance of drug resistance (first-line drugs) (Van Deun, *et al*., 2010). This MGIT960 system performs incubation and reading of the tubes continuously inside the machine using a predefined algorithm to interpret the fluorescent signal and giving the results as positive or negative (Tortoli, *et al*., 1999). The automated step in the MGIT 960 is the reading of the tubes, while all other preparatory steps are manual. Consequently, the main advantage is the reduction in turnaround time to a few days though the overall workload is not significantly less than the regular manual method. Besides, the reading throughput is substantially higher (O'Grady, *et al*., 2011; Van Deun, *et al*., 2010). However, it requires expensive laboratory infrastructure, high bio-safety precautions and specialized staff. Thus, it is not implemented widely though WHO recommends for rapid detection of MDR-TB (Raviglione, *et al*., 2012).

2.8.1.2. Genotypic DST methods

Unlike phenotypic DST, it is aimed to detect gene mutations known to be associated with drug resistance. The molecular detection of resistance-conferring gene mutations associated with drug resistance has been developed recently. A number of molecular assays have been

designed to detect the presence of *M. tuberculosis* and to detect resistance to isoniazid and/or rifampin. The common utilized tests include real-time PCR with hybridization probes, probe hybridization, while DNA sequencing remains the reference standard. For example, DNA probe and DNA sequencing of MTB gene such as catalase (*katG*) or RNA polymerase (*rpoB*) and mutations in these genes are associated with resistance to isoniazid and rifampicin respectively (Palomino, 2009; Drobniewski *et al*., 2012).

The genotypic methods provide remarkable advantages over phenotypic one that molecular tests are very rapid (few hours), highly sensitive and specific, however, this comes at a high cost, requires high expertise thus it is with practical reservations especially in low-resource countries (Palomino, 2009; Drobniewski *et al*., 2012). In addition, it may not differentiate active infection as DNA from a dead organism during antibiotic treatment can be detected and amplified by PCR (Dinnes, *et al*., 2007), and also molecular methods do not reveal the proportion of the resistant bacteria in the sample (Drobniewski *et al*., 2012). Moreover, a key concern is the fact that the data on resistance-inducing mutations aren't comprehensive, and not all mutations are translated into phenotypic resistance, thus absence of a mutation does not necessarily mean the isolate is phenotypically susceptible to the drug. This is also responsible for false negative results. It is a common finding that isolates demonstrate phenotypic drug-resistance while molecular testing shows they lack known resistance-conferring mutations (Moore and Shah, 2011). Drug resistance detection is dependent on the particular mutation and required between 65% and 100% mutant DNA to be present in the sample for 95% certainty of resistance detection (Blakemore *et al*., 2010).

GeneXpert MTB/RIF assay

It is an automated, real time nucleic acid amplification technology and one of the commonly used molecular testes for rapid diagnosis of TB and drug resistant TB. The assay is used for diagnosis of TB and MDR-TB by concurrently detecting *M. tuberculosis* and rifampicin resistance conferring mutations in a closed system, suitable for use outside conventional laboratory settings in 2hrs, directly from sputum samples. It also detects DNA in other sample types (i.e. pleural, lymph node aspirate or tissue, CSF, gastric fluid and tissue other than lymph node) (WHO, 2011; Drobniewski, *et al*., 2012).

The assay provides accurate results for RMP resistance (a proxy marker for MDR-TB) and allows rapid initiation of MDR-TB treatment, pending conventional culture and DST results. Besides, can overcome many of the operational difficulties, and thus it is recommended since 2010 as a first line/initial diagnostic test in endemic countries when rapid results are crucial for TB diagnosis in children, HIV infected patients or for appropriate management of MDR-TB cases (WHO, 2011; WHO, 2015). Although WHO advised to replace smear microscopy with this rapid assay (WHO, 2011), the cost of purchasing and maintenance of the test platform and cost of each kit ($25-30 per test) (Molicotti, *et al*., 2014), is prohibitive for developing nations like Ethiopia though nations are used the subsidized cost ($ 9.95).

Studies on performance of GeneXpert assay are diverse and mostly its performance has been evaluated against existing reference standards, microscopy and culture, for TB testing and phenotypic DST for rifampicin resistance testing. Like many other molecular assays, it works well only with smear-positive specimens; it has higher sensitivity for TB detection in smear positive than smear-negative patients, which is most problematic in sub-Saharan Africa, where many HIV-infected patients with TB are smear-negative. Nevertheless, this test may be valuable as an add-on test in smear negative patients (Helb, *et al*., 2010; WHO, 2014). According to 2014 WHO report, a single GeneXpert test directly on sputum detected 99% of smear-positive patient's but only 80% sensitive for the identification of smear-negative culture positives (WHO, 2014). A single direct GeneXpert assay was also shown to be 91% sensitive for the identification of culture-positive isolates (as compared to 59.5% smear microscopy in culture positive cases). The test had a reported specificity of 99.2% (Boehme, *et al*., 2010). Rifampicin resistance was detected with 95.1% sensitivity and 98.4% specificity. Moreover, the mean time required for GeneXpert in diagnosis of TB and RMP resistance is less than 1 day, whereas, 17 days for liquid culture and more than 30 days for solid culture. It is very rapid (<1day) compared with an average of 75 days for phenotypic DST in detection of rifampicin resistance (WHO, 2014).

2.8.2. Emerging DST Methods

Globally, about 60% of TB patients living in remote and peripheral healthcare centers where lacking the resources and infrastructure for advanced and molecular diagnostic systems

(Batz, *et al*., 2012). To satisfy the demands of resource-limited settings, considerable effort has been invested and several novel assays have emerged to circumvent the shortcomings of high cost and long turnaround time (TAT) of conventional culture DST, some of which have already gained WHO endorsement, while others demands sufficient evidences for approvals. These alternative phenotypic methods are designed to improve the existing methods, and are simple, rapid and affordable. Some of these are the microscopic observation broth-drug susceptibility assay (MODS), thin-layer agar (TLA) and MDR-/XDR-TB color plate test. MODS has been endorsed by the WHO, however, evidences are considered to be insufficient to recommend the use of the TLA assay and TLA based MDR-/XDR-TB color plate test for rapid screening of MDR-TB patients, despite this assay is a promising diagnostic technique and further research is encouraged (Minion, *et al*., 2010).

2.8.2.1. Microscopic observation broth-drug susceptibility assay (MODS)

MODS are a rapid liquid culture-based method, relied on the observation of the characteristic cord formation of *M. tuberculosis* when growing in a liquid medium (Caviedes *et al*., 2000). The test was first provided by Caviedes and his co-workers (2000) for concurrent detection and DST of *M. tuberculosis* in sputum samples, and showed the validity of direct detection of resistance to RMP and INH with sensitivity of 92% and specificity of 93%.

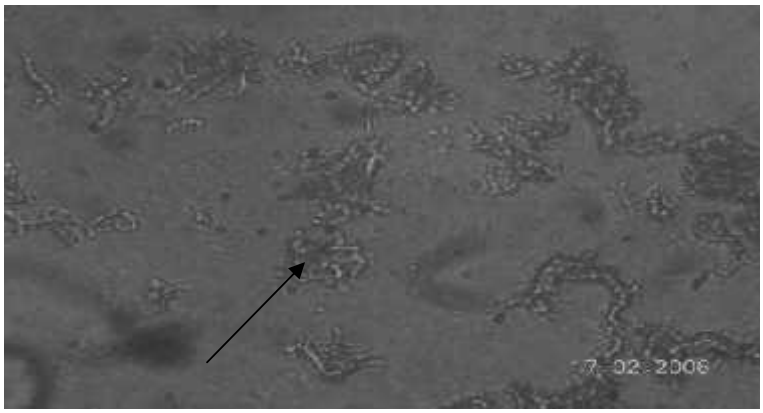

Figure 2: Characteristic Cord-like Appearance of MTB under the Microscope (inverted) in MODS (20x). Reprinted from (Shiferaw, *et al*., 2007)

To perform DST, decontaminated sputum samples (or isolate bacterial suspensions) are inoculated into supplemented Middlebrook 7H9 broth with and without drugs (INH and RMP at critical concentrations of 0.4 and 1µg/mL, respectively), in 24-well microtiter plates. The plates are sealed in zip-lock bags and incubated for 7 days at 37 ºC in a CO_2 incubator. Then, reading of the plates is performed with an inverted microscope at 40× magnification to identify the typical cord formation of *M. tuberculosis*. Growth in the drug-containing wells and in growth control (drug-free well) is interpreted as resistance. If no characteristic MTB cords are detected in the drug-free wells, the reading is repeated every other day up to 21 days, after which the sample is declared negative (Moore, *et al*., 2006).

The recent meta-analysis (9 studies) found MODS to have pooled sensitivities of 98% (95%CI 94.5-99.3) and pooled specificity of 99.4% (95.7-99.9) for detection of RMP with 1µg/mL cutoff. Besides, INH resistance with 0.1µg/mL cutoff, pooled sensitivity was 97.7% (95%CI 94.4-99.1) and 95.8% pooled specificity (88.1-98.6), but with a 0.4µg/mL cutoff, sensitivity decreased to 90.0% (84.5-93.7) and specificity increased to 98.6% (96.9-99.4), with an average TAT of 9.9 days (95%CI 4.4-15.8) (Minion, *et al*., 2010). MODS have also been performed in Ethiopian setting, and promising results were reported for diagnosis of MDR-TB. Shiferaw and his colleagues (2007, 2008) reported sensitivity above 90% and almost 100% specificity, and 98% accuracy of MODS for detecting MDR-TB. Both studies reported that concurrent culture detection and DST results obtained in a median of 9 days.

Although the MODS assay is accurate to detect MTB resistance, especially to RMP using short TAT, inexpensive and simple, it is best suited for larger laboratories that already have an existing infrastructure for TB diagnostic testing due to the requirement of an inverted microscope to read the plates and bio-safety concerns related to the use of liquid medium in 24-well plates. Furthermore, this test method is still labor intensive and technically demanding, particularly in inoculation, where contamination risk is greatest,7.4% (Minion, *et al*., 2010; Moore and Shah, 2011; O'Grady, *et al*., 2011). Care should also be taken when using MODS for direct testing in regions with high NTMs prevalence as the evidence is still lacking with respect to the assays ability to differentiate them from MTB, particularly *M. chelonae*. *M. tuberculosis* complex is distinguished from other NTM that exhibit cording,

such as *M. chelonae*, by the more rapid growth of the latter despite some studies have been reported more-rapid growth of *M. tuberculosis*, which could potentially lead to misidentification of an isolate as *M. chelonae*. Thus, the inclusion of PNB-containing well which would specifically inhibit MTB growth is proposed as a solution (Michael and Wilson, 2010; Minion, *et al*., 2010; Van Deun, *et al*., 2010).

2.8.2.2. Thin-layer agar (TLA) method

The thin layer agar (TLA) method is a low technology technique, also known as the micro-colony method, but unlike MODS assay it is performed on Middlebrook 7H11 agar (solid media). It was originally described for the rapid detection of mycobacterial growth and later applied as a rapid DST method (Welch *et al*., 1993; Mejia *et al*., 1999). This method uses quadrant agar plates with drug-free and drug-containing quadrants with critical concentrations. The quadrants are inoculated with the processed sputum and incubated at 37 ^{o}C with 5% CO_2. The plate is then examined under a light microscope with 10x magnifications for cording characteristic of the colony growth (Robledo, *et al*., 2008; Martin, *et al*., 2009; Van Deun, *et al*., 2010). One version of TLA includes PNB in one of the quadrants to guarantee growth is MTB complex rather than NTM **(**Robledo, *et al*., 2008). The recent meta-analysis identified 3 assessment studies of TLA, all evaluations showed 100% concordance with the reference proportion method for INH, RMP and MDR-TB detection, with the mean TAT of 11.1days (10.1-12.0). Also, a thorough cost analysis is not yet available (Minion, *et al*., 2010). Hence, further studies are necessary for endorsement of its worldwide use (Drobniewski, *et al*., 2012).

2.8.2.3. The MDR-/pre-XDR-TB color plate test (color plate test)

This assay is based on TLA technology, for rapid concurrent culture and direct DST method on a single agar plate. This particular TLA assay is based on color changes of the colony on the 4 quadrants of the plate, with 1 quadrant for detection of growth and the other 3 quadrants for DST, each quadrant for INH, RMP, and ciprofloxacin (CFX) (Minion, *et al*., 2010). The media is incorporated with an oxidation-reduction indicator: 2, 3 diphenyl-5-(2-thienyl) tetrazolium chloride (STC) (Lee *et al*., 2006; Lee *et al*., 2007), and four colour-coded quadrants to simplify the reading of the DST results. The addition of STC to the

media results in the growth of red MTB colonies, which makes them observable to the naked eye at early stages of growth or micro-colonies. This makes daily faster checking of the plate, as there is no need to check every plate under the microscope, although microscope is still needed to confirm a positive diagnosis of MTB based on chording colony morphology (Martin, *et al*., 2009). Growth of MTB on drug-free media indicates a positive culture, whereas growth of MTB on both drug-free (>1%) and drug-containing media with critical concentration indicates resistance.

There is only one published data on the performance characteristics of the MDR/XDR-TB color test using indirect inoculates. The preliminary work has demonstrated that the color test has been showed an excellent performance for the diagnosis of MDR-TB and pre-XDR-TB. Toit and colleagues (2012) conducted the color plate test for drug susceptibility testing of MTB and they found better performance having an average time to positivity 13 days. Moreover, the color test detected drug resistance with 98% sensitivity for INH, RMP and CFX and 99% for MDR-TB; 100% specificity for INH, 91% and 90% specificities for RMP and CFX respectively. Agreement between the color test and BACTEC MGIT 960 were respectively 98%, 96%, 94% and 97%. Despite limited data, they indicated that the color test is useful especially in low resource settings with prevalent drug resistance rate, as it is economical, simple technique, and rapid alternatives to conventional methods for direct testing MTB strains for drug resistance (Toit, *et al*., 2012).

3. OBJECTIVE OF THE STUDY

3.1. General Objective

The aim of the current study was to evaluate the diagnostic performance of color plate test as a rapid test for the concurrent diagnosis of pulmonary TB and multidrug-resistant TB compared to the gold standard culture-based drug susceptibility test, and the molecular test GeneXpert MTB/RIF assay in morning sputum specimen at St. Peter's and ALERT hospitals, Addis Ababa, Ethiopia.

3.2. Specific Objectives

- To determine the sensitivity, specificity, positive and negative predictive values of the color plate test for concurrent detection of MTB and multidrug-resistant TB;
- To establish the percent agreement between the color plate test and the gold standard LJ culture and indirect proportional drug susceptibility test;
- To determine the percent agreement between the color plate test and GeneXpert MTB/RIF assay
- To estimate the average time to positivity (turnaround time) of the color plate test

4. MATERIALS AND METHODS

4.1. Study Area

The study was conducted at ALERT hospital and St. Peter's TB referral hospitals, Addis Ababa, Ethiopia. The hospitals located in the capital city of the country, are government-owned, referral hospitals under the federal ministry of health (FMOH). Both hospitals provide referral services for the diagnosis and treatment of TB and MDR-TB patients from different regional states of the country. The MDR-TB inpatient services are given in MDR-TB care centers for at least a month. ALERT hospital has 12 and 24 beds for TB and MDR-TB inpatients respectively while St. Peter's hospital has provided 24 and 36 wards for management of TB and MDR-TB inpatients respectively. Currently, both hospitals provide extra medical services in addition to TB diagnosis and TB care management (ALERT, 2014; St. Peter, 2015).

4.2. Study Period

The study was conducted from March 2016 to February 2017.

4.3. Study Design

A cross-sectional, hospital-based diagnostic test validation study was conducted to evaluate the performance of the new rapid color plate test for simultaneous detection of *Mycobacterium tuberculosis* (MTB) and MDR-TB. Non-probability, a convenient sample of patients was recruited that considered at high risk for drug-resistant TB. MDR-TB suspected patients were defined by either history of previous treatment (previously treated for >1 month) and/or had contact with an individual with known or suspected drug resistant TB, according to world health organization criteria (WHO, 2010). According to the national TB and MDR-TB diagnostic algorithm, patients who are at risk of MDR-TB are prioritized for testing with GeneXpert assay (FMOH/EPHI, 2014). Moreover, the two hospitals were purposively selected based on increased number of MDR-TB patient flow to the health facilities (FMOH, 2015).

4.4. Source of Population

All patients suspected of possible pulmonary TB who were visiting ALERT hospital and St. Peter's TB referral hospital, Addis Ababa, Ethiopia during the study period were the source of population.

4.5. Study Population

The study population was patients with pulmonary tuberculosis enrolled in the two hospitals and those were suspected to have MDR-TB and requested to be examined by GeneXpert assay during the study period.

4.6. Inclusion and Exclusion Criteria

Patients were eligible for inclusion in the study if they were suspected to have multidrug-resistance TB (new or previously treated) attended at both hospitals. Patients who couldn't produce enough sputum sample (<3ml per tube) for laboratory analysis, and extra pulmonary tuberculosis were excluded from the study. Moreover, patients who were receiving tuberculosis treatment/re-treatment regimen for >2 weeks period during sample collection were also excluded from the study.

4.7. Study Variables

The dependent variables included the test status namely sensitivity, specificity, positive and negative predictive values whereas time to test positivity in color plate test and culture and DST were the independent variables.

4.8. Sample Size Determination and Sampling Technique

4.8.1. Sample Size Determination

The sample size required for this study was calculated using the formula estimating sample sizes for evaluating sensitivity and specificity of diagnostics test (Buderer, 1996; Jones *et al*., 2003). To determine the sample size, 95% anticipated sensitivity and specificity with absolute precision of less than 5% at 95% confidence interval (CI) was used. The prevalence of MDR-TB of 46.3% was used from previous study in the TB referral hospital

of Ethiopia (Abate *et al*., 2012). With this given data, 145 study participants were required to obtain sensitivity of 95% (precision of 5% and alpha error of 0.05) and to attain a specificity of 95%, 130 study subjects were required. By taking the larger one (145) and considering 10% contingency, the sample size was calculated and 154 study participants were recruited in the study. The Buderer's formula:

$$\text{Sample size } (n) \text{ based on sensitivity} = \frac{Z^2_{1-\alpha/2} \times S_N \times (1 - S_N)}{L^2 \times Prevalence}, \text{ and}$$

$$\text{sample size } (n) \text{ based on specificity} = \frac{Z^2_{1-\alpha/2} \times S_P \times (1 - S_P)}{L^2 \times (1 - Prevalence)},$$

where n = required sample size,
S_N = anticipated sensitivity,
S_p = anticipated specificity,
α = size of the critical region ($1 - \alpha$ is the confidence level),
$z_{1-\alpha/2}$ = standard normal deviate corresponding to the specified size of the critical region (α), and
L = absolute precision desired on either side (half-width of the confidence interval) of sensitivity or specificity.

4.8.2. Sampling Technique

Non-probability, convenient sampling technique was used to enroll patients. All patients who consented to participate in the study submitted an additional early morning sputum sample. For the present study, paired morning expectorated sputum samples were obtained on the same day from the same patients by using 50 ml falcon tube and the minimum acceptable volume of sputum was 3ml in each collection tube. One of the samples that used for diagnosis purpose was tested directly by GeneXpert assay at each respective hospital laboratories. Whereas, the other sputa were refrigerated (4-8^{o}C) to inhibit growth of unwanted micro-organisms and transported using on ice in ice-box to Armauer Hansen Research Institute (AHRI) TB laboratory once a week where the new color plate test and the gold standard test (LJ culture and DST) were conducted. Transportation method was in accordance with the national standards for transport of biological substance, category B

(UN 3373) using triple packing materials (falcon tube +zip lock bag+ ice box) (Appendix 3).Then, it was stored at 20°C for two weeks period before started the sample processes.

4.9. Demographic and Clinical Data

After having a signed informed consent, demographic data and clinical history of the study participants were collected from TB registration book of the hospitals using standardized data collection sheet (Appendix 5).

4.10. Laboratory Methods

In the current study, three laboratory tests were included namely (1) the color plate test (new test) (2) the conventional LJ culture and indirect proportional DST and (3) GeneXpert MTB/RIF assays (molecular test).

4.10.1. Color plate test

It is a non-commercial direct method for simultaneous culture and DST of *M. tuberculosis* (MTB) on a single agar plate. This particular color plate assay is based on color changes of the colony in the 4 color-coded quadrants of the plate to simplify the reading of DST results, with 1 quadrant for detection of growth (clear color) and the other 3 quadrants for DST, each quadrant for green-INH, yellow-RMP, and blue-PZA (figure 3) Growth of MTB on drug-free media indicates a positive culture while growth of MTB on both drug free and drug-containing media indicates resistance.

4.10.1.1. Preparation of color plate test

It was performed according to MDR/XDR-TB color plate test standard operating procedure version 7, 20/5/2010 (SOP, 2010). Middlebrook 7H11 agar was used to prepare the color test plates. The medium was supplemented with oleic acid albumin dextrose complex (OADC) supplement (10%), Mycobacteria Selectatab (Kirchner, Mast Laboratories Ltd, Merseyside, UK) and 50µg/ml of STC. Quadrant petri-dishes were prepared with approximately 4ml of agar per quadrant, one with 0.2µl/ml INH and green food coloring, one with 1µl/ml RMP and yellow food coloring, and one with 100µl/ml PZA and blue food coloring. All food coloring were filtered and sterilized using a 0.22µm pore size

filterer. The remaining quadrant contained no drug concentration and acted as the control for growth detection. The plates were screened for contamination every time the medium was prepared. Internal quality control was performed with a susceptible (H37Rv) and resistant strain (laboratory origin). For the present study, the color plate test was prepared at the Ohio State University medical laboratory, USA submitted to Ethiopia for the research purpose. Then, it was stored in cold room temperature (2-8^{0}C) for 2-4 weeks before the test was started.

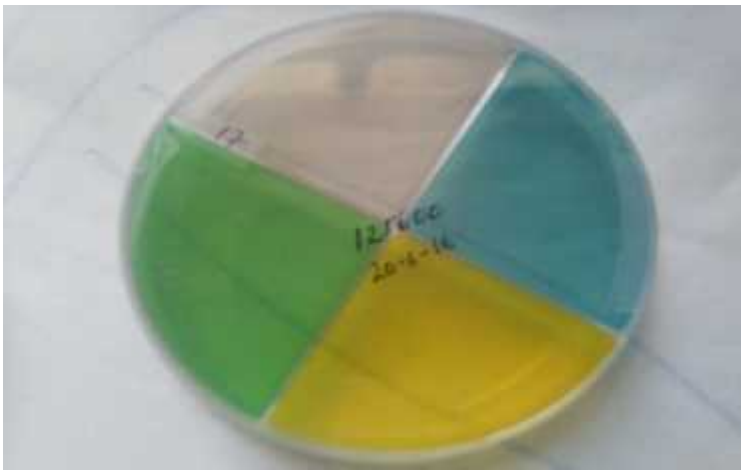

Figure 3: Color plate test photography: growth detection quadrant (clear, top), Isoniazid (green), Rifampicin (yellow) and Pyarazinamide (blue)

4.10.1.2. Preparation of disinfectant for sputum processing

It was prepared at AHRI TB laboratory based on MDR/XDR-TB color plate test standard operating procedure version 7, 20/5/2010 (SOP, 2010). The colour plate test disinfectant was used to decontaminate sputum samples before inoculation of sputum onto the culture medium. Unlike Petroff's method (NaOH), it uses a combination of four different chemicals namely trisodium phosphate (200g), ammonium sulphate (5g), magnesium sulphate (0.5g), and ferric ammonium citrate (0.25g), and all were dissolved in 1000ml of sterile distilled water by heating and stirring with a magnetic stirrer for 2 hours. Filtered twice by applying vacuum, 1st filtration to remove large particles through 2 filters (Whatman 3M) and the 2nd filtration with GELMAN filter (0.22µm pore size). Then, autoclaved at 121°C for 15 minutes; removed from the autoclave and cooled at room temperature. Penicillin (100 IU/ml) was added and mixed for 10 seconds. Finally, 1 ml of red food colouring was added

that used to reduce pipetting errors by providing clear observation which quadrants of the plate have had the sputum-disinfectant mixture added. The disinfectant is stable for at least 30 days at room temperature after use.

4.10.1.3. Carrying out the color culture plate test

a. Sputum decontamination and inoculation

Sputum samples were treated with the decontaminant solution (disinfectant) of the color test. The disinfectant was added using 2:1 ratio of the sputum samples and sputum-disinfectant mixture was maintained for 1:30 to 1:45 hours at room temperature. Then, using a disposable 1ml sterile plastic pipette mixed the sputum-disinfectant contents by pipetting up and down 3or 4 times and the last time, approximately half filled the disposable pipette with the sputum-disinfectant mixture (as with all sample processing, this procedure was done within the biosafety cabinet). Then after, open a new colour test plate and inoculated using two drops per quadrant in a clockwise order and close the lid of plates. After labelling of the plates with sample number and date of inoculation, the plates were double-sealed for safety reason (with parafilm and placed in zip-lock plastic bag) then, plates were transferred to the incubator for incubating at 37°C (SOP, 2010).

b. Plate Reading and Interpretation

The plates were read visually three times weekly until at least 50 colonies appeared in the drug-free control quadrant, for a maximum of 6 weeks period. Growth was detected as red colony with positive samples. A small (<3mm) point of red colony was examined for the characteristic cording cauliflower-like morphology of mycobacterial growth by using a conventional light microscope (magnification 40×) without removing the plastic bag and without opening the petri-dish. The plates also observed to check for contamination and any observations of growth or contamination were registered in a record sheet. Thus, the result from the detection quadrant was interpreted as positive for MBTC growth, contaminated and negative after 6 weeks of incubation. Furthermore, when mycobacterial colony growth of >50-500 colonies were first detected in the detection quadrant, the drug-containing quadrants were also examined concurrently. A strain was classified as resistant to a drug

when >1% of colonies appeared in a drug quadrant compared to the control quadrant (drug-free quadrant) and categorized as susceptible if the number of colonies is <1% and/or no mycobacterial growth in drug-containing quadrants (SOP, 2010). Moreover, the median time to positivity, the time to detection elapsed from the date of inoculation to the earliest date of visible colonies (50 colonies) was also recorded.

4.10.2. Conventional mycobacterium culture and DST

At AHRI TB laboratory, the other sputum sample was processed. After mixing manually, the sputum specimens were homogenized (pipeting for 1min and vortexed for 2min) to assure uniform distribution of bacilli and, then the sputa were divided into two aliquot equally (in consistency, color, appearance). The first aliquot was subjected to color plate test and the second one was cultured on solid LJ media. All MTB isolates from LJ media were harvested and stored in a deep freezer at -80^{0}C for 3 months till tested for the reference drug resistance profiles or patterns.

4.10.2.1. Specimen processing and mycobacterium culture

Conventional LJ medium was prepared as described by Canetti, *et al*., (1963) and Hans, *et al.,* (1998). Every new batch of LJ media was controlled for sterility after 48 hours incubation and growth using reference strains of laboratory origin (Appendix 4).

The sputa specimens were decontaminated using the modified Petroff method, N-acetyl L-cysteine (NALC) -Sodium hydroxide (NaOH) decontamination method which is used routinely in Ethiopian national TB reference laboratory (Kent and Kubica 1985; EHNRI/EPHI, 2013). Briefly, an equal volume of sputum was added to NALC-NaOH, the mixture was vortexed for 1 min until homogenized and then left to stand for 15 min at room temperature, after which the specimens were neutralized using sterile phosphate-buffered saline (PBS, pH 6.8) and centrifuged at 3,000 rpm for 15 minutes. Then, decanting the supernatant and the pellet (sediment) in each tube was suspended again in sterile PBS to 2 ml and mixed well. This re-suspended pellet (100µl) was inoculated into Lowenstein- Jensen (LJ) slant tubes; each slant was properly labeled with the sample number and date of inoculation. Then after, the cultures were incubated at 37°C for 8 weeks; they were

inspected first after 48hrs and then weekly. If mycobacterium growth colony was confirmed on LJ media, a culture was considered as positive and if not recorded as negative. A culture was also recorded as contaminated if LJ media demonstrated contamination and contaminated samples were re-inoculated with stored samples (sediment) for one more time. The positive isolates were confirmed by combination of colony morphology and microscopic observation of acid fast bacilli using Ziehl-Neelsen (ZN) staining method. The median time to positivity, the time to detection elapsed from the date of inoculation to the earliest date of visible colonies was recorded. All positive cultures were kept at-80°C with freezing medium as a backup for further anti-TB drug susceptibility testing using indirect proportion method (IPM) and mycobacterial growth indicator tube (MIGIT) for PZA resistance test.

4.10.2.2. DST using indirect Löwenstein–Jensen (LJ) proportional method

The reference culture-based DST was performed using LJ indirect proportional methods (LJ IPM) with primary isolates obtained from LJ media. First line anti-tubercular drugs namely isoniazid (INH), rifampicin (RMP), streptomycin, (SM) and ethambutol (EMB) were obtained as a powder from national TB reference laboratory (EPHI, Ethiopia). Each drug was prepared at a concentration of 10mg/ml in sterile distilled water with the exception of RMP, which was dissolved in di-methyl-formamide (DMF). Stock solutions were filter (0.45µm) sterilized. The drug media (LJ IPM) was performed according to established procedures on LJ medium with critical concentrations of 0.2µg/mL for INH, 40 µg/mL for RMP, 4.0 µg/mL for SM, and 2.0 µg/mL for EMB (Appendix 4).

Frozen isolates were cultured again on LJ media and the active phase of growth (2-4 weeks) of mycobacterium subjected to drug susceptibility test. Bacterial suspensions for DST were prepared by adding approximately 4mg(2/3 loopful 3mm internal diameter wire loop) of moist weight of a representative sample of mycobacterium colonies/mass in 200µl (0.2ml) of sterile distilled water in a bijou bottle with 4 to 5 glass beads, then vortexed for about 30 seconds to obtain a uniform solution. To obtain 1mg/ml suspension, 3.8 ml sterile distilled water was then added to the bottle and allowed to settle for about 30 minutes before softly aspirating the upper part into a fresh bijou bottle (S1 suspension). The S1 bacterial

suspension was adjusted with distilled water to a turbidity matching a McFarland No1, approximately equivalent to $3x10^8$ CFU/ml (appendix IV, C, 2.3). Then, S1 was further diluted 10-fold to obtain S2-S4. Test media containing INH, RMP, SM, and EMB were inoculated with 100µl (0.1ml) of S2 (10^{-2}) dilution while plain media were inoculated with both S2 (10^{-2}) and S4 (10^{-4}) dilutions. The first reading of DST results was done at 4weeks (28 days) of incubation and continued reading of susceptible results till 6 weeks (42 days). A strain was classified as susceptible to the particular drug only if the number of colonies that grew on the drug-containing medium is <1% of the number of colonies that grew on the drug free control tube and resistant if the number of colonies is >1% (Canetti, *et al*., 1963; Hans *et al*., 1998). Furthermore, the median time taken to DST result was registered.

4.10.3. GeneXpert MTB/RIF Assay

Of the paired sputa samples submitted by study participants, one sample that was used for diagnosis purpose directly tested by GeneXpert MTB/RIF assay at St. Peter's and ALERT hospital laboratories (routine-funded by local government), and internally validated.

It was performed as described by Cepheid GeneXpert MTB/RIF assay manual. Briefly, sputum samples were treated with sample reagent (SR) containing NaOH and isopropanol. The SR was added using a 2 to 1 ratio of the sputum sample, the closed specimen container was manually agitated twice (homogenized) during a 15-min incubation period at room temperature. Then, the treated sample is transferred into the cartridge, the cartridge is loaded into the GeneXpert machine and the automatic process completes the remaining assay steps. The results were recorded as the presence or absence of *M. tuberculosis* complex and the presence or absence of RMP resistance (Boehm, *et al*., 2010; Cepheid, 2011; FMOH/EPHI, 2014).

4.11. Data Management and Analysis

The demographic data and treatment history of the study participants were collected from TB registration book, entered to excel spreadsheet and analyzed by Statistical Package for Social Sciences (SPSS) version 20.0 (SPSS Inc., Chicago, IL, USA). Laboratory test results data for GeneXpert assay, gold standard LJ culture and DST, and color plate test were

collected on paper forms and subsequently entered into a secured electronic database for analysis. The results were interpreted in terms of sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV). The turnaround time and contamination rates of the color test were compared with the gold standard test. A kappa correlation statistical analysis was applied to test agreement between the DST result of the color plate test, the GeneXpert assay and the reference LJ culture and DST. In all analyses, $p<0.05$ was taken to indicate statistical significance.

4.12. Quality Assurance

To ensure the quality of data, sputum specimen and patient's profile data collection was conducted after the participant was informed the purpose of the study and consent obtained. Demographic data and treatment history were collected from the TB registration book by principal investigator and checked it on daily basis in order to update and maintain its accuracy and completeness. Each subject was given a study ID number and all the three tests were labeled accordingly. The qualities of laboratory works were assured by using well known instruments, known reference strain (positive and negative controls), reagents and chemicals from recognized companies on top of following latest and standard operating procedures (SOP) of the host laboratory which is in line with WHO guidelines. Reference strains of *M. tuberculosis,* H37Rv (susceptible) and known MDR strains (laboratory origin) were used for DST. Besides, prior to the actual work, pre-tests were conducted and reagents were checked for proper functioning and handled according to standard procedures. To avoid subjective interpretation of test results, all testes results were each interpreted blindly, with the readers being unaware of the results of the other test. Furthermore, an appropriate data collection procedures and analysis software's were implemented to make sure that the data was well organized.

4.13. Bio-safety Measures

The sputa specimen were collected in triple package and transported with cool box. All steps for preparation of LJ media, plain (no drug) and drug containing media, culturing and reading of the reference test results were done in the Biosafety class II cabinet (BSC-2), with the operator wearing N-95 mask, and it was carried out at P-3 TB conventional laboratory

equipped with negative pressure room. For the color plate reading and documentation, the plates were always maintained in the sealed zip lock bags, and the N-95 mask was always on when the plates were removed from the BSC to be examined under the microscope and photographed. Autoclave was utilized at 121°C for 20-30 minutes for disinfecting all society, the media and oven were utilized to clean and sterile all glass products at 200°C for 2 hours (Kent and Kubica, 1985; AHRI, 2012). Phenol 5% and ethanol 70% were utilized to clean and purify the seats and hood (Enarson, *et al.*, 1996; AHRI, 2012).

4.14. Ethical Consideration

Ethical clearance (DRERC/003/08, February 1, 2016) was obtained from the ethical and research review committee of Department of Microbiology, Immunology and Parasitology, College of Health Sciences, School of medicine, Addis Ababa University. The study also approved and obtained permission from the study sites, St. Peter's and ALERT TB referral hospitals, Addis Ababa, Ethiopia. Study participants received full explanation about the study including the purpose and benefits of the study. Informed consent was obtained from all participants prior to sample collection. Study subjects can withdraw from the study at anytime. All efforts were made to maintain confidentiality and ensure that data will not be used for other purpose (Appendix 1, 2). Results from conventional LJ method and color plate tests were for only research purposes and did not have impact on clinical decision making or alter treatment course.

5. RESULTS

5.1. Demographic characteristic and Recruited Patients

Among 154 subjects enrolled, a total of 17 subjects were excluded from analysis. Initially, 7 subjects were excluded for the reason that two subjects were received prior TB therapy for one month and five subjects did not provide sufficient sputum for both LJ culture and color plate test. Followed by additional 10 subjects were excluded due to invalid results of color plate tests (insufficient growth, <10 colonies, appeared in the growth control quadrants). None of the color plates (n=147) were contaminated while contamination rates of 8.2 % (12/147) were observed in the LJ culture. At initial testing, 93.2% (137/147) of the color plate tests gave interpretable or valid results. But, the ten invalid tests couldn't be repeated due to absence of backup sputum samples. Whereas, 88.4% (130/147) of the LJ culture results were valid during the first testing. However, repeated LJ culture (from backup frozen sediment) added 4.1% (6/147) of the contaminated culture, and 2.1% (n=3/147) of insufficient growth of MTB, giving a total of 94.6% (139/147) valid results. A total of 137 patients had all three test results and were included in the final analysis (Figure 4).

Among the 137 in the study, 88 (64%) subjects were diagnosed with new TB disease and 49 (36%) were diagnosed as re-treatment cases. The median age of the participants was 30 years old (range 8-72) and 56% male and 44% female.

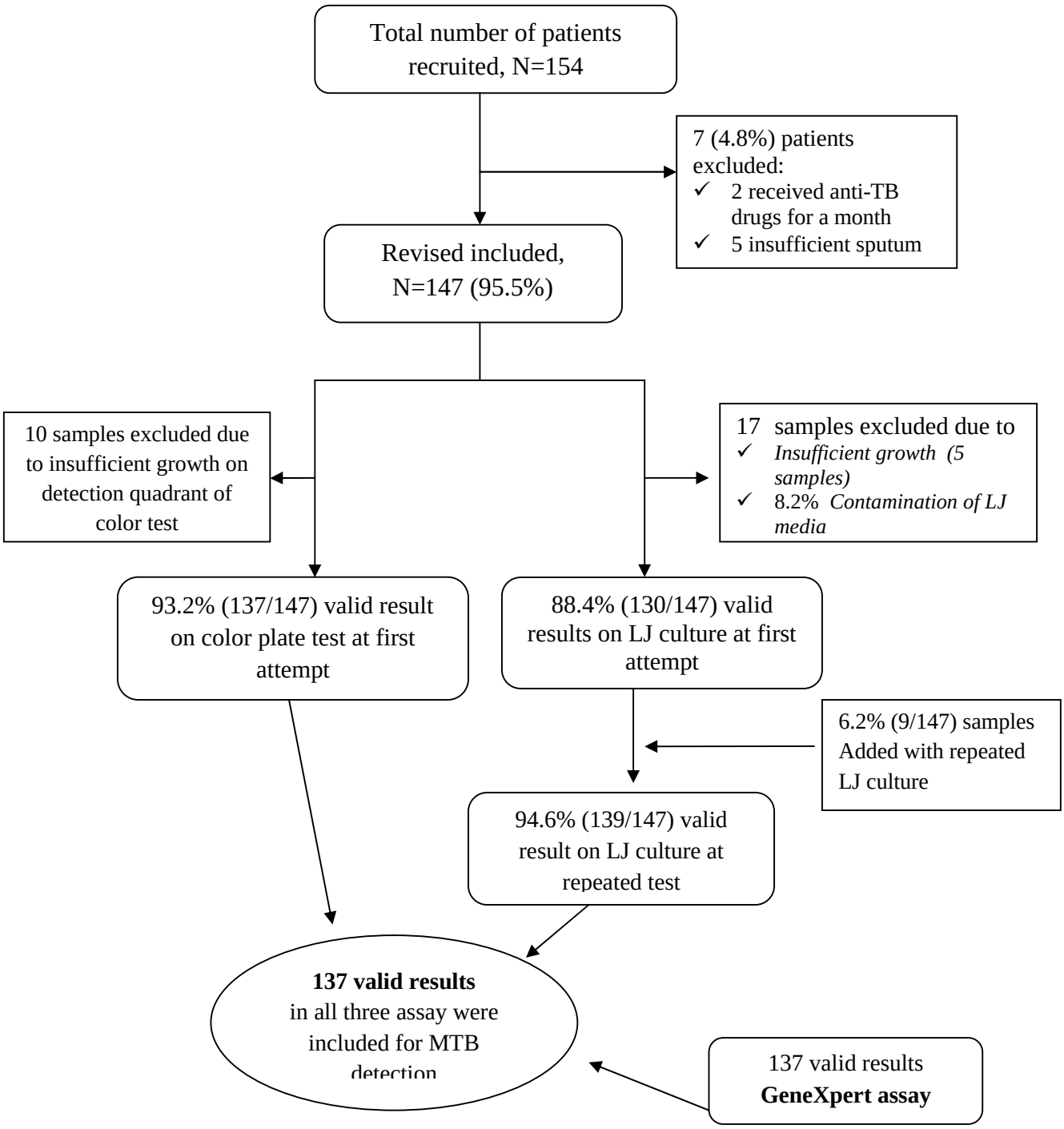

Figure 4: Flow Chart Showing Patient Enrollment and MTB detection performed

5.2. Time to MTB Culture Growth Detection and DST Results

It was calculated in terms of days from the date of inoculation to date of interpretable/valid positive results, and the result was compared between the color plate test and conventional diagnostic test (Table 1, 2). The date of positive-result availability for each sample was registered as a turnaround time (TAT). The TAT for color plate test (concurrent MTB detection and susceptibility results) were ranged from 5 to 23 days with a median and mean time of 13 days while the median TAT for reference DST using indirect proportional method was 22 days (range 15-35) after obtaining a primary isolate from LJ culture (median TAT 28 days).

Table 1: Median time to MTB Culture Growth Detection and DST Results on color plate test and solid LJ culture and indirect proportional DST in sputum samples of MDR-TB suspected patients

N= 137 cases	Culture growth/isolates for DST	Median time to positivity (days)	Minimum/ Maximum	*P* value
Color plate test	73 (53.3%)	13	5-23	< 0.001
Solid LJ culture	78 (56.9%)	28	12-46	< 0.001
LJ proportional DST	70 (89.7%)	22	15-35	< 0.001

Notes: Growth means culture positive by seeing growth; MDR-TB= Multidrug-resistant tuberculosis; LJ= Löwenstein-Jensen; DST= Drug Susceptibility Test; *P* value for comparison of color plate test Versus LJ culture and DST

Table 2: Frequency table of color plate test turnaround time (TAT)

Color test TAT (no. of days)	Frequency	%	Cumulative%
5	2	2.7	2.7
7	0	0	2.7
9	6	8.2	10.9
11	12	16.4	27.3
13	18	24.7	52.0
15	24	32.9	84.9
17	6	8.2	93.1
19	3	4.1	97.2
21	1	1.4	98.6
23	1	1.4	100.00
Total	73	100.00	

5.3. *Mycobacterium tuberculosis* (MTB) Growth Detection

5.3.1. Diagnostic accuracy of Color Plate test compared to solid LJ culture

In color plate test, *M. tuberculosis* complex was detected as red micro-colonies growth on drug-free quadrant while growth of buff/pale yellow color colonies on LJ medium indicated positive culture (Figure 5, 6).

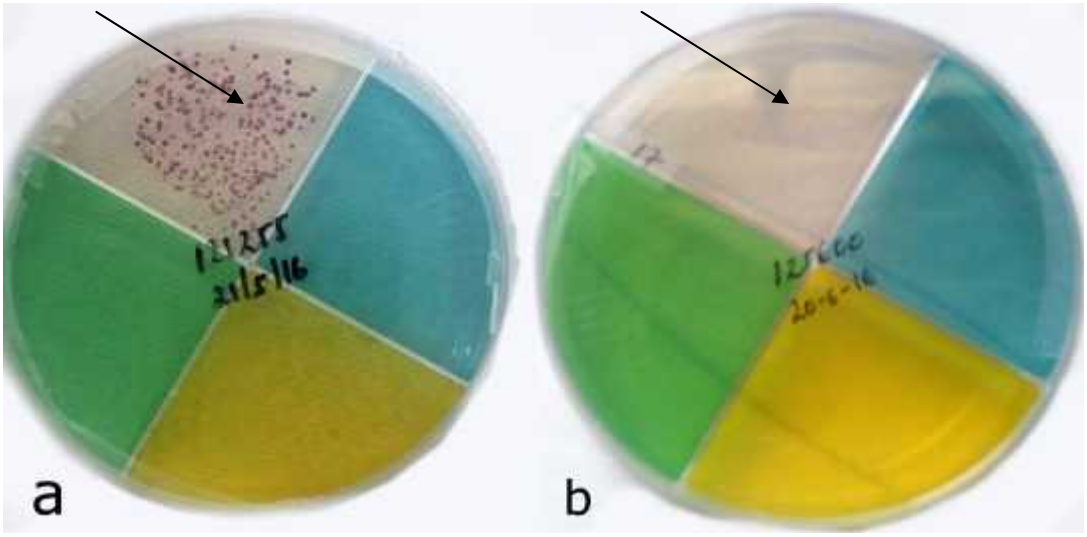

Figure 5: Photograph showing *M. tuberculosis* complex growth detection on color plate test (arrow indicated)

(a) Red micro-colonies appeared on drug-free quadrant (on day 12), **positive** for *M. tuberculosis* complex growth

(b) Red micro-colonies did not appear on drug-free quadrant till 6weeks, **negative** for *M. tuberculosis* complex growth

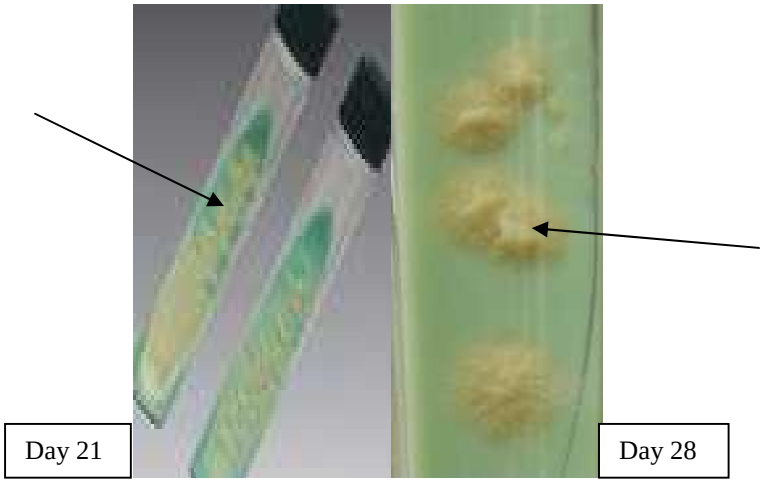

Figure 6: Colonies of *M. tuberculosis* (buff/pale yellow color, rough) on LJ culture medium

Among the 137 MDR-TB suspected patients, 78 were reference LJ culture-positive and 59 were reference LJ culture-negative. Of the noted reference culture-positive, the color plate test detected 73 of these cases and five cases were missed to be detected. Of the 59 reference LJ culture-negative, the color plate test was negative in 58 sputa specimen and positive in one of these cases. The sensitivity and specificity of the color plate test for detection of *M. tuberculosis* was 93.6% and 98.3% respectively when compared with the reference LJ culture. The positive predictive value (PPV) and negative predictive value (NPV) of the color plate test were 98.6% and 92.1% respectively. A strong agreement (95.62%, kappa 0.9114) was observed among color plate test and LJ culture for detection of MTB (Table 3).

Table 3: Diagnostic accuracy of color plate test compared to the reference LJ culture method

Types of Assay				**Sensitivity (%)**	**Specificity (%)**	**PPV (%)**	**NPV (%)**	**Prev. (%)**	**Test Efficiency (%)**
	Solid LJ culture								
Color Plate Test	Pos	Neg	Total						
Pos	73	1	74	93.6	98.3	98.6	92.1	57	0.956
Neg	5	58	64						
Total	78	59	137						

Agreement of color plate test with solid LJ

Agreement	Expected Agreement	Kappa	Std. Err.	Z	Prob>Z
95.62%	50.56%	0.9114	0.0853	10.69	0.0000

Note: PPV= positive predictive value, NPV= negative predictive value, LJ= Löwenstein-Jensen, Pos= positive, Neg=Negative, Prev=Prevalence, the kappa **(k)** value is a measure of test reliability, interpreted as follows: <0.4 = poor; 0.4 -0.75 = fair to good; >0.75 = strong.

5.3.2. Diagnostic Accuracy of Color plate test compared to GeneXpert MTB/RIF Assay

Among the total 137 MDR-TB suspects, 73 were detected by the color plate test, 78 were LJ culture-positive (reference standard), and 75 detected by GeneXpert assay. Of the five-color plate negative and LJ positive, four of them were positive by GeneXpert assay. Among 59 LJ culture-negative, 58 were also negative by color plate test while GeneXpert assay was negative in 47. The one color plate test positive and LJ negative was positive by GeneXpert assay. Of the 12 GeneXpert positive and LJ negative, 11 of them were negative by color

plate test. Despite MTB detection performance variation, strong agreement (89.05%, kappa 0.7756) had showed between color plate test and GeneXpert assay. Figure 7 indicated that the diagnostic accuracy of the color plate test and GeneXpert assay in reference to LJ culture.

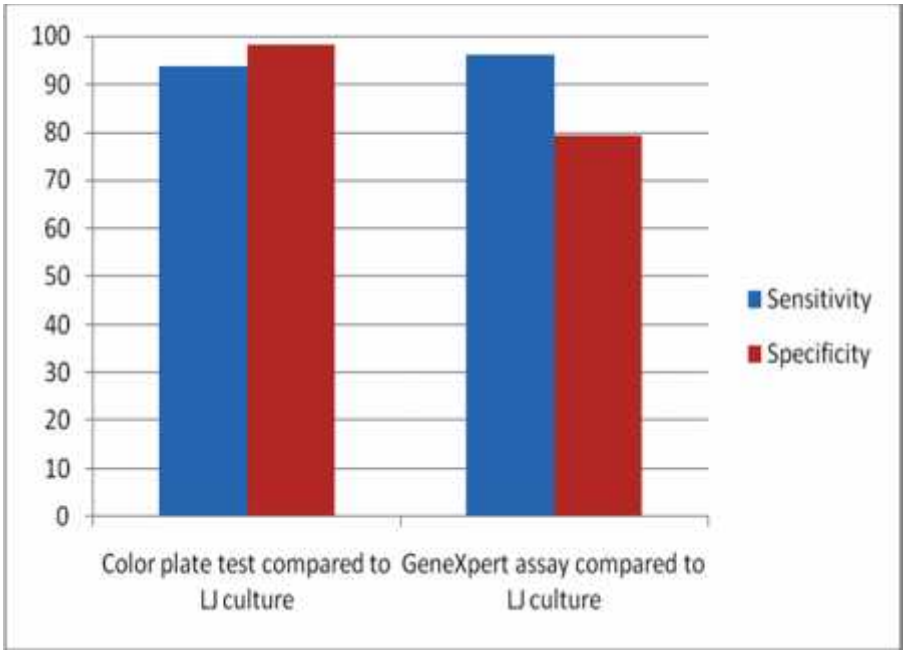

Figure 7: Diagnostic Accuracy of Color plate test compared to GeneXpert Assay

5.3.3. Diagnostic accuracy of GeneXpert MTB/RIF assay compared to solid LJ culture

Among the 137 MDR-TB suspected sputum samples, 78 were reference LJ culture-positive, and the GeneXpert assay detected 75 of these cases; and 59 specimens were reference culture-negative, and the GeneXpert assay was negative in 47. The sensitivity and specificity of GeneXpert assay for the detection of MTB were 96.2% and 79.7% respectively when compared to LJ culture. The positive predictive value (PPV) and negative predictive value (NPV) of the GeneXpert assay were 86.5% and 97.9% respectively. A strong agreement (90.51%, kappa 0.802) was observed between the color plate test and the LJ culture for detection of MTB (Table 4).

Table 4: Diagnostic accuracy of Genexpert MTB/RIF assay compared to the reference LJ culture method

Types of Assay				Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Prev. (%)	Test Efficiency (%)
	Solid LJ culture								
Color Plate Test	Pos	Neg	Total						
Pos	75	12	87	96.2	79.7	86.5	97.9	57	0.890
Neg	3	47	50						
Total	78	59	137						

Agreement of color plate test with solid LJ

Agreement	Expected Agreement	Kappa	Std. Err.	Z	Prob>Z
90.51%	52.08%	0.8020	0.0842	9.52	0.0000

Note: PPV= positive predictive value, NPV= negative predictive value, LJ= Löwenstein-Jensen, Pos= positive, Neg=Negative, Prev=Prevalence, the kappa **(k)** value is a measure of test reliability, interpreted as follows: <0.4 = poor; 0.4 -0.75 = fair to good; >0.75 = strong.

5.4. Drug Susceptibility Test (DST) Results

The performance of color plate test was also compared with the reference indirect proportional DST and GeneXpert assay results for detection of drug-resistant TB. From the reference LJ culture, 78 isolates (all LJ culture-positives) were subjected to reference DST (first line anti-TB drugs) by using LJ indirect proportional method (LJ-IPM). From 78 isolates, 93.6% (n=73) valid results were obtained, and five of them (6.4%) were invalid results due to contamination and/or 'no growth' on control (drug-free) tubes thus excluded from the analysis. Of the 73 valid results obtained by reference DST method, 70 isolates were valid with color plate test while 72 isolates were valid with GeneXpert assay and considered for analysis (figure 8). Besides, streptomycin drug susceptible test results were excluded from analysis due to more than one-third of invalid results observed on susceptible control test (H37Rv).

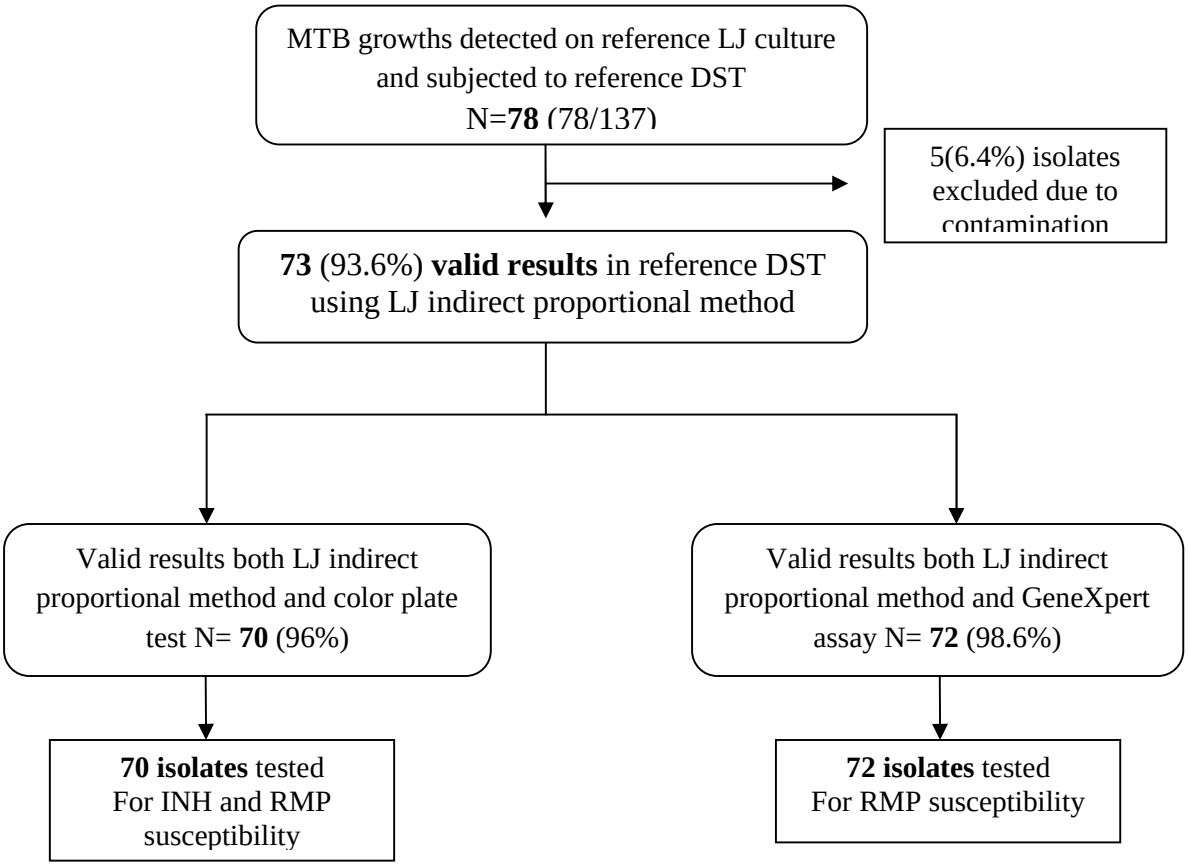

Figure 8: Flow Chart Showing Drug Susceptibility Assays Performed

5.4.1. Performance of Color Plate Test for Detection of Drug-resistant TB

The MTB growth was detected on 73 color plate test of 137 MDR-TB suspected sputa specimens. As indicated in figure 9, red MTB colonies were grown on drug-containing quadrants and constituents >1% of the 50-500 colonies of concurrent red MTB growth on the detection quadrant (clear, top), and thus the isolates or strains were classified as resistant to the particular anti-TB drug. Of the 70 color plates (isolates) tested for susceptibility, mono-resistance to INH, RMP, and PZA were detected in 24, 64 and 19 isolates respectively; drug resistance to both INH and RMP were observed on 19 color plate tests. Besides, 19 isolates were resistant to INH, RMP, and PZA.

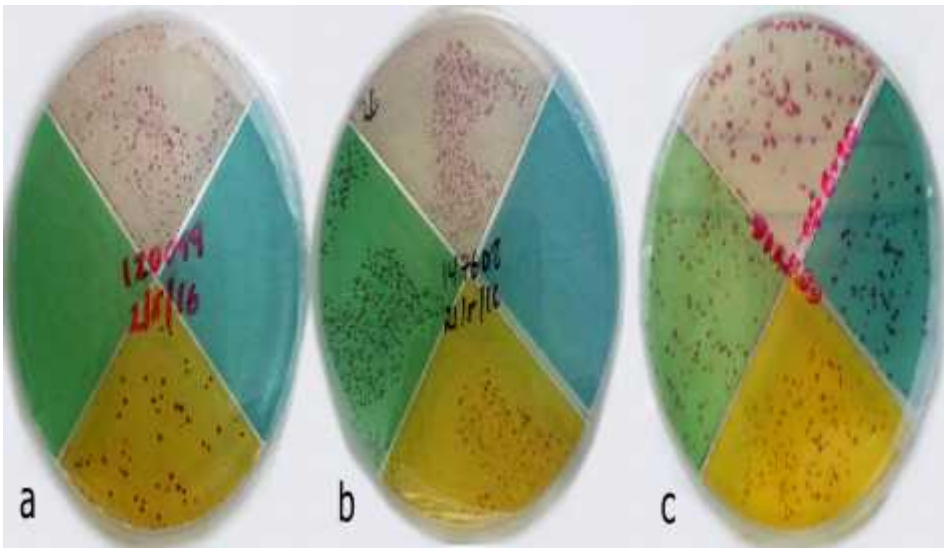

Figure 9: Photograph showing drug-resistant tuberculosis detection on color plate test

a) **Mono resistant to RMP**: Concurrent MTB colony growth on drug-free quadrant (Top, clear) and yellow rifampicin-containing quadrant (>1%).
b) **MDR-TB**: Concurrent MTB colony growth on drug-free quadrant (top, clear), green isoniazid-containing quadrant and yellow rifampicin-containing quadrant (>1%).
c) **MDR-TB + PZA resistant**: Concurrent MTB colony growth on drug-free quadrant (top, clear), green isoniazid-containing quadrant, yellow rifampicin-containing quadrant and blue pyrazinamide-containing quadrant (>1%).

Of the 70 isolates tested for INH susceptibility, results obtained by color plate test and LJ indirect proportional DST were in agreement for 65 isolates (93%; 44 susceptible, 21 resistant; kappa= 0.8399). Among five discordant samples, two strains tested susceptible with color plate test but resistant with LJ indirect proportional DST, and three strains tested resistant with color plate test and susceptible with LJ indirect proportional DST. Whereas, among the 70 isolates tested for RMP susceptibility, results obtained by color plate test and LJ indirect proportional DST were in agreement for only 27 strains (39%; 6 susceptible, 21 resistant; kappa= 0.0877). All 43 discordant strains tested resistant with color plate test but susceptible with LJ indirect proportional DST (Table 5).

Table 5: Comparison of drug susceptibility test performance results for *M. tuberculosis* isolates as determined by color plate test and LJ indirect proportional DST (n=70)

Color plate test	LJ IPM		Agreement % (95% CI)		kappa value (95% CI)	*P value*
	Susceptible	Resistance	Concordant	Discordant		
INH color test						
Susceptible	44 (63%)	2 (3%)	65 (93%)	5 (7%)	0.8399	< 0.001
Resistance	3 (4%)	21 (30%)				
RMP color test						
Susceptible	6 (9%)	0 (0%)	27(39%)	43 (61%)	0.0877	< 0.001
Resistance	43(61%)	21(30%)				

Note: LJ IPM = LJ indirect proportional DST; the kappa (k) value is a measure of test reliability, interpreted as follows: <0.4 = poor; 0.4 -0.75 = fair to good; >0.75 = strong.

The false-resistant and false-susceptible rates for color plate test compared with the LJ indirect proportional DST are shown in Table 6. The sensitivity (i.e. the ability to detect true INH resistance) for INH was 91.3%, and the specificity i.e. the ability to detect true INH susceptibility), for INH and RMP were 93.6% and 12.2% respectively.

Table 6: Performance characteristic (accuracy and reliability) of color plate test compared with LJ indirect proportional DST for detection of drug-resistant MTB isolates (n=70)

drugs	Cases	n	Prevalence	Sensitivity	Specificity	PPV	NPV	Test efficiency
INH	New	38	5 (13%)	80%	97%	80%	97%	
	Previously treated	32	18(56%)	94.4%	85.7%	89.5%	92.3%	
	Combined	**70**	23 (33%)	**91.3%**	**93.6%**	**87.5%**	**95.7%**	0.928
RMP	New	38	4(11%)	100%	14.7%	12.1%	100%	
	Previously treated	32	17(53%)	100%	6.7%	61.3%	100%	
	Combined	**70**	21(30%)	**100%**	**12.2%**	**35.9%**	**100%**	0.385

Note: INH= isoniazid; RMP= rifampicin; PPV = positive predictive value; NPV = negative predictive value; MTB= *Mycobacterium tuberculosis*

5.4.2. Performance of RMP-Color Plate Test Compared with GeneXpert RMP assay

The performance of color plate test was compared with GeneXpert assay result for detection of rifampin drug resistance. Of 70 isolates tested for RMP resistance, the RMP color plate test was in low agreement with the GeneXpert assay (38.57%, kappa 0.0773) for detection of RMP drug resistance.

5.4.3. Performance of GeneXpert Assay for Detection of RMP Drug-resistance

The performance of GeneXpert assay was also compared with the reference LJ indirect proportional method results for detection of RMP drug resistance (Table 7, 8). Among the 72 cases tested for RMP resistance, GeneXpert assay was in agreement with LJ indirect proportional DST result for 69 cases (96%). The sensitivity of the assay for the detection of RMP resistant MTB isolate was 90%, while 98% of specificity to detect RMP susceptible isolates.

Table 7: Comparison of drug susceptibility test performance of GeneXpert assay in reference to LJ indirect proportional method for rifampin drug resistance detection (n=72)

RIF Xpert assay	**RIF LJ IPM**		**Agreement % (95% CI)**		**kappa value** (95% CI)	**P value**
	Susceptible	Resistance	Concordant	Discordant		
Susceptible	49 (69%)	2 (3%)	69 (96%)	3 (4%)	0.8675	< 0.001
Resistance	1 (1%)	20 (27%)				

Table 8: Performance characteristic (accuracy and reliability) of GeneXpert MTB/RIF assay compared to reference LJ indirect proportional method (n=72).

Test drug	Cases	n	Prevalence	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Test Efficiency
RMP	New	40	4 (10%)	75%	100%	100%	97.1%	
	Previously treated	32	17(53%)	93.3%	94.1%	94.4%	86%	
	Combined	72	21(29.2%)	90%	98%	95.2%	94%	0.958

5.4.4. Pyrazinamide (PZA) Drug-resistance Test Results

Among the 137 MDR-TB suspected patients, 73 were culture-positive on color plate test. From the noted figure, concurrent detection of PZA-resistance (>1% of colonies on detection quadrant) were observed on 19 color plate testes. All of them were belongs to previously treated patients and occurred together with INH and RMP resistant rather than as mono-resistant to PZA (Figure 8). The 19 PZA-positive isolates and 19 PZA-negative isolates (randomly selected) were subjected to reference drug susceptibility testing using MIGIT DST though the result not yet revealed.

5.5. Cost Estimation

The estimates costs, simple totals of supplies and reagents needed per specimen were also compared. The retail cost for materials for one color plate test was 4-5 US$. According to Molicotti, *et al*., (2014), for culture based DST (LJ DST) costs about 7-12 US$ and 25-30 US$ required for GeneXpert MTB/RIF assay though the developing nations are used the subsidized cost (9.9 US$).

6. DISCUSSION

The present study was designed to evaluate the performance characteristics of color plate test in comparison with conventional LJ culture and indirect proportional DST, and the molecular GeneXpert assay. Due to limitation of published date, reports of TLA assay which is the basis for the current test, and MODS assay (comparable test) were also used to compare with the present findings. The characteristics assessed in evaluation of the new test are discussed and presented below as diagnostic and operational characteristics.

Laboratory contamination of *Mycobacterium tuberculosis* cultures has a great impact on quality of the test and may result in the misdiagnosis of tuberculosis. Despite the decontamination process, some contamination of culture media is to be expected. Liquid media is more susceptible to contamination than solid media; the acceptable rate for liquid media is 5-8% and 3-5% for solid media. Higher percentages may indicate incomplete processing or the use of contaminated reagents, media or equipment while lower percentages may indicate the use of harsh reagents or excessive processing (Dunlap, *et al*., 1995; Bauer, *et al*., 1997; Ribeiro, *et al*,; Forbes, *et al*., 2009).

In the present study, the contamination rates and interpretable results of the new color plate test were compared to the reference LJ culture method. Of the 147 sputa samples each tested on both color plate test and LJ culture, none of the new test was contaminated, and 92.5% gave interpretable results with a single culture attempted. Conversely, higher contamination rates of 8.2% were observed at first culture on LJ culture, but it provided similar interpretable results (94.6%) latter after repeated testing. In previous studies, reports have shown comparable and inconsistent results with the present findings of the contamination rates and interpretable result.

Similar to the current study, Toit *et al*., (2012) reported that any of the color plate tests were not contaminated despite indirect inoculates was used (n=197 isolates). However, higher contamination rates (4.1%) for TLA assay was reported by Robeldo and colleagues (2008) though the contamination rate for TLA assays appeared to be significantly lower than that of either solid or liquid media and MODS assay too (Minion, *et al*., 2010). These variations

might be due to the fact that the color plate test used different disinfectant chemical and decontamination protocol (prolonged sputum-disinfectant time) than that of TLA assay, MODS assay and solid or liquid culture method, where sputum specimen were processed using Petroff-method (NALC-NaOH; very short sputum-disinfectant time). Besides, the new color plate test showed zero or much lower contamination rate than that of an alternative direct agar DST. An alternative direct agar DST is a recognized cheap method that can provide DST results within 3 to 4 weeks (Kent, *et al*., 1985; Libonati *et al*., 1988). However, it can be confounded by bacterial/fungal contamination (under or overgrowth in controls), which invalidates about 15% of tests. Direct agar DST provided reportable results in only 41% of smear positive and 62% of culture-positive cases, according to Libonati *et al*., (1988) report. The higher contamination rate (8.2%) of LJ culture in this study could be delays of the sputa for two weeks before processed instead of cultured right away.

In spite of repeated attempts, Toit *et al*., (2012) reported superior interpretable result (98%) than the current study, which produced 93.2% (137/147) valid results during the first attempt. The discrepancy might be mainly due to variation in the types of specimen used among the two studies in addition to the re-culturing effects (repeated test). The current study used direct specimen (sputa) for inoculation where decontamination step is a pre-requisite for sputa inoculation. Decontamination process by itself is fatal to mycobacterium (Metchock, *et al*., 1999), particularly in the current test (color plate test) with prolonged decontaminaton process, more losses may be expected which leads to reduce the number of bacilli that did not yield enough colonies (valid/interpretable >50 colonies) on the growth control quadrants of the new test. This may have accounted for the 6.8% (10/147) invalid results of the color plate test. Whereas, Toit and colleague (2012) were done on indirect inoculates (MTB isolates), where culturing was done exclusive of decontamination step.

Similar to any other diagnostic tests, the basic performance characteristics of the color plate test designed to distinguish TB infected from uninfected and resistant TB from susceptible one are sensitivity and specificity. The two other key measurements of test performance are positive predictive value (PPV) and negative predictive value (NPV) (TDR, 2010).

Our present study revealed that the color plate test has demonstrated a very good diagnostic performance for the detection of *M. tuberculosis* (MTB) when compared to the reference solid LJ culture and the molecular diagnostic tool GeneXpert assay. The sensitivity and specificity of the color plate test was 93.6% and 98.3% respectively. Moreover, it had showed strong agreement (95.62%, kappa 0.9114) with LJ culture and GeneXpert assay (89.05%, kappa 0.7756) for detection of MTB.

Among the five (6.4%) reference culture positive and color plate negative TB cases (i.e., "false-negatives"), four of them were positive by GeneXpert assay. Besides, three of them (3/5) were grown on reference LJ media with scanty growth (< 5colonies) following prolonged incubation (>25 days). As to the one (1.7%) color plate test positive sample and reference culture negative (i.e., "false-positives"), it was also positive by GeneXpert assay. The probable reasons for false-negative results could be the use of different decontamination protocols among the color plate test and reference LJ method. Unlike the LJ culture method, the color plate test does not concentrate the sputa and inoculation was made with diluted sputa-disinfectant mixture at 1:2 ratios. Sputum concentration prior to culturing or inoculation is one of the approaches to improve the sensitivity of TB diagnostic tests (Parson, *et al*., 2011). In addition, in this study the sputa-disinfectant contact time is too long that may causes loss of viability of the bacilli (Metchock, *et al*., 1999). One false positive result may be due to laboratory cross-contamination during sputum collection and/or culturing. All laboratories are capable of producing false-positive results; the meta-analysis showed that 3.1% of positive results for MTB from laboratories are false positives (Bauer, *et al*., 1997; Burman and Reves 2000). A possible false-positive result due to environmental contaminant with overgrown of NTM was excluded as it was also detected by highly MTBC specific assay, GeneXpert assay.

Comparable MTB growth detection result was obtained in a study undertaken in Colombia using TLA method, 91.3% of sensitivity was reported by Robeldo *et al*., (2008) on patients with smear positive sputa (n=95). However, slightly higher growth detection sensitivity of 96.9% in MODS assay was observed on smear positive patients in Ethiopian setting (Shiferaw *et al*., 2007). This discrepancy could be due to different methodologies applied

between the studies such as variation in recruited patients (MDR-TB suspected vs smear positive), the test protocol differences and the diagnostic performance variation between the solid (color plate test) and liquid (MODS) assays for detection of MTB growth. In contrast to all culture-based tests including MODS assay that are used concentrated sediment for inoculation, the color plate test protocol does not allow to concentrate the sputa rather inoculated with diluted sputa-disinfectant mixture, this may result in decreasing the diagnostic sensitivity of the color plate test. Besides, the liquid culture system is recovered more mycobacterium and has better sensitivity than solid culture for MTB growth detection (FIND, 2006).

Our finding also indicated that GeneXpert assay has showed a good diagnostic performance characteristics compared with the reference solid LJ culture for detection of MTB. The sensitivity of GeneXpert assay in detecting pulmonary tuberculosis in MDR-TB suspected patients was higher (96.2%), but the specificity for excluding pulmonary tuberculosis was lower (79.6%).

The current result of sensitivity was in agreement with previous studies undertaken in a high TB burden country such as India and Ethiopia. In India, Sharma *et al*., (2015) reported comparable sensitivity of 95.7% for GeneXpert assay in detecting culture positive cases (n=1406). However, in Ethiopia, comparatively lower sensitivity of 88.2% was recorded in detecting smear positive cases (n=525) (FMOH/EPHI, 2014). Similarly, WHO (2014) also revealed lower pooled sensitivity of 88% (95%CI 84-92%; 22 studies, 9008 participants). The difference with regarding to the sensitivity performance might be related to the sample size and target population variation. In the present study, small size was used and the target population was solely based on MDR-TB suspected patients.

The specificity 79.6% of the present study is lower than Sharma et *al*., (2015) and WHO (2014) results reported as 99.8% and 99% respectively. Among 12 (20.4%) GeneXpert assay positive patients but reference culture negative, eleven of them were also negative to color plate test, and the dominant patients were belongs to previously treated groups who had been on treatment for up to two weeks.

The relatively lower specificity result in the current study might be attributed to medication reason. In fact, the use of GeneXpert is not Food and Drug Administration (FDA)-approved in patients who have received more than 3 days of anit-TB therapy (Cephalid, 2011). Twelve patients in this study were on anti-TB medications (range of 1day to 9 days), 7 (11.9%) had discordant results between GeneXpert and cultures (7 GeneXpert positive, 7 LJ negative, and 7 color plate negative). Anti-TB treatment kills and reduces the live TB bacilli load per ml of sputum, and probably the dead bacilli might be detected by GeneXpert assay as it can detect and amplify DNA of a single bacillus even from a dead organism during antibiotic treatment (Dinnes, *et al*., 2007). However, it may not be detected or grown on culture media as it requires 10-100 viable bacilli per ml of sputum (Molicotti, *et al*., 2014). Additionally, the use of two different specimens for GeneXpert and culture can affect the results. It is possible a variation in bacilli load between the sputum used for GeneXpert and the cultures (LJ and color plate culture) though paired morning expectorated sputum samples were collected on the same day from the same patient though prior studies have shown that the difference bacilli load variation is minimal (Hamid, *et al*., 2012; Ssengooba, *et al*., 2012).

In this study, the accuracy of color plate test for diagnosis of drug-resistant tuberculosis was also assessed in comparison with the reference phenotypic DST (LJ-indirect proportional method) and the molecular DST, GeneXpert assay in terms of sensitivity, specificity, PPV and NPV. For testing INH, 0.2µg/mL critical concentrations were applied for both reference DST and color plate test while 1µg/mL and 40µg/mL critical concentrations of RMP were used for color plate test and reference DST respectively.

The ability to detect INH resistance is important for treatment. For patients with INH resistance, the continuation phase of drug treatment would include RMP, PZA, and EMB rather than RMP and INH as for drug susceptible. Thus, if one does not know INH drug resistance, during continuation phase, mono-RMP therapy leads to increase RMP drug resistance and the development of MDR-TB. For Ethiopia, Biadglegne, *et al.,* (2014) reported INH resistance of 3.2% in new cases (previously untreated) and up to 56.1% in retreatment cases (previously treated).

Of the 70 isolates tested for INH resistance in the current study, the color plate test detected drug resistance with 91.3% sensitivity for INH and specificity of 93.6%, and the DST agreement between color plate test and the reference DST was strong (kappa value of 0.84 and concordance 93%). Despite few studies were available, previous assessment of INH resistance with the color test and TLA assay yielded higher performance than did the color plate test in the current study. Toit and colleagues (2012) reported that an excellent performance of color plate test for INH resistance detection with 98% sensitivity and 100% specificity although indirect inoculates were used (n=197 isolates). Similarly, Robeldo and colleagues (2008) were reported that 100% sensitivity and specificity for INH resistance detection using TLA assay on smear-positive sputa (n=95) from MDR-TB suspected patients. Several reasons can support the difference between our finding and their findings: the type of specimen used (direct sputa vs indirect isolate), the sample size and the choice of reference DST methods (solid LJIPM vs liquid MIGIT).

Studies have showed that direct inoculation (sputa specimen) was associated with lower sensitivity and specificity for resistance detection to INH and RMP though did not differ significantly between the type of inoculates (Minion *et al*., 2010). This may have accounted for the current relatively lower sensitivity and specificity for INH resistance detection using sputum sample (direct inoculates). Additionally, previous studies used the liquid reference DST method (MIGIT) with two weeks incubation period while the current solid reference DST used protracted incubation period (6 weeks), this may have also attributed for reduced INH susceptibility detection as the test drug (INH) might be inactivated potentially during prolonged incubation (Libonati, *et al*., 1988); and the liquid culture based DST is more rapid (fast growth) and sensitive than solid culture DST for drug susceptibility testing of *M. tuberculosis* (FIND, 2006).

Of the 70 isolates tested for RMP resistance, the DST agreement between the color plate test and the reference DST method (LJ IPM) was very low, below half (kappa value of 0.08 and concordance 39%). Thus, the current color plate test has misclassified majority of the RMP susceptible isolate as RMP resistant. Unlike our findings, different investigators reported an excellent performance of RMP resistance detection using color plate test and TLA assay.

Toit and colleagues (2012) reported that 98% sensitivity and 88% specificity for RMP detection with 96% agreement between the color test and the reference DST, BACTEC MGIT 960. Besides, all assessments (sensitivity, specificity, PPV and NPP) of RMP resistance with the TLA assay was produced 100% using both direct (two studies) and indirect (1 study) specimens (Minion, *et al*., 2010). This key discrepancy could be strongly associated with the critical concentration of RMP used in color plate test (RMP quadrant). Even though the color plate test (RMP-quadrant) was prepared with a standard critical concentration (1µl/ml RMP) in the current study, the critical concentration could be inactivated potentially and lowered at some step before sample inoculation (from preparation to storage of the plates). This might be supported by susceptible quality test result done at AHRI TB laboratory in Ethiopia. Unlike on INH and PZA quadrants of the color plates, the conventional susceptible MTB isolate (H37Rv) were RMP resistant on RMP quadrants (3% of the color plates) at the first and second step (two fold) bacterial dilution (1:100 and 1:1000), but became fully susceptible at the third step dilution (1:10000).

The performance of color plate test for detection of RMP resistance was also very low with poor agreement (kappa value of 0.07 and agreement 36%) when compared to the molecular RMP resistant DST from GeneXpert MTB/RIF assay. In our study, the sensitivity of GeneXpert assay to detect RMP resistance in sputum samples was 90% and the specificity for excluding RMP resistance was 98%. It had also showed strong agreement with the reference DST method (LJ IPM) (kappa value of 0.87 and concordance 96%) for RMP detection. Among the 3(4%) discordant isolates, two were GeneXpert RMP susceptible and phenotypic LJ-IMP resistant while one was GeneXpert RMP resistant and phenotypic LJ-IMP susceptible isolate. It is a common finding that isolates demonstrate phenotypic drug-resistance while molecular testing shows they lack known resistance-conferring mutations and not all mutations are translated into phenotypic resistance, thus absence of a mutation does not necessarily mean the isolate is phenotypically susceptible to the drug (Moore and Shah, 2011).

Our findings are consistent with previous studies. According to the recent WHO report (2014), pooled sensitivity and specificity of the GeneXpert assay for RMP resistance

detection were 95.1% and 98.4% respectively. In India, Sharma (2015) (n=422) also reported sensitivity of 94.5% and 97.7% specificity. In Ethiopia, the sensitivity and specificity of GeneXpert assay in detecting MDR cases were 96.8% and 92.9% respectively (FMOH/EPHI, 2014). The relatively lower sensitivity of the current GeneXpert assay to detect RMP resistance could be due to small amount of mutant DNA (*rpoB* gene) presented in the sample. Drug resistance detection is dependent on the particular mutation and required between 65% and 100% mutant DNA to be present in the sample for 95% certainty of resistance detection (Blakemore, 2010).

The operational characteristic of diagnostic tests mainly includes the time taken to perform the test (turnaround time), technical simplicity of the test (ease of use), the risk of potential biohazard and the fee required for the test. The ease of use depends on how difficult it is to train staff to do the test and interpret the results correctly (TDR, 2010). Along with the accuracy of the test, it is also a crucial factor for decisions regarding the appropriateness and adoption of a new diagnostic test for/in specific settings (Minion, *et al*., 2010).

The rapid concurrent detection of TB and drug-resistant TB is vital to ensure optimal anti-TB therapy and helps to reduce empirically treated cases as it may enlarges the proportion of bacteriologically confirmed patients. Thereby, possible to improve the patient's outcome and minimize the exposure of public from the risks of undiagnosed and untreated patients. In present study, the use of color plate test enables the rapid concurrent detection of *M. tuberculosis* complex and its pattern of resistance to the three anti-TB drugs namely INH, RMP and PZA directly from sputa specimen in a mean and median time of 13 days, with ranges of 5 to 23 days. Interestingly, the fastest growth was observed with two sputa samples on day 5 of incubation that was belongs to XDR-TB and total drug resistant tuberculosis (TDR-TB) patients. Results for 85% of 73 plate culture-positive sputa were available within 15 days. However, the reference method required an average of 50 days for DST, a median time of 28 days for growth on LJ and then an additional 22 days was required to detect drug resistant TB using gold standard LJ indirect proportional method. The primary reason for rapid detection of the new color plate test over the conventional LJ DST is the chemistry of STC indicator that was incorporated in color plate test media. The

STC indicator is reduced prior to colony formation and result in the growth of red TB colonies. The color contrast provided by the red colonies makes them observable to the naked eye at early stages of growth or micro-colonies, in 2 weeks instead of the normal 3 weeks incubation (Lee, *et al*., 2006). The direct sputum inoculation onto drug containing quadrants allows simultaneous detection of MTB growth and DST results also contributing to a shorter TAT of the new test. Of course, the molecular assays such as GeneXpert assay is the most rapid tool for simultaneous detection of MTB and rifampicin resistance within 2 hrs (Cepheid, 2011; Boehme, 2010) though the molecular tests can be cost prohibitive and may not be available.

Despite few published studies on the new color plate test, our median TAT was comparable to one color plate test study and three TLA assay studies. In Estonia setting, 13 days of mean turnaround time was recorded with MDR/pre-XDR-TB color plate test using indirect inoculates (n=197 isolates) (Toit *et al*., 2012). Martin and colleagues (2009) reported median TAT of 10 days with TLA assay using indirect inoculates (n=147 isolates). Two additional TLA studies used direct sputa specimen for inoculation and reported a median TAT of 11.1 days (Minion, *et al*., 2010).

Additional advantages of the color plate test besides presenting rapid DST results are providing low risk of biohazard. Biohazard risks of the color plate test are similar or less than conventional methods. The sputum digestion-decontamination procedure was performed in aerosol-contained falcon tubes, which is common to all culture-based tests including the reference LJ culture and the GeneXpert assay. The risks are minimized during pipetting by not emptying while mixing to reduce the risk of aerosol production, and the use of a disposable plastic pipettes rather than reusable glass pipettes to reduce potential biohazard from broken glass. In addition, the culture plates were double-sealed after initial sputa specimen inoculation and all reading performed with its double sealed plastic bag and for disposal, the plates are discarded without opening (SOP, 2010). Lastly, due to results are obtained quite rapidly (short TAT), culture materials are discarded in less time than in the conventional methods. Therefore, without the need to handle cultured isolates, it could be done with low biohazard risks and the color plate test may be safer than the conventional

method (LJ culture and indirect proportional DST), which requires manipulation of an amplified culture of *M. tuberculosis.* In addition to safety, both MTB culture and direct DST for the color plate test are performed on a single agar plate as a result the new color plate test would provide least labor and reduced incubator space as compared to the conventional method.

Furthermore, the new color plate test may take an operational characteristic advantage over the liquid MODS assay, which is rapid, low technology and endorsed for rapid screening of MDR-TB. Due to the color contrast, it is possible to carry out daily faster checking of the plate visually or with light microscopy for micro-colony detection. But, MODS assay needs an expensive inverted microscope to interpret the results and continuous monitoring of the culture plates for the formation of mycobacterial cord (Coronel, *et al*., 2008). Moreover, one of the main innovations of the color plate test is flexible enough to be adjusted for provide a rapid MDR-/pre-XDR-TB diagnosis with three drug containing quadrants (INH, RMP, fluoroquinolone) with one culture plate format. Color plate studies prepared ciprofloxacin (CFX) culture medium in one of the quarters instead of PZA quarter and found an excellent accuracy of CFX resistance detection, which is a marker for XDR-TB (Minion, *et al*., 2010; Toit, *et al*., 2012). The PZA plates were developed by Ohio State University (OSU) as a surrogate test for *M. bovis* specifically for use in detection of bovine TB in pastoral areas where human and animal transmission may occur (Gumi, *et al*., 2011; Ameni, *et al*., 2013; Torres-Gonzalez, *et al*., 2013). But, clinically, PZA drug resistance may be important in areas of low MDR-TB prevalence. PZA in combination with INH, RMP, EMB is WHO recommended first line drug for drug susceptible TB. The addition of PZA with INH and RMP forms the basis of modern TB therapy, shortening the therapy from previously 9-12 months to current 6 months (Zhang, *et al*., 2003). If a patient is not able to take PZA in the first two months either due to intolerance or PZA drug resistance, treatment course is extended from 6 months to 9 months due to increase treatment relapse. So, the ability to detect PZA resistance may be of benefit to improving outcomes of patients with drug susceptible TB.

The new test has an additional merit that can perform with low cost relatively. The estimate costs required for the new test were also compared though the costs are not a true representation of the total expenses of the color plate test rather simple totals of supplies and reagents needed per specimen. The cost per test result for color plate test was minimal (4-5 US$) and is comparable to the currently available inexpensive tests (Caviedes, *et al*., 2000). It is also relatively low cost per test result as compared to the conventional culture-based indirect DST and more-cheaper than molecular GeneXpert assay (Molicotti *et al*., (2014).

Despite providing rapid diagnosis, comparatively low cost per test result, and apparent simplicity of use, the new color plate test has important limitations. One of the practical limitations of the test is that as it is a closed system (double-sealed from inoculation to discard), obtaining isolates for further testing may be difficult. However, a routine back-up of isolates can obtain with conventional solid media either by sub-culturing or using parallel inoculation of sputum, which may help laboratories to have additional isolates if further tests are required (DST for second-line drugs, species identification, molecular typing and storage of strains). Thus, the color plate test is may be suggested to provide only rapid diagnosis.

Another drawback of the new test could be lack of specificity of micro-colony cording detection. Similar to the other comparative assays (TLA, MODS and BACTEC-MGIT), identification of *M. tuberculosis* complex in the color plate test is relay on cording (cord formation) properties of the organism but that is not confirmatory identification, still lacking of and unable to differentiate MTBC from non-tuberculosis mycobacterium (NTM) particularly *M. chelonae* that also exhibits cording (Yagupsky, *et al*., 1990; Caviedes, *et al*., Sharma, *et al*., 2007; 2000; Minion, *et al*., 2010).

Limitation of the Study

Sputa samples for this experiment were collected from patients suspected of MDR-TB cases and has small sample size (n=137). Thus, the findings may not be extended to patients who are not MDR-TB suspect. The operational and logistic difficulties limited our ability to increase the sample size and to link the color plate test performance with smear-positive and smear-negative patients. Specifically, the difficulty is because most of the MDR-TB suspected patients were examined by only GeneXpert assay as initial diagnostic tool instead of smear microscopy. The other drawback of the study was related to the type of sputum specimen used for the research. Due to resource constraints, it was impossible to conduct direct comparison between the color plate test and GeneXpert assay using pooled sputa specimen. Instead, paired morning expectorated sputum samples were collected on the same day from the same patient. Although the bacilli load variation is minimal among the samples, the use of two different specimens for color culture plate and GeneXpert assay can influence the results. Similarly, it is a possible in bacilli load variation among spot and morning sputum specimen thus the results may be affected with spot sputa specimen. The third limitation, the sputa specimens were not cultured right away rather frozen (20^0C) for two weeks which decrease the sensitivity and increase contamination, as specimen quality can be affected by storage. Reading of the color plate tests were performed at intervals every other day (e.g., 5, 7, 9, 11...) that was considered the laboratory work load but daily reading of the plates would yield the fastest result. Finally, though the conventional pyrazinamide DST is ongoing using Mycobacteria Growth Indicator Tube™ (MGIT) method, we are unable to perform the test on timely bases due to resource limitation. Thus, the accuracy of color plate test for detection of pyrazinamide resistant TB was not included in this thesis paper.

7. CONCLUSION AND RECOMMENDATIONS

TB has existed for millennia and is still a major global health concern. Despite the fact that TB is curable completely, the emerging drug-resistant tuberculosis particularly MDR-and XDR-TB is a potential and continuing challenge for global TB control program. Optimal anti-TB treatment is initiated based on timely TB diagnosis and detection of drug-resistant TB. Unfortunately, the existing diagnostic tools are slow and unaffordable for resource-limited countries with high TB burden. In the present study, we evaluate the diagnostic performance of a low cost-technology assay namely color plate test for the rapid concurrent detection of *Mycobacterium tuberculosis* (MTB), and multidrug-resistant TB (MDR-TB), and compared it to the conventional LJ culture and indirect proportional DST, and the molecular GeneXpert assay directly from morning sputum specimen of MDR-TB suspected patients.

The present finding have demonstrated that the color plate test is highly sensitive (93.6%) and specific (98.3%), for the detection of MTB, strong agreement with both the conventional LJ culture and GeneXpert assay. Despite the suboptimal performance for RMP resistance detection, it does show a good performance for detection of INH resistant-TB with sensitivity of 91.3% and 93.6% specific. The results of the color plate test for concurrent detection of MTB and drug-resistant TB can be obtained much more rapidly than conventional indirect DST method (13 days vs 50 days). It is also affordable than GeneXpert assay. Besides, the color plate test showed insignificant contamination rate, and easy to perform with minimal bio-safety risk relatively. Thus, the color plate test was found to be a good alternative method for screening of TB and selective drug-resistant TB in a timely and affordable way in resource-limited settings despite limitations of the test need to be addressed before the test approval.

RECOMMENDATIONS:

Based on our findings we forwarded the following recommendations:

- The critical concentration of rifampicin that determined for rapid evaluation of rifampicin susceptibility of M. *tuberculosis* complex using color plate test should be

tested again and/or revised, which may improve the suboptimal performance of the test for detection rifampicin-resistant TB and ultimately for effective diagnosis of MDR-TB.

- Further study should be done by including large sample size and different TB patient categories such as smear-positive, smear-negative and spot sputa specimens so as to elucidate the diagnostic performance of the color plate test on aforementioned cases.

- The color plate test could use para-nitrobenzoic acid (PNB) inhibition as a definitive identification of MTBC along with the characteristic micro-colony morphology. For this advancement, the color plate format may be designed to include PNB-containing quadrant, a specific inhibitor of MTBC growth, which helps to ruling out false-positive results produced by the growth of Non-Tuberculosis Mycobacterium (NTM).

- The standard operating procedure (SOP) of the color plate test need to be optimized with respect to decontamination protocol that the sputum-disinfectant contact time should be precise so as to prevent the bacilli from harshly decontaminated, moreover, the inclusion of centrifugation step in the SOP may facilitate for culturing of concentrated bacilli, and thereby can enhance the sensitivity of the color plate test.

- Further study on direct comparison using pooled sputa specimens is warranted between the new color plate test and GeneXpert MTB/RIF assay in order to draw more rational conclusion among the two diagnostic assays.

- Molecular strain identification need to be carried out on an extraordinary two MTB isolates, belongs to XDR-and TDR-TB cases, that were grown swiftly on day 5 onto the new color plate test.

- Further studies need to be done on thorough cost analysis and laboratory work load required for the new test so as to extract additional advantage of the test.

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APPENDICES

Appendix 1: Informed consent form (English version)

Dear Sir/Madam

My name is Biruk Mekonnen, I am currently a student of Addis Ababa University, School of Medicine, College of Health Science, Department of Microbiology, Immunology and Parasitology, and undertaking a master's degree (MSc) in the field of medical microbiology. I am going to carry out a scientific study on the **Performance Evaluation of Multidrug-Resistant Tuberculosis Color Plate Test (color plate test),** which is a new TB diagnostic instrument. The aim of this study is to evaluate the diagnostic performance of color plate test as a rapid test for the concurrent detection of pulmonary TB and MDR-TB compared to the current gold standard culture based DST and GeneXpert Assay in MDR-TB suspected patients at St. Peter's and ALERT hospitals, Addis Ababa, Ethiopia.

You are kindly invited to participate in this study which would take 6 months to 1year. All comments, both positive and negative, are welcome. Therefore, your honest and genuine participation is highly appreciated and helpful to attain the aim of the study. If you are voluntary, you will be asked to give additional morning sputum samples and to give permission to access of your medical record. Your socio-demographic data and medical history will be taken from your record. We would like to indicate that your name will not be written in the form and we assure you all the information you give will be kept strictly confidential. If you are unable to continue an interview, I may stop the interview process at any time. I greatly appreciate your cooperation.
Are you willing to participate in the interview? If yes, precede; if no, thanks and stop here.

Consent Form
Name _____Identification Code _____

I have read the information sheet (or it has been read to me); I have understood that this study is about **"Performance Evaluation of Multidrug-Resistant Tuberculosis (MDR-TB) Color Plate Test, Addis Ababa, Ethiopia"**. I have asked some questions and clarification has been given to me. I have given my consent freely to participate in the study, and I hereby to approve my agreement with my signature.

Participants: signature_____ Date _____
Investigators: signature_____ Date _____
Witness: 1. Signature _____ Date _____
2. Signature _____ Date _____

Appendix 2: **Informed consent form (Amharic version)**

<u>የፈቃደኝነት መጠየቂያ ቅፅ</u>

ስሜ ቡሩክ መኮንን ይባላል በአዲስ አበባ ዩኒቨርሲቲ ጤና ሣይንስ ኮሌጅ የድህረ ምርቃ ተማሪ ነኝ፡፡ የቲቢ ምርመራን በተሻለ ጥራት ለመስራት የሚያስችል አዲስ የምርመራ ዘዴ ለመጠቀም አሁን ያሉትን የምርመራ ዘዴዎች ከአዲሱ አንጻር በመገምገም ጥናት እያደረኩ እገኛለሁ፡፡ የእርስዎ ትብብርና ፍቃደኝነት ለጥናቱ ጠቀሜታ ስላለው የምርመራ ናሙና (አክታ) እና መረጃዎችን በመስጠት ትብብርዎን እየጠየኩ ከዚህ በመቀጠል ጥያቄዎች የማቀርብልዎ ሲሆን ትብብሮ በፈቃደኝነት ላይ የተመሰረተ እንዲሁም መመለስ የማይፈልጉት ጥያቄም ካጋጠመዎ አለመመለስ ይችላሉ፡፡ እንዲሁም በመረጃ መሠብሠቢያው ላይ ስምዎ የማይጠቀስ እና መረጃውም በሚስጥር የሚያዝ መሆኑን እገልፃለሁ፣ ፣

እኔም የጥናቱ ተሳታፊ ይህንን በመገንዘብ በጥናቱ ላይ ለመሳተፍ ተስማምቼያለሁ፣ ፣

ፊ ር ማ: _____
ቀን፡_____

መረጃውን የሰበሰበው ግለሰብ ስም: _____
ፊ ር ማ: _____
ቀን፡_____

Appendix 3: **National Standard operating procedures for biological sample transportation**

Purpose

This standard operating procedure provides the general technical requirements and Operational guidelines for the proper collecting, packing, and shipping of biological specimen to testing laboratories for analysis. This SOP includes the guidance and regulatory requirements that ensure proper collecting, packing, and shipping of sputum samples classified as "hazardous material"

General Consideration

Potential hazards associated with the planned tasks are thoroughly evaluated prior to conducting laboratory activities. The laboratory safety manual provides a description of potential hazards and associated safety and control measures.

Materials

• Falcon tube, Cetylpyridinium chloride, triple package and absorbent cotton swab

Sample Type: Sputum
Amount: 3-8 ml
Collection:
• Paired purulent /muco purulent early morning sputum specimen for GneXpert and culture and DST.

Storage: Store the sputum specimen at 2 to 8^{o}C up to 5 days and kept at -20^{o}C until started processing (for 15 days)

Transport: Use triple packaging and the sample must reach to the testing site within 5 days after collection

Stability: Cold chain must be maintained using Ice pack and the Ice pack must be changed at the transit site after 12 hours.

Specimen Rejection:

- ✓ •Specimen is unlabeled or mislabeled.
- ✓ Specimen without request form.
- ✓ Specimen name and request form does not match.
- ✓ Specimen container breakage or leakage.

Safety precautions

- ✓ Patients should produce sputum in sputum coughing designated area
- ✓ Avoid shaking of the tube
- ✓ Wear gown and glove when handling the sputum

Procedures: **Sputum Specimen Collection Procedure**

Instruct the patient

- ✓ To collect in a separate, ventilated room or preferably outdoors/ produce sputum in sputum coughing designation area/
- ✓ To keep both hands on hips, cough forcibly and collect sputum in the mouth

- ✓ To spit the sputum carefully into a wide-mouthed, leak proof container
- ✓ To collect 3–5ml in volume, although smaller quantities are acceptable if the quality is satisfactory.

Sputum Sample Packaging and Shipment

- ✓ Obtain samples in the laboratory-specified containers and verify the completeness of the sample identification information on the label and keeping record.
- ✓ Verify custody seals on sample containers and/or bags are intact and have been initialed and dated.
- ✓ Place samples in re-sealable plastic bags and then into the cooler.
- ✓ Place ample amounts of wet ice contained in doubled re sealable bags inside the garbage bag/liner in cooler.
- ✓ Write the shipper's tracking number

Triple Packaging Materials

All specimens should be appropriately packaged within a triple packaging system: primary, secondary and outer packaging and should contain all relevant documentation:

A. Primary Receptacle: A primary watertight, leak-proof receptacle containing the specimen.
B. Secondary Packaging: Zip locks Bag with pouch
C. Outer packaging: protects their contents from outside influences while transit.

Source: (AHRI, 2012; EHNRI, 2013; FMOH/EPHI, 2014).

Appendix 4: Laboratory tests procedures:

4.1. Acid fast staining or Ziehl Neelsen's staining method

The quality of work in AFB diagnostic microscopy depends on a number of factors like specimen collection, quality of reagent, staining technique, reading of smear, reporting and recording and training of technician. However, collecting a suitable specimen and making a good smear are critical as quality of rest of the procedure depends upon it. Smear preparation must be performed carefully and with attention to detail.

Composition:

1. Concentrated carbol fuchsin
   - Basic fuchsin 1 gm
   - Absolute alcohol 10 ml
   - Phenol 5% aqueous solution 100 ml
   - Dissolve the dye in alcohol and add phenol solution
2. Acid alcohol
   - Ethyl alcohol (95%) 97 ml
   - Concentrated hydrochloric acid 3 ml
3. Alkaline methylene blue
   - Methylene blue saturated alcoholic solution 30 ml
   - Potassium hydroxide (1% aqueous sol.) 1 ml
   - Distilled water 99 ml
   - Add potassium hydroxide solution in distilled water and mix methylene blue solution and filter.

Procedure:

- Make a smear of on slide and fix it over the flame methanol fixation.
- Flood the smear with carbol fuchsin and heat from the below till steam comes out.
- Allow the hot carbol fuchsin to act for 3 to 5 min. Do not boil the stain or allow it to dry on the slide.
- Wash the slide with tap water.
- Decolorize with acid alcohol for about 15-20 seconds until the bacterial smear appears faint pink or colour less. Wash it with tap water.
- Counter stain with methylene blue for about 30 seconds.
- Wash with tap water, blots dry the slide.
- Examine the slide under microscope with oil immersion objective
- Acid fast bacteria will take pink / red colour while non acid fast stain blue

WHO Grading Scale

Reports	AFBs seen
Negative	No number of AFB seen in at least 100 fields
Scanty	1-9 AFB / 100 fields
1+	10-99 AFB / 100 fields
2+	1-10 AFB / fields in at least 50 fields
3+	More than 10 AFB/ field in at least 20 fields

Source: (AHRI, 2012)

4.2. Standard Operating Procedures (SOPs) for GeneXpert MTB/RIF assay

1. Principles and Procedures of GeneXpert MTB/RIF assay

Test principle:

The GeneXpert MTB/RIF assay system integrates automates specimen processing, nucleic acid amplification, and detection of the target sequences in specimens using real-time PCR and reverse transcriptase PCR. The system consists of an instrument, barcode scanner, computer, and preloaded software for running tests and viewing the results. The systemic use and requires single disposable GeneXpert cartridges preloaded with the liquid buffers and lyophilized reagent beads necessary for sample processing and host of the PCR process (DNA extraction, and heminested real-time PCR). The primers in the GeneXpert system have amplified a portion of the *rpoB* gene containing the 81 base pair "core" region. The probes are can able to differentiate between the conserved wild-type (*B. globigii,* internal control*) nucleic* acid sequence and mutations in the core region that are associated with RMP/RIF's detection (Cepheid, 2011).

Sample preparation:

The sputum samples are treated with sample reagent (SR). The sample reagent contains NaOH and isopropanol. The SR is added at a 2:1 ratio to the sputum sample and incubated for 15 min at room temperature. The treated sample is transferred into the cartridge, the cartridge is loaded into the GeneXpert instrument, and an automatic process completes the remaining assay steps.

Assay cartridge: it contains Lyophilized *Bacillus globigii* spores which serve as an internal sample processing and PCR control, Processing chamber, Valve body and Reaction tube

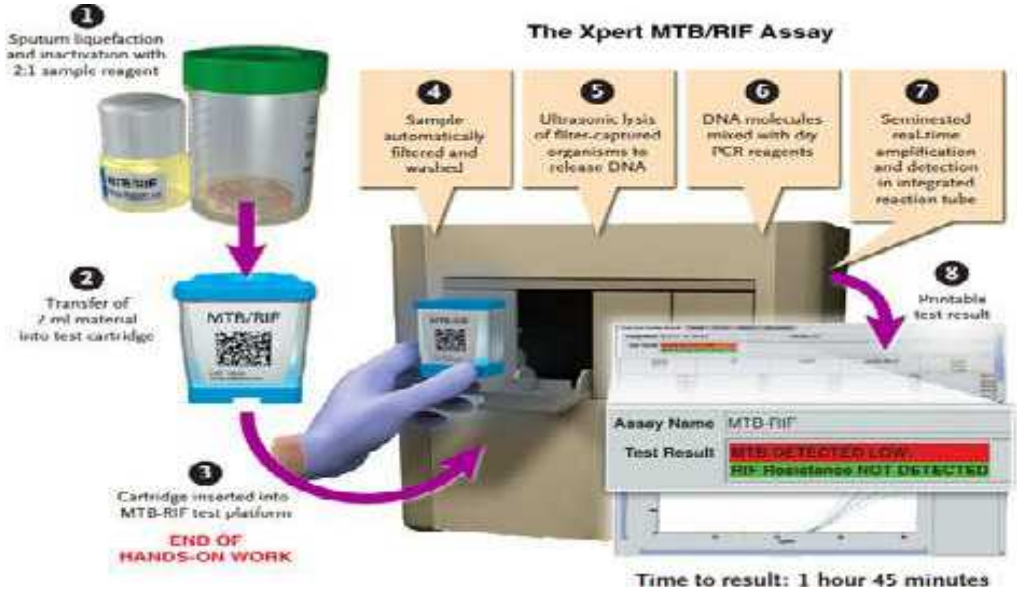

Figure 10: GeneXpert MTB/RIF Assay

Result and Interpretation

1. ***M. tuberculosis* (MTB) detected (Positive)**
    - MTB target DNA is detected.
    - MTB detected - The MTB result will be displayed as High, Medium, Low or Very Low depending on the Ct value of the *M.tb* target present in the sample.
2. ***M. tuberculosis* (MTB) not detected (Negative)**
    - MTB target DNA is not detected
3. **Rifampicin (RMP) resistance detection:** if and only if MTB is detected
    - RMP resistance DETECTED: a mutation in the *rpo* B gene has been detected
    - RMP resistance NOT DETECTED: no mutation in the *rpo* B gene has been detected
    - RMP resistance INDETERMINATE: the MTB concentration was very low and resistance could not be determined.
4. **INVALID results:** Presence or absence of MTB cannot be determined, repeat test with extra specimen. SPC does not meet acceptance criteria, the sample was not properly processed, or PCR is inhibited.

Source: (Boehme, *et al*., 2010; Cephalid, 2011; FMOH/EPHI, 2014)

4.3. Standard Operating Procedures (SOPs) for TB culture and DST

4.3.1. Löwenstein-Jensen (LJ) media preparation (plain, dug-free)

Purpose:

Lowenstein Jensen Medium (LJ Medium) is a selective medium that is commonly used for the cultivation and isolation of **Mycobacterium** (notably *Mycobacterium tuberculosis*) from specimens containing mixed flora. The medium is light green in color with solid consistency. Slants will be inoculated with decontaminated and concentrated sputum specimens. Once good growth is obtained, these positive slants will be stored in a cool, dark place to archive the positive *M. tuberculosis* isolates.

Composition

1. **LJ Medium Base:**

    L-Asparagine = 3.6 g
    Monopotassium Phosphate = 2.5 g
    Magnesium Sulfate = 0.24 g

Sodium Citrate = 0.6 g
Malachite Green = 0.4 g
Potato Flour = 30 g

2. **Supplements:**
   Glycerol = 12 ml
   Egg suspension = 1000 ml

Procedure:

1. Clean and disinfect the work area
2. Dissolve 37.3 g of the medium in 600 mL of purified water containing 12 mL of glycerol.
3. Heat with frequent agitation to completely dissolve the medium.
4. Autoclave at 121°C for 15 minutes.
5. Prepare 1000 mL of a uniform suspension of fresh eggs under aseptic conditions. Avoid whipping air into suspension during the collection and mixing.
6. Aseptically mix the 1000 mL of egg suspension with 600 mL of the sterile Lowenstein-Jensen Medium cooled to 50 – 60°C, avoiding air bubbles.
7. Dispense the finished medium into sterile screw-cap test tubes. Place the tubes in a slanted position and heat/inspissate at 85°C for 45 minutes.
8. Then the media was dated and incubated at 37^{o}C for 24-48hrs to check for sterility.

Source: (Canetti, *et al*., 1963; Hans *et al*., 1998; AHRI, 2012; EHNRI/EPHI, 2013)

4.3.2. Sputum specimen cultivation on solid media (LJ)

The sputa sample cultivation was performed according to Petroff's decontamination method

1. Preparation of Petroff's solutions

a. Phenol red (1%) indicator
   By adding 1g of phenol red to 100 ml of sterile distilled water.
b. NaOH (4%) solution
   By adding 4 g of sodium hydroxide (NaOH) to 100 ml of sterile distilled water.
c. Neutralizing solution
   Prepared by adding 72 ml of HCl (73%) to 1 ml of phenol red (1%) and the volume made up to 1L with sterile distilled water**.**

2. Procedure of sputum culturing upon (LJ) solid media

- ✓ The sputum sample was transferred to cap centrifuge tube
- ✓ NaOH buffer (4%) was added to volume equivalent the amount of sputum sample and left for 15 minutes to less of liquefaction sampling.

- ✓ The sample was centrifuged at 3000 RPM speed for 15 minutes, refrigerated centrifugation used to utilize of increase recuperation of Mycobacterium.
- ✓ The supernatant was emptied precisely into a reasonable compartment containing a mycobactericidal disinfectant
- ✓ Neutralizing buffer was added by Pasteur pipette until the shading is yellow (balance point) or (neutralizing point)
- ✓ Two vials of L.J medium used and added about 2-3 drops of the pellet and incubated in slant position and in semi-closed vial for 3 days at 37°C
- ✓ After this time period, the vials were tightly closed and incubated vertically at 37°C for 6 weeks
- ✓ Then, the outcomes were recorded as positive or negative.
- ✓ Positive result means development and growth of *Mycobacterium tuberculosis*.

Source: (Kent and kubica, 1985; EHNRI/EPHI, 2013).

3. Preparation of MacFarland standard suspension

Principle:

A MacFarland standard is prepared by adding barium chloride to aqueous sulfuric acid. The density of the resulting barium sulfate precipitate is used as a proxy to approximate the colony count of bacterial suspensions. MacFarland No.1 is the equivalent of approximately $3x10^8$ CFU/ml. The MacFarland standard is the most widely used standard for the visual comparison of bacterial suspensions. This allows the adjustment of the turbidity so that the number of bacteria in the suspension will be within the expected range.

Procedure:

1. **Preparation of 1% sulphuric acid**
   - ✓ Add 1 ml H_2SO_4 to 99 ml distilled water
   - ✓ Always add acid to water
2. **Preparation of 1% barium chloride**
   - ✓ Dissolve 1 g of $BaCl_2$ by 100ml of distilled water

Preparation of 1 MacFarland Standard

- ✓ Add 0.1 ml 1% barium chloride solution in to 9.9 ml of 1% sulphuric acid
- ✓ Label the suspension and indicate the last day of use, in a 1 month delay
- ✓ It has to be resuspended just before use

Source: (EHNRI/EPHI, 2013).

4.3.3. Drug susceptibility testing (DST) for MTB

The DST for MTB was tested upon (LJ) solid media
The proportional methods are used in this study to detect of drug susceptibility testing upon solid media (LJ). The Lowenstein- Jensen medium was prepared and added to 4 sterile volumetric flasks (200 ml for each) and mixed with different volume antibiotic as shown in table.

Antibiotic	Abbreviation	Concentration in LJ Media	Concentration of stock solution In solvent	Prepared of antibiotic dilution	Amount of 200 ml LJ preparation
Isoniazid	INH	0.2	100 µg/ml Ethyl Glycol	20 µg/ml	2 ml of dilution
Rifampicin	RMP	40	100 µg/ml Ethyl Glycol	20 µg/ml	2 ml of dilution
Streptomycin	SM	4	2000 µg/ml D. W	4000 µg/ml	1.6 ml of dilution
Ethambutol	EMB	2	1000 µg/ml D. W	20 µg/ml	4 ml of dilution

The mixture of media was added to a screw-cap bottle for slant position and dispensed at 85^{o}C for 45 minutes

DST Test procedure

The test starts with taking representative colonies, and the bacterial suspension is adjusted with sterile distilled water to a turbidity matching a McFarland standard No1 (approximately equivalent to $3x10^{8}$ CFU/ml). Then, two serial dilutions (10^{-2} and 10^{-4}) made from the original suspension are used for inoculation. Finally, 100µL (0.1ml) of the bacterial suspension are inoculated in both drug-containg media and drug-free media, test media containing the drug INH, RMP, SM, EMB are inoculated with dilutions 10^{-2} while two growth control (drug-free) media are inoculated with 10^{-2} and 10^{-4} (to get countable colony) and incubated at 37 ^{o}C. The first reading of drug susceptibility test results is done at 4 weeks (28 days) of incubation, and the number of colonies in each tube is counted. Resistance is expressed as the percentage of colonies on drug-containing media with recommended critical concentration in comparison to the growth on drug-free media. If the proportion exceeds 1% for all first line drugs, the isolate is designated resistant. In order to designate an isolate as susceptible, incubation must be extended and on the 42nd day (6weeks) if the proportion does not exceed 1%, the isolate is considered susceptible (Hans L, *et al*., 1998; O'Grady, *et al*., 2011). The test is repeated instead of "borderline resistance" (about 1% growth on drug containing medium).

Source: (EHNRI/EPHI, 2013).

Appendix 5: Tuberculosis Laboratory Requesting and Reporting Form

1. Patient ID

Patient Full Name: _____Age (Yrs): _____Sex (M/F): _____
Region:_____Zone/Subcity:_____Woreda:_____Kebele:_____
House No: _____ Tel.: _____
Referring Health Facility: _____ Co-infection: _____

2. DISEASE TYPE & TREATMENT HISTORY:

Site: Pulmonary Extra pulmonary (specify_____
Registration Group: New Relapse After default after failure of 1st treatment
After failure of retreatment other
Previous TB drug use: New First line second line MDR TB contact

3. REQUEST FOR TESTING AT TB LABORATORY:

Reason: Diagnosis: If diagnosis, presumptive TB / RR-TB/ MDR-TB? Yes No
Follow up: If Follow up, at _____ months during treatment
Follow up at ___ months after treatment
Specimen: Sputum Other (Specify): _____
Date specimen collected: ____/____/____ (Ethiopian Calendar)
Requested tests: Microscopy Xpert MTB/RIF test Culture DST Line probe assay
Person requesting examination: Name: _____ _Date_____

4. LABORATORY RESULT:

Sample Number: _____ Date specimen received: ____/___/____ (Ethiopian Calendar)
Date of result: ____/____/___ Examined by (name and signature): _____

a. Microscopic examination result (Ziehl Neelsen (ZN):

Negative	**Positive**			
	1-9(Scanty)	1+	2+	3+

b. GeneXpert MTB/RIF test result (*to be completed in the laboratory)*

Date sample collected: ____/___/___ Date of result: ____/___/___
Examined by (name and signature): _____
M. tuberculosis: Detected Not detected Invalid / No result / Error
Rifampicin resistance: Detected Not detected Indeterminate result

c. TB Culture result:

Date	Media used (liquid or solid)	Lab. S.No.	Result (Tick One)						
			Negative	1-9 (<10 C)	+ (10-100 C)	++ (>100 C)	+++ confluent growth)	NTM[1]	Contaminated

[1] Non-tuberculous mycobacteria.,C= colonies **Source:** (ALERT and St. Peter's hospitals, 2015

Appendix 6: MDR-TB Color Plate Test (color plate test) Result Collection Sheet

S. no.	Sample ID	Sample collection Date	Sample processing date	Date of first positivity (or contamination)	**If contaminated** write BACTERIAL or FUNGAL or MIXED & leave the counts blank	On date of first positivity Enter the count of colonies below				**Photograph** taken on date of first positivity (TICK)	Interpretation of DST	Also read on day **21** (date_____) Enter the count of colonies below					Interpretation of DST
						Detection Clear (Top)	INH Green (Right	RMP Yellow (Bottom	PZA Blue (Left			**Date**	Detection Clear	INH Green	RMP Yellow	PZA Blue	

INH= Isoniazid, RMP=Rifampicn, PZA=Pyrazinamide Source: (SOP, 2010)

Appendix 7: LJ Culture Result Collection Sheet

S. no.	Sample ID	Sample Collection date	Sputum culture date	Results											Remark
				Positive for Mycobacterium Tuberculosis Complex (MTBC)								Non tuberculosis mycobacterium (NTM)	Negative	Contaminated	
				1st week	2nd week	3rd week	4th week	5th week	6th week	7th week	8th week				

Note:

- **1-9 colonies actual count**
- **10-99 colonies (+1)**
- **≥ 100 colonies (+2)**
- **Confluent growth (+3)**

Source: (FMOH/EPHI, 2014)

Appendix 8: **LJ indirect proportional DST result recording sheet**

S. no.	Patient ID	Sample collection Date	Isolate tested date	4^{th} weeks reading						6^{h} weeks reading						Result Interpretation (R, S)
				Growth control		Test drugs				**Growth control**		Test drugs				
				10^4	10^2	INH 10^2	RMP 10^2	SM 10^2	EMB 10^2	10^4	10^2	INH 10^2	RMP 10^2	SM 10^2	EMB 10^2	

INH= Isoniazid, RMP=Rifampicn, EMB= Ethambutol, SE Streptomycin
R= resistant
S= susceptible

Source: (FMOH/EPHI, 2014)

Appendix 9: Operational definitions

1. **Presumptive MDR-TB**: refers to a patient who presents with symptoms or sign suggestive of MDR TB.
2. **New patients**: have never been treated for TB or have taken anti-TB drugs for less than 1 month.
3. **Previously treated patients**: A patient who has received 1 month or more of anti-TB (first line or second line) drugs in the past. They are further classified by the outcome of their most recent course of treatment as follows:
    - **Relapse patients** have previously been treated for TB, were declared *cured* or *treatment completed* at the end of their most recent course of treatment, and are now diagnosed with a recurrent episode of TB (either a true relapse or a new episode of TB caused by re-infection).
    - **Treatment after failure patients** are those who have previously been treated for TB and whose *treatment failed* at the end of their most recent course of treatment.
    - **Treatment after loss to follow-up patients** has previously been treated for TB and was declared lost to follow-up at the end of their most recent course of treatment. (These were previously known as *treatment after default* patients.)
4. **Wild type bacteria**: is the original strain of bacteria that has not undergone any form of mutation genetically or phenotypically.
5. **Mutant bacteria**: is a strain that has undergone mutational changes naturally or due to the presence of mutagenic agents such as drugs
6. **Drug-susceptible isolate:** an organism's ability to be killed by a particular drug; the wild type bacteria, not previously exposed to a drug.
7. **Drug-resistant isolate**: an organism's ability to grow despite the presence of a particular drug, if more than 1% of the bacteria exhibit resistance to that drug.
8. **Mono-resistant TB**: TB that is caused by strains of *M. tuberculosis* (MTB) that is resistant to one first-line anti-TB drug only.
9. **Isoniazed-resistant TB**: TB caused by strains of MTB that are resistant to only INH
10. **Rifampicin-resistant TB**: resistance to RMP detected using phenotypic or genotypic methods, with or without resistance to other anti-TB drugs.
11. **Pyrazinamide-resistant TB**: TB caused by strains of MTB that are resistant to only PZA.
12. **MDR-TB**: TB caused by strains of MTB that are resistant to at least INH and RMP.
13. **XDR-TB**: resistance to any fluoroquinolone, and at least one second-line injectable agent (amikacin, kanamycin, and/or capreomycin), in addition to MDR-TB.
14. **TDR-TB**: is a complete drug resistance where patients of TB develop resistance to all available drugs although WHO has not yet defined it.
15. **Reference standard**: It is used as the reference method for assessing the performance characteristics of another test method; the best available approximation of a true result, generally indicating a test method that is currently accepted as reasonably, but not necessarily, 100% accurate.
16. **Confidence interval**: the confidence interval quantifies the uncertainty in measurement; usually reported as the 95% confidence interval, the range that we can be 95% certain covers the true value.
17. **Accuracy**: the percentage of correct results obtained by the test under evaluation compared with the results of a reference or 'gold standard' test. Usually it expressed as the number

of correct results (true positive and true negative) divided by the total number of results, multiplied by 100.

18. **Reliability**: yielding the same or compatible results is measured by a percentage and if you get exactly the same results every time then they are 100% reliable.
19. **Sensitivity**: the probability (percentage) of the screening test correctly identifying diseased subjects (determined by the result of the reference or 'gold standard' test)
20. **Specificity**: the probability of the screening test correctly identifying non-diseased subjects (determined by the result of the reference or 'gold standard' test)
21. **Positive predictive value (PPV):** is the probability that subjects with a positive screening test truly have the disease or the probability that a positive result accurately indicates the presence of infection.
22. **Negative predictive value (NPV):** is the probability that subjects with a negative screening test truly don't have the disease or the probability that a negative result accurately indicates the absence of infection.
23. **False negative**: is a test result that indicates a person does not have a disease or condition when the person actually does have it
24. **False positives** are test results reported as positive for a disease eg. MTB species not present in the patient specimen.
25. **Direct DST**: DST performed on primary clinical specimens without prior bacterial culturing.
26. **Indirect DST**: DST performed on pure isolates cultured from primary clinical specimens
27. **Critical concentration** is the amount of drug in the medium that inhibits the growth of susceptible organisms (wild type) but not that of resistant mutants. It is used as a cut off in qualitative drug susceptibility assays.
28. **Turnaround time**: pertaining to the time elapsed between the dates of inoculation to date of interpretable/valid positive results.
29. **Bio-safety** is defined as the discipline addressing the safe handling and containment of infectious microorganisms and hazardous biological materials.
30. **Quality assurance** (QA): an ongoing process of monitoring a system for reproducibility or reliability of results, with which corrective action can be instituted if standards are not met.
31. **Blinding:** Interpreting a test result without knowledge of a patient's condition or previous test results.

Source: (TDR, 2010; WHO, 2008; WHO, 2010; Zhang and Yew, 2009; WHO, 2013)

CurriclumVitae (May, 2017)

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II. Educational Backgrounds

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- Lecturer, College of Veterinary Medicine, Mekelle University, Ethiopia, Giving Lecture, involving research works and community services since September 2010.
- Technical Assistant, College of Veterinary Medicine, Mekelle University, Ethiopia. Handling and assisting practical classes and involved in different research activities, (2004 -2005).

2. Clinical duty experiences

- Agricultural office worker as Animal health assistant, Prevention and control of animal diseases plus promotion of public health, Ministry of Agriculture, S. Wollo Agriculture office, P.O. Box 80 Dessie, Ethiopia from 2001-2003.

IV. Adviser ship activities/Student supervision (DVM students):

I have been advised students during their senior seminars and DVM thesis research works with different topics/titles:

- Prevalence of Ovine Haemonchosis in and around Bahir Dar, Ethiopia.
- Major Husbandry and Health Care Practice of Small Scale Poultry Farms in and around Mekelle, Ethiopia.
- Prevalence of Bovine Hydatidosis and its Economic Significance in Dire Dawa Manucipality Abattior, Ethiopia.
- Prevalence and Economic Significance of Bovine Fasciolosis in Mekelle Municipal Abattoir, Ethiopia.
- Analysis of Current Problems facing Hatchery Operation in Mekelle Poultry Farms, Mekelle, Ethiopia.
- Prevalence of small ruminant lung worm infection in and around Mekelle, Ethiopia.
- Economic Importance of Newcasstle Disease in Village chickens, Ethiopia.
- Major Respiratory Disease of Camel and its Managments in Ethiopia.
- Isolation and Identification Of *Ornithobacterium Rhinotracheale* In Chickens In Local Market And Farms Of Addis Ababa, Ethiopia

V. Major community/voluntary services:

- I have been participated on different community services such as vaccination campaign, clinical services, animal welfare and public health promotion while celebrating major professional holidays (celebration of world rabies day and world veterinary day) and periodically.
- I have been also participated on breeding, feeding, husbandry and health managements of dairy and poultry in Tigray region, Ethiopia.

VI. Research Activities:

- I have been conducted the following researches both as Principal investigator and Co principal investigator:
  - ✓ Study on Epidemiology (Sero and Molecular) of Newcastle Disease Virus isolates in Village Chickens in Selected Woreda of Tigray Region, Ethiopia.
  - ✓ Pilot Study on the Prevention and Control of Newcastle Disease Using Combined Conventional Methods and Participatory Learning and Action in Two Selected Villages of Enderta Wereda, Tigray, Ethiopia.
  - ✓ Promotion of food security by breeding improvement through Synchronization of dairy cattle in selected Woredas of Tigray region, Ethiopia.

VII. Publication in Peer-reviewed Journals:

1. Miheret, M., **Biruk, M**., Habtamu, T., Ashwani, K. (2013): Prevalence of Bovine Hydatidosis and Its Economic Significance in Dire Dawa Manucipality Abattior, Ethiopia. *Momona Ethiopian Journal of Science*, **5**:1.
2. Kassaw Amssalu Tadesse **Biruk Mekonnen Woldie**, Yohannes Tekle Assfaw, Habtamu Tassew Tarekegn, Edlu Jorga and Essayas Gelagay Leykun: Evaluation of oral vaccicinationof village chickens against newcastel disease with I-2 vaccine coated parboiled cracked maize in Enderta district, Tigray Ethiopia. *African journal of Biotechnology*. Vol. 13(43), pp 4183-4187, 22 october, 2014.
3. Sero-Prevalence Study Of Newcastle Disease In Local Chickens In Selected Woreda Of East Showa Zone, Ethiopia (2010) (ongoing, as PI)
4. Study on Epidemiology (Sero and Molecular) of Newcastle Disease Virus isolates in Village Chickens in Selected Woreda of Tigray Region, Ethiopia. (2012) (ongoing, As PI)
5. Isolation and Identification Of *Ornithobacterium Rhinotracheale* In Chickens In Local Market and Farms Of Addis Ababa, Ethiopia (2014) (ongoing, co- Author)
6. Prevalence of Ovine Haemonchosis in and around Bahir Dar, Ethiopia. (2012) (ongoing, co-Author)

IV. References:

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DECLARATION

Statement of Author:

I, the under signed, declare that this thesis is my original work and that all sources of materials used for this thesis have been properly acknowledged. This thesis has been submitted in partial fulfillment of the requirements for MSc. degree at Addis Ababa University, School of Medicine, College of Health Sciences, Department of Microbiology, Immunology and Parasitology and is deposited at the University or College library to be made available to borrowers under rules of the library. I solemnly declare that this thesis is not submitted to any other institution anywhere for the award of any academic certificate, diploma, or degree.

Name: Biruk Mekonnen Signature: _____ Date of Submission: _____

Approvals of Academic Advisors:

This thesis has been submitted for examination with our approval as University Advisors:

Dr. Tamrat Abebe (MSc, PhD) Signature: _____

Dr. Adane Mihret (DVM, MSc. PhD) Signature: _____