ADDIS ABABA UNIVERSITY SCHOOL OF GRADUATE STUDIES

EVALUATION OF DIRECT COLORIMETRIC MTT ASSAY FOR DETECTION OF RIFAMPICIN AND ISONIAZID RESISTANCE IN *MYCOBACTERIUM TUBERCULOSIS* UNDER PROGRAM CONDITION

By

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List of Abbreviations

AHRI	Armauer Hansen Research Institute
ALERT	All Africa Leprosy and Tuberculosis Research, Rehabilitation and Training Center
CDC	Centers for Disease Control
CFU	Colony Forming Unit
CTAB	Cetyltrimethylammonium bromide
DMF	Dimethyl formamide
DMSO	Dimethlysulphooxide
DNA	Deoxyribose nucleic acid
DST	Drug susceptibility test
E	Ethambutol
EHNRI	Ethiopian Health and Nutrition Research Institute
EMB	Ethambutol
H	Isoniazid
HIV	Human Immunodeficiency Virus
INH	Isoniazid
IUATLD	International Union Against Tuberculosis and Lung Disease
LJ	Lowenstein-Jensen
MDR-TB	Multi-drug Resistant Tuberculosis
MOH	Ministry Of Health
MTT	3-(4,5- Dimethylthiazol –2-Yl)-2, 5- Diphenyl Tetrazolium Bromide
NHS	National Institute for Health and Clinical Excellence
NCCLS	National Committee for Clinical Laboratory Standards
OADC	Oleic acid Albumin Dextrose Catalase
OD	Optical Density
PANTA	Antibiotic mixture (Polymyxin B, Amphotericin B, Nalidixic acid, Trimithoprim, Azlocillin)
PBS	Phosphate Buffered saline
PCR	Polymerase Chain Reaction

PZA	Pyrazinamide
R	Rifampicin
RIF	Rifampicin
RODU	Relative optical density unit
S	Streptomycin
SDS	Sodium Dodecyl Sulfate
TB	Tuberculosis
TE	Tris- EDTA
WHO	World Health Organization
XDR	Extensively drug resistant
Z	Pyrazinamide

Abstract

The spread of drug resistant tuberculosis especially MDR-TB in the world remains a major public health problem. Early diagnosis of TB and rapid detection of drug resistance is an urgent priority for proper patient management and to control dissemination of resistant strains. Simple, rapid and inexpensive methods of detecting drug resistant TB are essential for effective treatment. The MTT assay has been developed as a rapid, simple and inexpensive method for the detection of rifampicin (RIF) resistant *M. tuberculosis* with promising results. However, the method was not evaluated under program condition for the detection of RIF resistance and not yet standardized and evaluated for the detection of isoniazid (INH) resistance. The objectives of this study were thus to evaluate the direct MTT assay for detection of RIF resistance in *M. tuberculosis*, and to standardize and evaluate the method for INH resistance under program condition. Sputum samples were decontaminated with 4% NaOH and cultured on Lowenstein-Jensen (LJ) media and Middlebrook 7H9 (M7H9) broth media. Drug susceptibility test was done on LJ media using the proportion method. MTT assay was done on M7H9 broth containing 0.2 μg/ml INH, 2 μg/ml RIF and drug free control. Formazan production was quantified by measuring the optical density (OD) at 570 nm and relative optical density unit (RODU) was calculated as a ratio of drug containing tube to drug free control. A strain was defined as resistant if it has a RODU > 0.5 and susceptible if it has RODU < 0.2. Out of 115 isolates tested for RIF resistance, 6 were identified as RIF resistant using the MTT assay but 7 using the proportion method. 0.2 μg/ml INH concentration was taken as a critical concentration to be used for direct MTT assay for detection of INH resistance. Out of 18 isolates tested for INH resistance, 2 were found INH resistant both with proportion method and MTT assay. The direct MTT assay result matched 99% for RIF and 100% for INH with the conventional method. The MTT assay gave 94% interpretable result for rifampicin and 100% for isoniazid at 2nd week. In the conventional method it takes 7-14 weeks to get a final drug susceptibility test result. Therefore, this rapid, simple and inexpensive method could be an alternative method for drug susceptibility test under program condition. Further studies are required in regional labs to validate the method.

Key words: *Mycobacterium tuberculosis*, MDR-TB, MTT assay, Proportion method, Isoniazid, Rifampicin.

1. Introduction

1.1. *Mycobacterium tuberculosis*

Mycobacterium tuberculosis is an etiologic agent of tuberculosis. It is classified under the class actinomycetes, order actinomycetales and family mycobacteriaceae. *M. tuberculosis* is the member of the pathogenic *M. tuberculosis* complex along with, *M. bovis*, *M. africanum*, *M. microti*, *M. bovis* BCG and *M. canetti* (Cole, 2002; Miltgen *et al*., 2002).

Mycobacterium tuberculosis is an obligate an aerobe, non-spore forming, non-capsulated, and non-motile rod shaped bacterium with a length of 2-4 µm and a width of 0.2-0.5 µm (Haldane, 2000; Barrera, 2007). It is a facultative intracellular parasite, usually of macrophages, and has a slow generation time of 12-24 hours. The bacilli grow best at neutral pH and temperature of 37 ^{0}C (Barrera, 2007).

The cell wall structure of *M. tuberculosis* deserves special attention due to its unique property among prokaryotes (McMurray, 2004; Brennan, 2003). Over 60% of its cell wall is composed of complex lipids, which includes mycolic acids (long-chain fatty acids containing 60-to 90-carbon atoms), cord factor and wax D (Fu and Fu-Liu, 2002; Barrera, 2007). This unusual cell wall structure endows the bacterium with resistance to many antibiotics, acids, alkalis, osmotic lyses, lethal oxidations and dehydration. The resistance to acids and alkalis is useful in the isolation of *M. tuberculosis* from contaminated clinical specimens such as sputum (McMurray, 2004). Because this lipid-rich cell wall, which is relatively impermeable to various basic dyes, *M. tuberculosis* stained with carbol-fuchsin dye retains it when decolorized with acid-alcohol. This gives it the name, acid fast bacilli (AFB) (McMurray, 2004; Barrera, 2007).

The complete genome sequence of the best-characterized strain of *M. tuberculosis*, H37Rv, has been determined, and comprised of 4, 411, 529 base pairs containing 3, 995 predicated protein-coding sequences, over 4,000 genes and a relatively high G + C content of 65.6% (Cole *et al*., 1998; Camus *et al*., 2002). The knowledge of the

complete sequence of the *M. tuberculosis* genome has allowed many genetic approaches to study the physiology, pathogenicity as well as the biology of this slow growing organism (Smith, 2003)

1.2. Tuberculosis: the disease

Tuberculosis (TB) is a chronic, infectious disease that primarily attacks the lungs (pulmonary tuberculosis). It also affects intestines, bones and joints, skin, genitourinary tract, lymphatic, and nervous systems (extra pulmonary tuberculosis) (CDC, 2000). The disease has been neglected for several years by the international health community. However, due to its high incidence, mortality and morbidity rate in the 1990s a new understanding of the global tuberculosis burden and acceleration in tuberculosis control efforts worldwide came into picture (Raviglione, 2003). The World Health Organization declared tuberculosis a global emergency in 1993 in recognition of its growing importance as a public health problem (WHO, 1993).

1.2.1. Global epidemiology of tuberculosis

TB is the most common contagious and one of the top killer diseases worldwide. Approximately, one third of the world population is infected with the TB bacillus (WHO, 2005). According to WHO (2007) report, globally there were an estimated 8.8 million new TB cases and a total of 1.6 million deaths in 2005. TB accounts for 2.5% of the global burden of disease, for 26% of preventable deaths, and is a leading infectious cause of death among young women (WHO, 2001b). It is estimated that between 2002 and 2020, approximately 1000 million people will be newly infected, over 150 million people will get sick, and 36 million will die of TB if proper control measures are not instituted (Sharma and Mohan, 2004).

The incidence and mortality caused by TB is not the same world wide. Over ninety-five percent of all cases and 99 % of deaths occur in developing countries, with the greatest burden in sub-Saharan Africa and South East Asia. About 75 % of it occurs within the economically productive age group of 15-54 years (Dye, 2006; WHO, 2006). Three

regions of the world, Africa, Western Pacific and South-East Asia share the highest percentage (85%) (WHO, 2007). Of the 199 countries registered by WHO for case notification, 22 are notified as high-burden countries (HBC) for TB. According to WHO (2007) report, these countries account for approximately 80% of the estimated number of all forms of new TB cases and deaths arising worldwide each year.

1.2.2. Tuberculosis epidemiology in Ethiopia

Ethiopia is one of the HBC reported for tuberculosis. The country ranks 8th in 2005 based on estimated number of all forms of TB cases (WHO, 2007). According to WHO (2007) report, the incidences of TB of all forms and smear positive TB stands at 344 and 152 per 100,000 populations, respectively. The prevalence and mortality of TB of all forms estimated as 546 and 73 per 100,000 populations, respectively.

1.2.3. Transmission and pathogenesis

TB spreads from person to person through the air. Droplet nuclei are produced when persons with pulmonary tuberculosis cough, sneeze, speak, or sing. A single cough may generate up to 3000 droplets where each one contains 1-3 tubercle bacilli (CDC, 2000; Vijayan, 2002). These tiny particles with a size of 1-5 microns in diameter can remain suspended in the air for several hours depending on the environment. Transmission occurs when another person inhales air containing these droplet nuclei (CDC, 2000).

After inhalation, the infectious droplet nuclei passes down the bronchial tree and lodge in the lung alveoli in the peripheral airways (Frieden *et al.*, 2003). The bacilli are taken up by alveolar macrophages, which play a central role in tuberculosis, initiating a cascade of events that result in either successful clearing or inhibition of the initial infection or in progression to active disease (Cosma *et al.*, 2003; Frieden *et al.*, 2003). Subsequently, if it is contained, *M. tuberculosis* spreads through lymphatic channels to regional lymph nodes and through the blood stream to more distant sites (Lillebaek *et al.*, 2002: Doherty and Andersen, 2004).

At the cellular level, alveolar macrophages infected with *M. tuberculosis* interact with T lymphocytes via MHC molecules and several cytokines (Frieden *et al*., 2003). The infected macrophages release interleukins, which stimulate predominantly CD4-positive T lymphocytes. This, in turn, stimulates the phagocytosis and intracellular killing of tubercle bacilli in the macrophage (Kaufmann, 2002; Frieden *et al*., 2003; Doherty and Andersen, 2004). Activated T lymphocytes and macrophages are attracted and form granulomas that limit further replication and spread of the organism (Saunders and Cooper, 2000; Kaufmann, 2002; Cosma *et al*., 2003; Frieden *et al*., 2003).

Progression from TB infection to active disease occurs when the TB bacilli overcome the immune system defenses and begin to multiply (Cosma *et al*., 2003; Doherty and Andersen, 2004). Infection can progress to disease very quickly or many years after infection. In approximately 5% of all infected individuals disease occurs within 2-5 years of infection (CDC, 2000). In another 5%, infection progress to active disease once in their life. Overall, approximately 10% of all persons infected with *M. tuberculosis* develop active disease sometime during their life and the remaining 90% will stay infected, but free of disease, for the rest of their lives (CDC, 2000; Cosma *et al*., 2003). However, some factors such as HIV infection, diabetes and some medical conditions increase the risk that TB infection will progress to active disease. The risk may be 3 times greater to more than 100 times greater for persons who have these conditions than for those who do not (CDC, 2000).

1.2.4. Clinical manifestation

In patients where Pulmonary TB becomes an active disease, the symptoms include a productive and prolonged cough for more than three weeks, chest pain and bloody sputum, fever, chills, night sweats, appetite loss, weight loss and fatigue (CDC, 2000). These Clinical symptoms develop in only a small proportion (5-10 %) of infected healthy people while the majority (90%) with latent TB infection will have no symptom (McMurray, 2004).

1.2.5. Diagnosis

Accurate and early diagnosis of TB is crucial to effective patient management and TB control (Perkins, 2000; Nahid *et al*., 2006). Bacteriological methods such as AFB smear microscopy examination and growth on solid culture media are the most commonly used laboratory methods for routine diagnosis (Drobniewski *et al*., 2003). Conventional culture method (Löwenstein-Jensen medium) is the "gold standard" for the diagnosis of active TB especially in low-resource countries. AFB smear microscopy is rapid and inexpensive and is thus very useful method to identify highly contagious patients. It detects bacilli number in the range of 5,000-10,000 per ml sputum. Culture is used to detect cases with 10-1,000 bacilli per ml of sputum and for drug susceptibility testing (Prasad *et. al*., 2001; de Waard and Robledo, 2007).

Chest x-ray that reveals cavitations, calcification (healed disease), and nodes in the upper lobes is another diagnostic method for active pulmonary tuberculosis (Nahid *et al.*, 2006). Radiometric and non radiometric BACTEC liquid culture systems have been in common use in level III mycobacteriology laboratories in developed countries for more than a decade (Perkins, 2000). The new rapid molecular methods like PCR and nucleic acid amplification (NAA) are widely used in developed countries (Watterson and Drobniewski, 2000; Perkins, 2000; Nahid *et al.*, 2006).

1.2.6. Treatment

The overall goal of treating tuberculosis is to cure the patient and minimize its transmission. Thus, successful treatment of tuberculosis has benefits both for the patient and the community in which the patient resides. Regimens for the treatment of TB must contain multiple drugs to which the organisms are susceptible. The first line drugs used in the treatment regimen includes rifampicin (R), isoniazed (H), pyrazinamide (Z), ethambutol (E) and streptomycin (S). For most patients, the preferred regimen for treating TB disease consists of an initial 2-month phase of four drugs: isoniazid, rifampicin, pyrazinamide, and ethambutol followed by a 4-month continuation phase of isoniazid and rifampicin (6-month regimen) (CDC, 2000; CDC, 2003, WHO, 2003; NHS, 2006).

Alternatively, a 9-month regimen of isoniazid and rifampicin is acceptable for persons who cannot or should not take pyrazinamide (e.g., pregnant women). In this case a combination of isoniazid, rifampicin, and ethambutol are given in the initial 2-month phase followed by a 7-month continuation phase of isoniazid and rifampicin (CDC, 2003)

In Ethiopia, all the five drugs, streptomycin, ethambutol (E), isoniazid (H), rifampicin (R) and pyrazinamide (Z) are used as anti TB drugs. These drugs are implemented in three different categories of treatment regimens. Each regimen is recommended for a defined group of patients and their formulation includes the duration of treatment, type of drug and frequency of delivery.

Category I: Short course chemotherapy for smear positive pulmonary tuberculosis (PTB) and seriously ill smear negative PTB and extra-pulmonary tuberculosis (EPTB) cases. This regimen consists of 2 months treatment with E, R, H, and Z during the intensive phase followed by 6 months E and H in the continuation phase (2S(RHZ)/ 6(EH) or 2E(RHZ)/6(EH))

Category II: Re-treatment regimen. The treatment regimen for this category consists of 3 months treatment with E, R, H, and Z during the intensive phase followed by 5 months treatment with E, R, H in the continuation phase (2SE(RHZ)/1E(RHZ) $5E_3(RH)_3$) .

Category III: Short course chemotherapy for smear negative PTB and EPTB. The regimen consists of 8 weeks treatment with R, H and Z during the intensive phase followed by 6 months with E and H in the continuation phase (2(RHZ)/ 6 (EH)) (MOH, 2005).

1.3. Drug resistance in *M. tuberculosis*

Drug resistance is defined as a decrease in sensitivity to a sufficient degree to be reasonably certain that the strain concerned is different from a wild strains of human type that have never come in contact with the drugs (WHO, 1997). Generally, when one percent or more of organisms isolated in culture media are resistant to an anti-tuberculosis drug, therapeutic success is less likely to occur. It is then that the strain is considered resistant to the drug (Long, 2000).

Drug resistance can occur by random spontaneous mutation at any time during bacterial replication (Drobniewski and Balabanova, 2002). In any large population of tubercle bacilli, there will be several naturally occurring drug-resistant mutants (Pfyffer, 2000). Random mutation that confers resistance to five of the first-line anti-tuberculosis drugs occurs at low but predictable frequency in wild strains. The probability of incidence of drug resistant mutants is 10^{-8} for rifampicin and 10^{-6} for isoniazid, pyrazinamide, ethambutol and streptomycin (Rattan, 1998; Pfyffer, 2000). Since the mutations conferring drug resistance are chromosomal, the likelihood of a mutant being simultaneously resistant to two or more drugs is the product of individual probabilities. For instance, spontaneous mutation resulting in combined resistance to INH and RMP is developed at a rate of 1 in 10^{14} (10^6x 10^8) (Rattan, 1998).

A patient is said to have drug-resistant TB if the strain causing the disease is resistant to one or more of the first-line drugs (Long, 2000). Multidrug-resistant TB (MDR-TB) defined as resistant to at least isoniazid and rifampicin has been an area of growing concern in the last decade as it is one of the most important threats to tuberculosis control and management of patients in the era of HIV (Espinal, 2003; Prasad, 2005). Despite its global magnitude, the problem of MDR-TB has not been addressed adequately until 1997 (Prasad, 2005). The 1994-97 global project on anti-tuberculosis drugs resistance surveillance initiated by WHO and International Union Against Tuberculosis and Lung Diseases (IUATLD) has enlightened the scientific community regarding the magnitude of

MDR-TB world-wide. It is now clear that MDR-TB is ubiquitous and its presence in some countries has reached epidemic levels (Espinal, 2003).

Moreover, the problem of extensively drug resistant (XDR) strains has recently been introduced. XDR-TB is defined as TB strains showing resistance to rifampicin and isoniazid plus resistance to any fluoroquinolone and at least one of the three injectable second-line drugs (capreomycin, kanamycin, or amikacin) used in anti-TB treatment (CDC, 2006). XDR-TB now constitutes an emerging threat for the spread of drug resistance, especially in HIV-infected patients (Gandhi, 2006).

1. 3.1. Causes of drug resistance

Resistance of *M. tuberculosis* to anti-tuberculosis drugs is a man-made amplification of the natural phenomenon (Pfyffer, 2000). Wild isolates of *M. tuberculosis* that have never been exposed to anti-tuberculosis drugs are virtually never clinically resistant. It is the combination of spontaneous drug resistance developed in bacteria and improper anti-tuberculosis drugs management that leads to the development of resistance (Drobniewski and Balabanova, 2002). Hence emergence of drug resistance is a result of the selection of pre-existing resistant mutants in the original bacterial population by "drug pressure" (Long, 2000)

Exposure to a single drug because of poor adherence to treatment, inappropriate prescription, irregular drug supply, and poor drug quality suppresses the growth of bacilli susceptible to that drug (WHO, 2000; Drobniewski and Balabanova, 2002). However it permits the growth of the small number of resistant organisms within that bacterial population which are arising by spontaneous mutation (Faustini *et al*., 2006). A population wholly resistant to a single drug then emerges, and continuing inadequate treatment goes on to select from among this population the small number of organisms which have mutated to have further drug resistance. Resistance to one drug may therefore become resistance to two drugs, and then sequentially to many drugs (Aziz and Wright, 2005; Faustini *et al*., 2006).

The emergence of drug resistant *M. tuberculosis* is also associated with a variety of management, health provider, and patient-related factors. These may include the lack of availability of a standardized therapeutic regimen, poor implementation, and frequent or prolonged shortages of drug supply in areas with inadequate resources or political instability (Nachega and Chaisson, 2003; Aziz and Wright, 2005). Use of drugs or drug combinations of unproven quality with low bioavailability is an additional concern in the development of drug resistant TB (WHO, 1997).

1.3.2. Type of drug resistance

Clinically, drug resistance in TB may be broadly classified as primary and acquired (Prasad, 2005). Primary drug resistance may be defined as drug resistance in a patient who has not received any anti-tuberculosis treatment in the past. The resistance that develops in a patient who has received prior chemotherapy is defined as acquired drug resistance (Sharma and Mohan, 2004; Prasad, 2005). Because of the difficulty to confirm the validity of the patients' past history of treatment, the World Health Organization and the International Union Against Tuberculosis and Lung Diseases (IUATLD), have replaced the term primary resistance by the term "drug resistance among new cases" and acquired resistance by the term "drug resistance among previously treated cases" (WHO, 2000).

1.3.3. Molecular mechanisms of drug resistance

The genetic bases of resistance development to most anti-tuberculosis drugs are established (Rattan *et al*., 1998). Resistance to first line anti-tuberculosis drugs has been linked to mutations in at least 10 genes; *katG, inhA, ahpC, kasA* and *ndh* for INH resistance; *rpoB* for RIF resistance, *embB* for EMB resistance, *pncA* for PZA resistance and *rpsL* and *rrs* for STR resistance (Johnson *et al*., 2006).

Rifampicin (RIF) is a highly efficient bactericidal drug. It interferes with the transcription of mRNA by binding to β-subunit of RNA polymerase (*rpoB*) (Telenti, 1998; Mokrousov *et al*., 2002). RNA polymerase is an essential enzyme composed of four different

subunits (α, β, β' and σ) encoded by *rpoA, rpoB, rpoC* and *rpoD* genes, respectively (Johnson *et al*., 2006). RIF binds to the β-subunit hindering transcription and thereby killing the organism. RIF resistance is caused by missense mutations, nucleotide deletions or insertions in an 81–base pair region of the *rpoB* gene, which codes for the beta subunit of DNA-dependent RNA polymerase (Nachega and Chaisson, 2003). Overall, more than 95% of all missense mutations are located in an 81-bp core region of the *rpoB* gene between codons 507–533. This results in the development of RIF resistance in a large majority of resistant isolates (70%) by decreasing Rifampicin-binding affinity (Mokrousov *et al*., 2002; Johnson *et al*., 2006).

In general mutations in the genome of *M. tuberculosis* that confers resistance to RIF is rare and occurs at a rate of 2.25×10^{-10}, resulting in resistance of 1 in 10^8 in a drug-free environment (Nachega and Chaisson, 2003; Johnson *et al*., 2006).

Isoniazid (INH) is a potent drug that inhibits the synthesis of mycolic acids which is an important component of the cell wall. It is a pro-drug that requires cellular activation by mycobacterial enzyme before exerting toxic effects on the bacillus (Barry *et al.,* 1998; Slayden and Barry, 2000). The enzyme that activates INH is the catalase peroxidase encoded by *KatG*. The activated INH is converted to several toxic products including 4-pyridylmethanol and isonicotinic acid. The toxic substance affects mycolic acid biosynthesis (Slayden and Barry, 2000). A lack of mycolic acid synthesis eventually results in loss of cellular integrity and death of bacteria (Barry *et al*., 1998).

Resistance to INH develops primarily through failure to activate the pro-drug due to mutations in the *katG* gene (Slayden and Barry, 2000). As indicated by Johnson *et al.* (2006), resistance to INH is most frequently arising due to mutation in mycobacterium isolates. Most mutations are found between codons 138 and 328 with the most commonly observed gene alteration being at codon 315 of the *katG* gene (Slayden and Barry, 2000). In addition to mutations in *katG,* mutations in other loci, such as the alkylhydroperoxidase (*AhpC)* and the enoylreductase (*InhA*), contribute to INH resistance (Barry *et al.,* 1998). It is found that about 42-58% isolates has mutation in the katG gene,

21-34% in the *InhA* gene and 10-15% in the *AhpC* gene (Telenti, 1998). The rate of mutation for isoniazid is 10^{-8} resulting in resistance in 1 out of 10^{6} bacilli (Long, 2000).

Pyrazinamide (PZA) is another prodrug which is converted to its active form, pyrazinoic acid (POA) by the pyrazinamidase (PZase) encoded by *pncA* (Somoskovi *et al*., 2001). Though the basis of resistance in most strains was identified, the exact mechanism of action of PZA has not been firmly established (Telenti, 1998). However Somoskovi *et al.* (2001), demonstrated that PZA target an enzyme involved in fatty-acid synthesis and is responsible for killing persistent tubercle bacilli in the initial intensive phase of chemotherapy. Moreover it is identified that mutations in the *pncA* gene are the major mechanism of resistance to PZA in *M. tuberculosis* (Lemaitre *et al*., 1999).

Ethambutol (EMB) is an anti-tuberculosis drug which inhibits enzyme involved in cell wall biosynthesis (Telenti, 1998). Resistance to EMB is associated with mutation in *embCAB genes.* These genes encode arabinosyltransferases involved in the synthesis of arabinogalactan and lipoarabinomannan which are unique mycobacterial cell wall components (Johnson *et al*., 2006). EMB resistance results from an overexpression of the *Emb* proteins and structural mutation in *Emb*B. Mutations, identified in up to 65% of clinical isolates of *M tuberculosis*, are associated with high level of EMB resistance (Telenti, 1998; Mokrousov *et al*., 2002; Johnson *et al*., 2006).

1.3.4. Epidemiology of drug resistant tuberculosis

The global distribution of drug-resistant TB was poorly defined until recently. After dramatic outbreaks of multidrug-resistant tuberculosis (MDR-TB) in the early 1990s, resistance became recognized as a global problem. In 1994, the IUATLD and WHO establish the global project on antituberculosis drug resistance surveillance (Nachega and Chaisson, 2003). Since the establishment of the WHO/IUATLD global project on anti-tuberculosis drug resistance surveillance, three global reports have been produced (WHO, 1997, 2001a, 2004). The main findings of the reports were that drug-resistant TB was present in all settings surveyed and MDR-TB was identified in most settings.

Multidrug-resistant tuberculosis is an increasing global problem. According to Dye *et al.* (2002), an estimated 273,000 (3.2%) new cases of MDR-TB occurred worldwide in 2000. More recently Zignol *et al.* (2006), has reported a world wide increase of MDR-TB cases. According to this report, out of the total estimated 8.8 million TB cases that occurred in the year 2004, 424, 203 (4.3%) were MDR-TB. Out of these, 242, 799 were estimate to have occurred among new cases and 181,408 among previously treated cases. The latest report on worldwide anti-tuberculosis drug resistance, prepared by the WHO/ IUATLD Global Project on anti-tuberculosis drug resistance surveillance, stated that MDR-TB has reached levels of up to 14% among new patients, and levels as high as 50% among previously treated patients in some settings (WHO, 2004).

The extent and burden of MDR-TB varies significantly from country to country and region to region (Ormerod, 2005). Eastern Europe, especially Estonia, Latvia, and Russia have the highest MDR-TB burden (Espinal *et al.*, 2001). Overall, three countries: China, India, and the Russian Federation accounted for 62% of the estimated MDR-TB global burden (Zignol *et al.,* 2006).

Surveillance data on patterns of resistance among isolates from Ethiopia are scarce, as *M. tuberculosis* culture and susceptibility testing are not routinely performed. However, different reports showed the existence of drug resistant TB. A study conducted in Addis Ababa in 1994 by Meaza Demissie *et al.* (1997), showed an overall prevalence of 15.6% drug resistance new cases of TB. Of these new TB cases, 10.2% were found resistant to streptomycin, 8.4% to isoniazid, 1.8% to rifampicin, and 1.2% MDR-TB. In contrast, a study done in Harar in 1995 showed higher overall prevalence of resistance in patients with new cases (32.5%) and previously treated cases (51.2%) of TB, and 0.4% MDR-TB (Mitike *et al*., 1997). Getahun Abate *et al.* (1998) also reported a 50% resistance to one or more of the first-line drugs and 12% multi-drug resistant in some retreated patients in Addis Ababa. Recently the percentage of MDR-TB in new cases was estimated to be1.7% (WHO, 2007).

1.3.5. Drug resistance detection methods

Early detection of drug resistance constitutes one of the priorities of TB control programs (Lemus *et al*., 2004; Palomino *et al*., 2007). To meet this requirement several methods have been developed.

1.3.5.1. Conventional methods

Conventional culture methods are performed on solid media such as Lowenstein-Jensen (LJ) and Middlebrook10/11. Proportions, absolute concentration and resistance ratio are the three conventional methods used for drug sensitivity test. The proportion method is the most commonly used method worldwide amongst the three methods mentioned above (Heifets and Cangelosi, 1999; Martin and Portaels, 2007).

The proportion method is based on the principle of calculating the proportion of resistant bacilli present in a strain. High and low dilutions of bacteria are inoculated on drug free and drug containing media, in order to provide numerable colonies. The ratio of the number of colonies growing on drug containing medium to that of drug free medium indicates the proportion of drug resistant bacilli present in the bacterial population. Resistance is defined as more growth (≥ 1%) in drug-containing media compared to the growth on drug-free control media containing 1 in 100 diluted bacterial suspensions (Heifets and Cangelosi, 1999; Sharma and Mohan, 2004; Martin and Portaels, 2007).

Although the method remains as "gold standard" to detect drug resistant *M. tuberculosis*, it has certain limitations. It is laborious and takes long period (6-12 weeks) to obtain the result. This time lag poses a significant danger to patients, the community, and health workers (Mshana *et al*., 1998; Martin *et al*., 2005).

1.3.5.2. Rapid methods

The newer rapid methods such as molecular (Line-probe assay, PCR-single strand conformational polymorphism (PCR-SSCP), DNA microarrays), BACTEC 460-TB

(Becton Dickinson, Sparks, MD, USA) and BACTEC MGIT 960 (mycobacterial growth indicator tube) allow early detection. They are rapid and reduce time lag by conventional methods.

Due to its reliability and speed, the BACTEC TB-460 radiometric system is the first to achieve worldwide recognition (Martin *et al.*, 2005). It detects the presence of mycobacteria based on their metabolism. A radioactive marker is present in the tube that is detected by the machine when growth occurs (Heifets and Cangelosi, 1999; Guillerm *et al.*, 2006). This method yields reliable results within 5–12 days (Tortoli, 2000).

The BACTEC MGIT 960 is working based on fluorescence detection of mycobacterial growth. It detects growth in a tube containing a modified Middlebrook 7H9 broth together with a fluorescence quenching-based oxygen sensor embedded at the bottom of the tube (Guillerm *et al.*, 2006). The method is rapid and gives reliable result with in 6-12 days (Martin *et al.*, 2005). The molecular methods on the other hand characterize genes that confer resistance to first-line antimicrobial drugs and gives result in few days and hours (Mohammadzadeh *et al.*, 2006).

The methods discussed above have a lot of drawbacks and disadvantages to implement them in low income countries such as Ethiopia. The expensiveness of the machine, the need of radioactive waste disposal, costly reagents and cross contamination limits the implementation of BACTEC 460-TB in low-resource countries (Heifets and Cangelosi, 1999; Piersimoni *et al.*, 1999; Kumar *et al.*, 2005). The rapid BACTEC MGIT 960 machine is extremely expensive (40,000 € in Europe, and up to 100,000 € in Africa). It also requires frequent technical support from the company for maintenance (Guillerm *et al.*, 2006). This makes it impossible to routinely use in developing countries, where most of the TB patients are found (Martin *et al.*, 2005). New molecular methods are promising, but their costs, need of skills, supplies, equipment and facilities have precluded their routine implementation in countries with low resources (Palomino *et al.*, 2002; Martin *et al.*, 2005; Mohammadzadeh *et al.*, 2006)

Therefore, there is a need for rapid, inexpensive and reliable methods for early detection of drug resistance TB in high-burden TB and low-resource countries. Colorimetric methods that use redox indicators (MTT, Alamar blue) or the nitrate reduction assay have received increasing attention because of their simplicity and the absence of any requirement for sophisticated equipment or highly trained personnel. Several studies have evaluated their accuracy and performance in comparison with reference standard methods, particularly for the detection of resistance to rifampicin and isoniazid (Martin *et al.*, 2005; Palomino, *et al.*, 2007).

1.3.5.3. MTT assay

MTT assay is a colorimetric method that uses a yellow tetrazolium salt, 3- (4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) which is converted by dehydrogenase enzyme in living cells to produce insoluble purple formazan crystals that, after solubilization can be measured with a spectrophotometer (Mosmann, 1983). The MTT assay was developed by Mossman in 1983 as a quantitative measure of mammalian cell survival and proliferation (Mosmann, 1983). It is also used to determine cytotoxicity of potential medicinal agents and other toxic materials.

The MTT assay was first suggested by Mshana and his colleagues (Getahun Abate *et al.*, 1998; Mshana *et al.*, 1998) for use in a colorimetric assay for indirect detection of rifampicin resistance in clinical isolates. These studies showed that there was a linear relationship between the number of viable mycobacteria and the ability to reduce MTT. Recently, the MTT test has also been applied in the detection of resistance to other anti-tuberculosis drugs with good results (Foongladda 2002; Morcillo *et al.*, 2004; Martin, *et al.*, 2005; Montoro, *et al.*, 2005). The sensitivity and specificity of this assay remained high when compared with the proportion method. It ranges from 90-100% for sensitivity to 91.7-100% for specificity (Martin, *et al.*, 2005).

More recently, and with the purpose of speeding up detection of drug resistance in *M. tuberculosis*, the MTT assay has been applied directly to sputum samples for the

detection of rifampicin resistance (Getahun Abate *et al*., 2004). The sensitivity and specificity of this direct MTT assay matched those of the standard indirect assay on 7H10 agar, with 98.5% of the samples giving interpretable results within two weeks (Getahun Abate *et al.,* 2004).

The MTT assay was previously standardized and evaluated for a direct detection of rifampicin resistant *M. tuberculosis* in sputum specimens (Abate *et al.,* 2004). However, it is not yet evaluated under program condition to be used in TB control program. The MTT assay was not standardized and evaluated for isoniazid, one of the most effective first line drug used in TB treatment regimens. Preliminary data indicates the assay could also be used for rapid detection of isoniazid resistance. Thus, this study was conducted with the objectives of standardizing the MTT assay for the detection of isoniazid resistance directly on smear-positive sputum samples and evaluating the assay for a direct detection of rifampicin and isoniazid resistant *M. tuberculosis* under program condition, at Ethiopian Health and Nutrition Research Institute mycobacteriology laboratory.

2. Objective

2.1. General objective

- ❖ To evaluate the applicability of direct colorimetric MTT assay for the detection of rifampicin and isoniazid resistant *M. tuberculosis* under program condition.

2.2. Specific objectives

- ❖ To evaluate direct MTT assay for detection of rifampicin resistant *M. tuberculosis* using conventional proportion method as gold standard.
- ❖ To standardize MTT assay for a direct detection of isoniazid resistant *M. tuberculosis*.
- ❖ To evaluate direct MTT assay for detection of isoniazid resistant *M. tuberculosis* using conventional proportion method as gold standard.

3. Materials and Methods

3.1. Study design

The study design for the evaluation of the MTT assay was cross-sectional. In this study single sputum was collected from smear positive new cases and re-treatment cases.

3.2. Sputum specimens

Sputum specimens were collected from those patients who came to St Peter's TB Specialized hospital for treatment between 19 November 2006 and 4 May 2007. The patients were smear positive new cases and re-treatment cases. A total of 153 sputum specimens were taken for the study. Among these one hundred thirty were used in the evaluation of MTT assay for detection of rifampicin resistance and the remaining 23 for INH resistance. Sputum specimens were collected by a laboratory technician and all patients' information was filled in the questionnaire (Appendix 1).

3.3. Sample size

The estimated sample size was calculated by taking the sensitivity and specificity of the MTT assay as 90% and 95%, respectively, at 80% power and 95% confidence interval. STATA soft ware was used for the calculation. Based on the given criteria the sample size used for the evaluation of direct MTT assay for rifampicin resistance was calculated to be 113 culture positive sputum specimens.

3.4. Sputum collection

A single sputum specimen from each patient was collected in a 20 ml screw capped universal tube from 153 smear positive new and re-treatment cases, and transported to Ethiopian Health and Nutrition Research Institute (EHNR) mycobacteriology laboratory. The samples were kept at 4°C and processed within three days.

3.5. Drug and reagent preparation

Two stocks of rifampicin and isoniazid solution, one for drug sensitivity test on Lowenstein-Jensen (LJ) media and the other for MTT assay were prepared. Stock solution of rifampicin (RIF) and isoniazid (INH) (Sigma) were prepared at concentration of 80 µg/ ml in ethanol/PBS and 1mg/ml in PBS respectively. The stock solutions were filter sterilized, aliquoted in eppendrofs and stored at -20^{0}C until use. Final concentration of 2 mg/ml RIF and 0.2 mg/ml INH were used in the MTT assay. For the drug sensitivity on LJ medium, stock solutions of RIF and INH (sigma) were prepared at 10 mg/ml in dimethlysulphooxide (DMSO) and 10 mg/ml in sterilized distilled water, respectively, and stored at -20^{0}C. From the stock solution, 1ml of RIF and 1:100 diluted 1ml of INH were dispensed to 500 ml LJ medium to get a final concentration of 40 µg/ ml RIF and 0.2 µg/ ml INH.

A stock solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenly tetrazolium bromide (MTT) (sigma) 5mg/ml was prepared in PBS, pH 6.8, filter sterilized with 0.2 µm filter and kept at 4^{0}C in the dark. A final concentration of 0.5 mg/ml was used in the assay. Formazan solubilization buffer was prepared by mixing 1:1 (vol / vol) 20% sodium dodecyl sulfate (SDS) and a solution of 50% N, N-dimethyl formamide (DMF) (sigma). The mixture was kept at 37^{0}C in water bath for one hour to dissolve SDS, and then kept at room temperature.

3.6. Media preparation

3.6.1. Middlebrook 7H9 broth media

Middlebrook 7H9 base (Becton Dickinson, France) was mixed with distilled water in a proportion of 4.7 gm to 900 ml. Then 5 ml glycerol (Sigma) was added to the solution and autoclaved at 121^{0}C for 15 minutes. The broth media was allowed to cool down to a temperature of 45^{0}C. Then, 100 ml Oleic acid Albumin Dextrose Catalase (OADC) enrichment (Becton-Dickinson) and 1 vial antibiotic mixture (PANTA), a standard antibiotic cocktail of Polymyxin B, Amphotericin B, Nalidixic acid, Trimethoprim and

Azlocillin, (Becton-Dickinson) were added to the broth medium. Finally, 3 ml of the broth was dispensed into screw capped culture tubes (20x125mm) and was kept at 4^0C.

3.6.2. Lowenstein-Jensen (LJ) Media

Monopotasium phosphate (2.4 g), magnesium sulphate (0.24 g), magnesium citrate (0.6 g) and asparagin (3.6 g) (Sigma) were dissolved in 600 ml distilled water. Then, 12 ml glycerol was added to the solution and autoclaved at 121^0C for 30 minutes. The LJ solution was kept at 4^0C until use. Malachite green, 2 gm, was dissolved in 100 ml distilled water and autoclaved at 121^0C for 10 minutes. For the preparation of LJ media, a well-shaken homogenate egg solution (1000 ml) and 2% malachite green were added to LJ solution (600 ml). The mixtures were vigorously shaken and dispensed in 7 ml volumes into 14 ml McCartney bottles. Finally the medium was inspissated in a slant position at 80^0C for 1 hour.

The drug containing media was also prepared in a similar way by adding 1ml of INH from1:100 diluted stock solution and 2 ml RIF from 2 mg/ml stock solution into two separate 500ml LJ medium to get a final drug concentration of 0.2 µg/ml INH and 40 µg/ml RIF. The medium was then dispensed in 7 ml volumes into 14 ml McCartney bottles and were inspissated in a slant position at 80^0C for 1 hour.

3.7. Bacterial isolation and culture

The sputum was digested and decontaminated using modified Petroff's method (WHO, 1998). Equal volume of 4% NaOH was added to the sputum in 50 ml screw capped bottle. Then it was vortexed for 1-3 minutes until the sputum and NaOH mixture became homogeneous and Kept for 15 minutes. After addition of 40-45 ml PBS (pH 6.8), the sample was centrifuged for 15 minutes at 3000 rpm. The supernatant was decanted and the pellet was resuspended in 3 ml PBS. An aliquot of 100 µl of the sample was then inoculated into two tubes containing Lowenstein-Jensen (LJ) media for primary isolation. The tubes were incubated at 37^0C and examined for bacterial growth until week eight. From the remaining resuspended sample, 500 µl aliquot was inoculated into each of the

six tubes containing 3 ml Middle brook 7H9 broth supplemented with OADC and PANTA. Three of the tubes contained rifampicin at final concentration of 2 mg/ml while the rest were drug free controls. They were then incubated at 37^0C, and MTT assay was done every week for three consecutive weeks.

3.8. MTT assay

3.8.1. Evaluation of MTT assay for direct detection of rifampicin resistance

MTT is a yellow tetrazolium salt that is converted into a purple formazan by dehydrogenases of live cells. The assay is based on the principle that the amount of formazan produced is directly proportional to the number of live cells (Mosmann, 1983). The assay was done each week as described by Getahun Abate *et al.* (2004). Briefly, 300 µl of 5 mg/ml MTT solution was added into Middlrbrook 7H9 broth medium. The tubes were vortexed and incubated for four hrs at 37^0C. The formazan produced was dissolved with solubilization buffer (20% SDS) in a 50% aqueous solution of DMF and the tubes were reincubated for 1 hr. The color change, as observed by the naked eye, was then recorded. A change in color from yellow to purple indicated the growth of bacteria. Optical density (OD_{570}) was then measured at 570 nm (Novaspec II photometer, Pharmacia Biotech Ltd, UK), against a blank containing 7H9 broth, PBS, MTT and solubilization buffer. The MTT results were defined as interpretable if OD of a control tube was ≥ 0.1. Relative optical density unit (RODU) values were calculated by dividing the OD of the drug containing tubes with the OD of drug free control. A strain was defined as rifampicin susceptible when the relative optical density unit (RODU) value was below 0.2 and resistant when it was above 0.5. RODU between 0.2 and 0.5 was considered indicative of borderline resistance. A test for bacterial contamination was performed on nutrient agar before performing the MTT assay. The experimental approach is summarized in Figure 1.

3.8.2. Standardization of MTT assay for direct detection of INH resistance

A stock solution of 100 µg/ml INH was prepared in PBS. From the stock solution, three different final concentrations of INH (0.1, 0.2 and 0.4 µg/ml) were used for the determination of the critical concentration to be used in the assay.

Ten clinical isolates of *M. tuberculosis* with a known INH susceptibility pattern were used to standardize the assy. The five isolates (G7SNW292, G6S594, G9S3, G10S6 and G8S593 whose susceptibility was already confirmed on LJ media and obtained from EHNRI) were INH susceptible and the remaining five isolates (LJ confirmed INH resistant G1R44, G2R549, G3R541, G4R4 and G5R207) were INH resistant. Reference strains, INH susceptible H37Rv and resistant ATTCC 35835 were also included. Each of the isolates was subcultured on two LJ medium and incubated for 3 to 4 weeks at 37^0C. Inoculums were prepared by suspension of colonial growth from 3-4 week old LJ cultures in sterilized distilled water to turbidity equal to that of a № 0.5 McFarland standard. Then, 0.5 ml of inoculum was dispensed into culture tubes containing 3ml 7H9 broth. For each isolates 12 culture tubes were used, among which 9 tubes contained INH at final concentrations of 0.1 µg/ml, 0.2 µg/ml and 0.4 µg/ml. For each drug concentration a duplicate of 3 test tubes were used. The remaining 3 tubes were drug free controls. All test tubes were incubated at 37^0C until the day of MTT assay.

The MTT assay was carried out as indicated by Getahun Abate *et al.* (1998). Briefly, 300 µl of 5 mg/ml MTT solution was added to culture broth. The tubes were shaken and incubated at 37^0C for 4 hours. A formazan solubilization buffer (500 µl) was added, and the tubes were mixed by vortexing and incubated at 37^0C for another hour. Absorbance was measured at 570 nm against a blank containing 7H9 broth, MTT, and solubilization buffer. Relative optical density unit (RODU) values were calculated by dividing the OD of the drug containing tubes with the OD of drug free control.

3.8.3. Evaluation of the MTT assay for direct detection of INH resistance

The MTT assay was evaluated for direct detection of INH resistant from sputum samples. Twenty three smear positive sputum samples (20 new and 3 re-treatment cases) were used for the evaluation. Based on the above standardization result a final concentration of 0.2 µg/ml INH was used in the direct MTT assay. Similar to the MTT assay for rifampicin, the lowest OD value that gave interpretable result was found to be 0.1. The MTT assay was performed as described above for Rifampicin. Briefly, 300 µl MTT solution was added into 7H9 broth medium to give a final concentration of 0.5 mg/ml. The tubes were vortexed and incubated for 4 hrs at 37^{0}C. The formazan produced was dissolved with SDS-DMF solubilization buffer, and the tubes were reincubated for 1 hr. Optical density (OD_{570}) was then measured at 570 nm, using a tube containing 7H9 broth, PBS, MTT and solubilization buffer as a reference. The MTT results were defined interpretable if OD of a control tube was ≥ 0.1. Relative optical density unit (RODU) values were calculated by dividing the OD of the drug containing tubes with the OD of drug free control. A strain was defined as INH susceptible, resistant and borderline resistance when the relative optical density unit (RODU) was <0.2, >0.5 and between 0.2 and 0.5 respectively. The experimental approach is summarized in Figure 1.

To determine the cut off value for isoniazid, after a thorough vortex, 100 µl of the bacterial suspension was transferred from the 7H9 control medium to the tubes containing LJ medium before doing the MTT assay. The tubes were incubated at 37^{0}C and observed each week for colony forming unit (CFU). The OD value from the MTT result was correlated with CFU to determine the lowest OD value that corresponds to the CFU. Visual determination of color change from yellow to purple in the control tubes also compared with CFU. A test for bacterial contamination was performed on nutrient agar before performing the MTT assay.

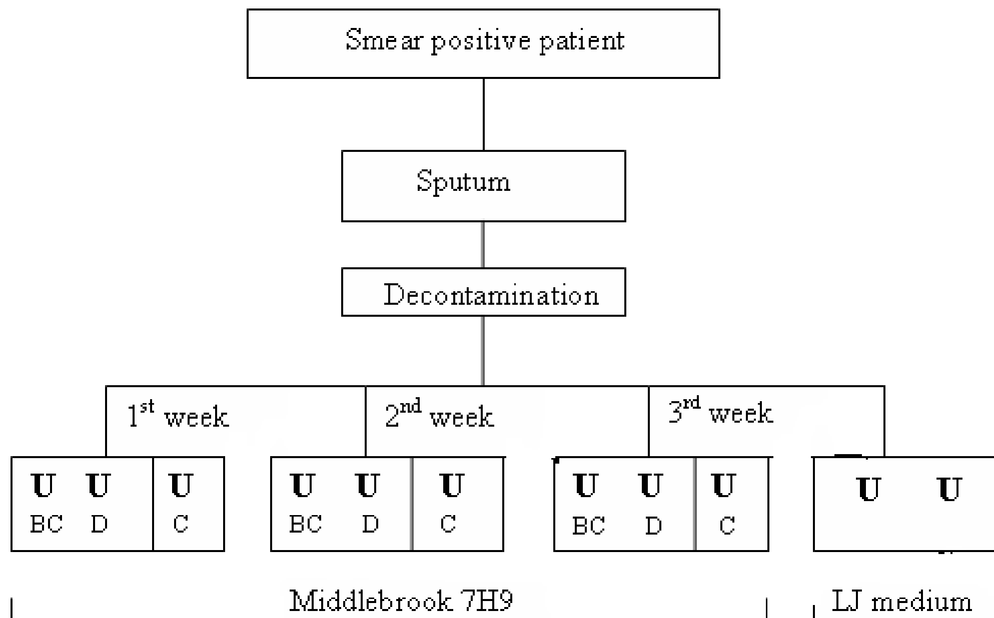


Figure 1: Flow chart of MTT assay. Abbreviations: BC, bacterial control without rifampicin/ isoniazid; D, tube with 2 µg of rifampicin/ml or 0.2 µg of isoniazid/ml; C, control without bacteria (blank control); LJ, Lowenstein-Jensen.

3.9. Drug susceptibility test (DST)

Standard indirect drug susceptibility test was done to rifampicin and isoniazid. The test was done based on the modified proportion method (Canetti *et al.*, 1969) using Lowenstein-Jenson media. Briefly, one loopful (approximately 5-10 mg) colony of *M. tuberculosis* from a primary culture was taken and vortexed (for 30-60 seconds) in a 16 x 125 mm screw-cup tubes that contained 6-8 glass beads and 2-3 drops of sterile distilled water. Then, 5 ml of sterile distilled water was added to bacterial suspensions and allowed to settle for 15 minutes so that the homogenized suspension could be taken easily and the large particle settled in the tube. A bacterial suspension with turbidity corresponding to McFarland turbidity standard-no1 (approximately $3x10^8$ bacterial suspensions /ml) was prepared in sterile distilled water. The concentration was then diluted to 1: 100 and 1:10000. From the 1:100 bacterial dilutions, 10 µl was transferred

into two drug containing tubes and one drug free control tube. About 10 μl of bacterial suspension from the 1:10000 dilutions was transferred into one drug free control tube. All the tubes were then incubated at 37^0C and examined for colony formation every week until the six week. One reference strain (H37Rv, RIF and INH susceptible) was included in each test batch as a negative control. This serves as a quality control check on the presence of drug in the medium.

The proportion of growth was calculated by dividing the number of colonies in a drug medium with the drug free medium when the numbers of colonies on drug free media were between 50 and 150. A bacterial growth of more than 1% was taken as resistant and less than 1% as susceptible. The proportion of growth could also be determined by comparing the number of colonies in a drug containing tube inoculated with 1:100 dilutions with a control containing 1:10000 diluted bacterial suspensions. A higher number of colonies in a drug containing media indicated resistance.

3.10. Species identification

3.10.1. Genomic DNA Extraction

The DNA extraction of mycobacteria was performed according to extraction protocol described by van Soolingen *et al.* (1995). Bacteria grown on Lowenstein–Jensen (LJ) media were resuspended in 400 μl of 1x TE buffer with pH 8.0. The mixture was heated in a water bath at 80°C for 30 minutes to kill the bacteria and cooled to room temperature. Fifty μl of lysozyme (10 mg/ml) [Sigma] was added to lyse the bacteria and the mixture was incubated in a 37^0C water-bath for one hour. In the lysosome treated samples, 75 μl of 10% SDS/proteinase K mix (10 mg/ml) (Sigma Chemical Corporation) was added and incubation was continued for 10 minutes at 65^0C. To remove inhibitors, 100 μl of 5M NaCl and 100 μl of cetyltrimethylammonium bromide (CTAB)/NaCl solution were added to the sample, vortexed and incubated for 10 minutes at 65^0C. Then, 300 μl a 25:24:1 mix of phenol: chloroform: isoamyl alcohol (Sigma) was added to each sample and vortexed for 5 seconds. After

centrifugation at 12,000xg for 5 minutes at room temperature, the upper phase was carefully removed and transferred to 1.5 ml eppendorf tubes. Isopropanol was added at 450 µl volume to each sample and kept at -20°C for 30 minutes. Subsequently, the samples were centrifuged at 12,000xg for 15 minutes. The DNA pellet was then washed with 1ml ice-cold 70% ethanol to remove CTAB and NaCl. After 5 minutes of incubation at -20 ^{O}C, the samples were centrifuged for 5 minutes. The ethanol was removed and the DNA pellets were dried at room temperature. The dried pellet were then re-suspended in 20 µl DNase/RNase free 1X TE buffer (pH = 8.0) and heated for 10 minutes in a 68^{0}C water bath to dissolve the pellet. Fifty-fold dilutions of DNA were used to determine the concentration spectrophotometrycally at absorption of 260 nm and dilutions of 1 µg/µl were prepared. The DNA samples were stored at -20 ^{O}C until used.

3.10.2. Polymerase Chain Reaction (PCR)

RD10 primer was used in the PCR to detect difference among species. A multi-primer PCR assay with three primers was used to detect RD10 deletion region. The primer sequences for RD10 flanking forward primer (5′-CTG-CAA-CCA-TCC-GGT-ACA-C-3′), RD10 flanking reverse primer (5′-AAG-CGC-TAC-ATC-GCC-AAG-3′) and RD10 Internal primer (5′-GAA-GTC-GTA-ACT-CAC-CGG-GA-3′) were used. Twenty five µl reaction mixture containing each of the two flanking primers at a concentration of 10 µg/µl, 10 µg/µl internal primer, and 2 µl (1 µg/µl) of DNA from bacterial cells were used. Hot start master mix (QIAGEN GmbH Germany) was used in all PCR reactions.
The cycling programme was: initial denaturation 1 cycle of 95°C for 15 minutes and 35 cycles of denaturation, 94°C for 1 minute; annealing, 55°C for 1 minute; elongation, 72°C for 1 minute and final extension at 72^{0}C for 10 minutes, in Thermo hybid thrmocycler.

The PCR products were visualized in 1.8% agarose (Sigma) gel with 0.5 µg/ml ethidium bromide. Five micro liters of each PCR product was loaded with 1 µl of 5x-loading buffers (0.25% Bromophenol blue, 0.25% Xylene cyanol FF, 50% Glycerol) on the gel. Three micro liters of 0.1 µg/µl molecular size markers was loaded as a marker. An

electrophoresis separation was performed at 100V and 50mA for 90 minutes. Pictures were taken using a UV trans-illuminator (UVP-imager) (In Epi- chemi II, darkroom, Upland), with camera attached to it and a computer.

3.11. Study setting and laboratories

The study was conducted in two institutions. Sputum isolation, culturing, MTT assay and drug sensitivity test was carried out at EHNRI mycobacterology laboratory. The optical density (OD) measurement, Genomic DNA extraction and PCR were done at AHRI.

3.12. Statistical analysis

Statistical analysis was performed using SPSS software version 13. Wilcoxon's singed ranks test and Mann-Whitney U test was used to detect statistically significant differences between the growth pattern of resistant and susceptible strains. 95% confidence level was used for the test. A probability of <0.05 was considered significant. Data are presented in form of tables and graphs.

3.13. Ethical consideration

Ethical clearance was obtained from Biology department, Addis Ababa University Ethical Committee and AHRI/ALERT Ethical Review Committee. After informing the study subjects about the benefit of the study, those volunteers to give their informed consent was included in the study. All results were kept confidential.

4. Result

4.1. Sputum specimens

In this study a total of 153 sputum specimens were collected of which 130 were used in the evaluation of direct MTT assay for the detection of rifampicin resistant *M. tuberculosis*, 21 in the evaluation of direct MTT assay for the detection of isoniazid resistant *M. tuberculosis* and 2 in both. The sputum was obtained from 146 new cases and 7 re-treatment cases. The mean age of the patients was 30.45 years ± 1.19 standard error (SE) (range: 7-80 years) and median age 25.5 years (Fig. 2). Among the patients 79 (51.63%) were male and 74 (48.37%) were female.

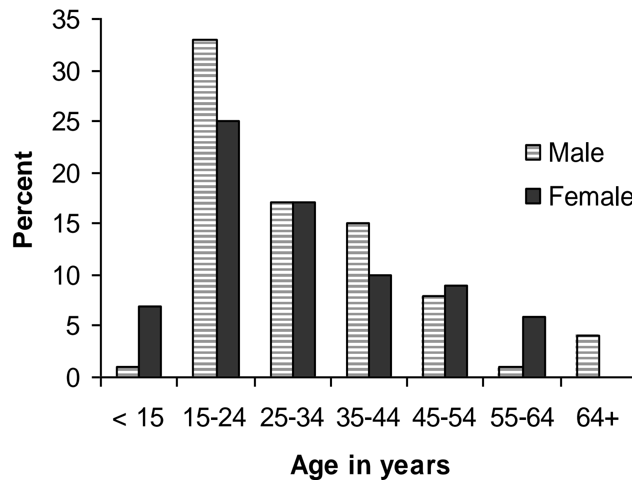


Figure 2: Age and sex distribution of patients

4.2. Evaluation of direct MTT assay for detection of rifampicin resistant *M. tuberculosis*

4.2.1. Culture

A total of 132 sputum specimens were used to evaluate the direct MTT assay for detection of rifampicin resistance in *M. tuberculosis*. The specimens were cultured on Lowenstein- Jensen (LJ) medium and Middle brook 7H9 broth medium (M7H9) used in MTT assay. As shown in Table 1, out of 132 sputum specimens, 117 (88.64%) specimens were found out to be culture positive and 8 (6.06%) were culture negative on LJ medium.

In M7H9 used in the MTT assay, 115 (87.12%) were culture positive and 10 (7.58%) were culture negative (OD_{570} <0.1). Among the 10 isolates that showed uninterpretable MTT result (OD less than 0.1 in the control medium), 2 had growth on LJ medium and were culture positive. The remaining 7 (5.3%) samples were contaminated.

Table 1: Culture result on LJ medium and Middlebrook 7H9 broth used in MTT assay for detection of rifampicin susceptibility test (n=132)

	Middlebrook 7H9 broth medium used in MTT assay			
LJ medium	Positive	Negative	Contaminated	**Total**
Positive	115	2	0	**117**
Negative	0	8	0	**8**
Contaminated	0	0	7	**7**
Total	**115**	**10**	**7**	**132**

4.2.2. Visual observation

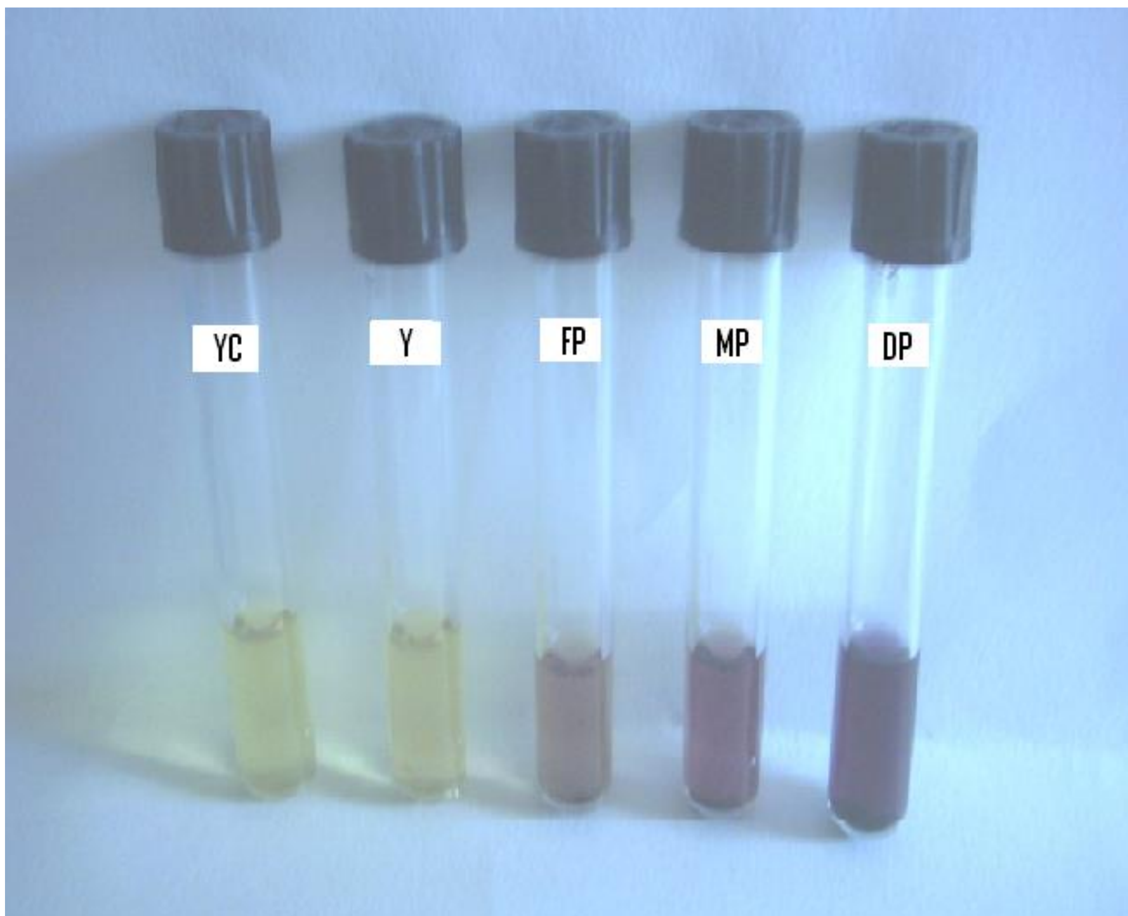
MTT assay was done each week by taking two culture tubes per isolates; one control with out rifampicin and one experimental with rifampicin. One reference control tube, without bacteria and rifampicin, was also included for each week batches. Every week color change in each tube was visually observed with the naked eye before the OD value was measured using the spectrophotometer (Fig. 3 J). The visually determined color changes were correlated with the optical density (OD) values measured with Novaspec II photometer at 570 nm. Cultures regarded as yellow through visual observation had OD values < 0.1 (mean OD= 0.06 ± 0.004 SE). As shown in Table 2 and Fig. 3 J cultures that gave interpretable result (OD_{570} >0.1) had a visual color change ranging from faint to dark purple. Observations visually interpreted as faint purple, medium purple and dark purple had mean OD values of 0.23 ± 0.015, 0.51± 0.027 and 0.99 ± 0.027, respectively. This shows that resistant and susceptible isolates could be easily identified with the naked eye (Fig. 3 L & K).

Table 2: Correlation of visually observed colors and the OD value of control tubes measured at 570 nm by Novaspec II photometer

Visually observed color change	OD value at 570nm*
Yellow	0.06
Faint purple	0.23
Medium purple	0.51
Dark purple	0.99

* First and second weeks mean OD value of control tubes measured at 570 nm

J



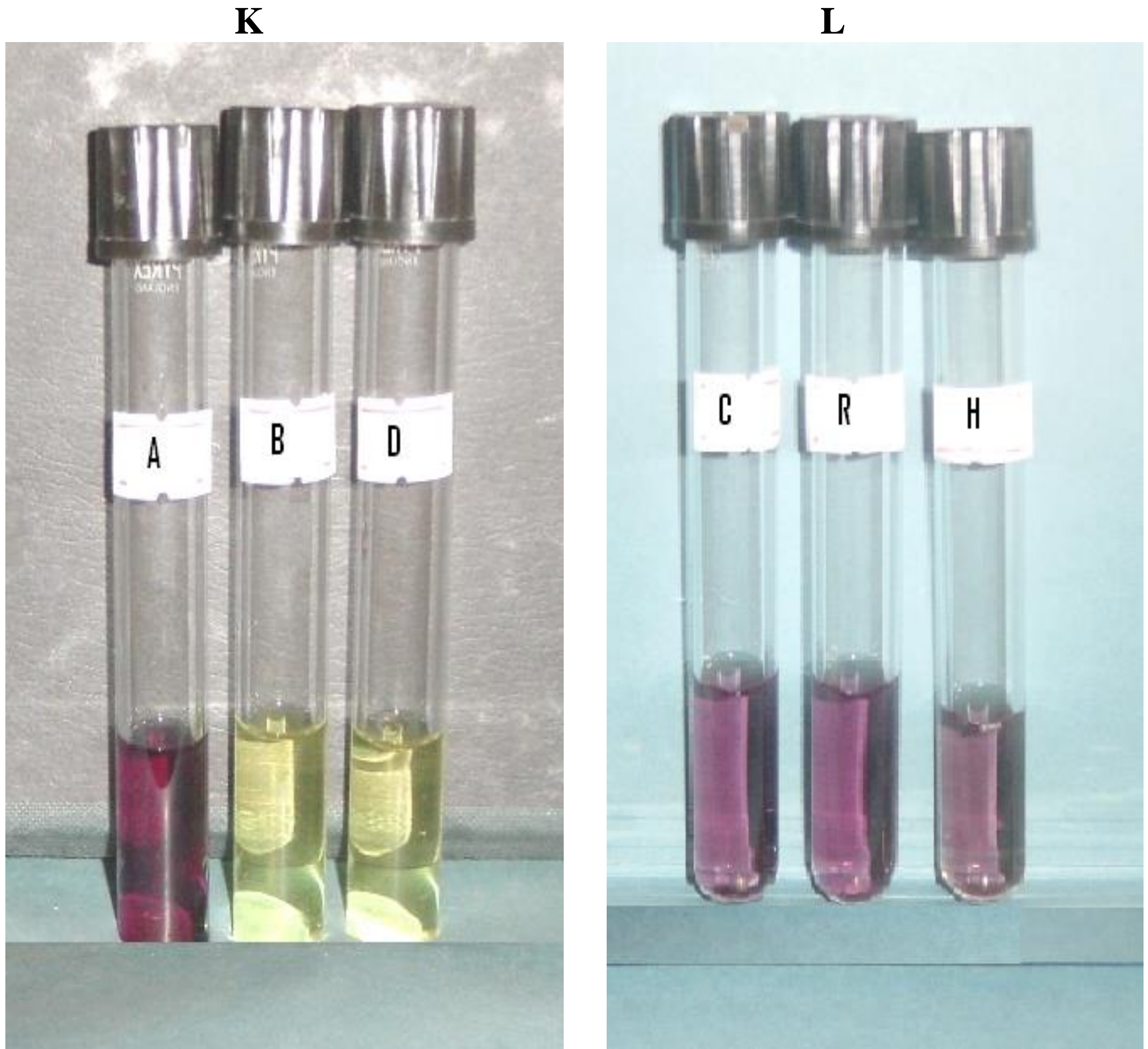


Figure 3: Visual observation of typical color change in the MTT assay: (**J**) Difference in color change; YC= blank control with yellow color, Y= Control tube with yellow color and corresponding OD value < 0.1, FP= Control tube with faint purple color and corresponding mean OD value 0.23 ± 0.015, MP= Control tube with medium purple and corresponding mean OD value 0.51± 0.027, DP= Control tube with dark purple color and corresponding mean OD value 0.99 ± 0.027. (**K**) Susceptibility visual determination; A= Growth in drug free control tube, B= Rifampicin susceptible, D= Isoniazid susceptible. (**L**) C= control tube, R= rifampicin resistant, H= isoniazid resistant.

4.2.3. Interpretation time and drug susceptibility test (DST)

MTT assay was done after one, two, and three weeks of incubation. Out of the 115 isolates analyzed, 61 (53.04%) gave interpretable result (OD value of control tube > 0.1) in the 1st week, 108 (93.91%) in the 2nd week and 115 (100%) in the 3rd week (Table 3). Of 109 susceptible isolates, 56% gave interpretable result at week one, 93.6% at week two and 100% at week three. Out of the 6 rifampicin resistant isolates detected by MTT assay, 83.3% gave interpretable result during the second week of incubation and 100% during the third week.

Table 3: Time for interpretation of a direct MTT assay to detect rifampicin resistance in *M. tuberculosis*

Week	No. of interpretable isolates (%)	No. of isolates giving interpretable result*	
		S	R
1	53.04	61	0
2	93.91	102	5
3	100	109	6
Total	**100**	**109**	**6**

* S, susceptible; R, resistance

The direct MTT assay was rapid and gave drug susceptibility test result within 3 weeks whereas the conventional method requires 7-14 weeks to yield the result (Table 4).

Table 4: Comparison for time required by direct MTT assay and conventional method in yielding susceptibility result

Methods	Time required for primary isolation (weeks)	Time required to yield DST result (weeks)	Total time (weeks)
LJ	3-8	4-6	7-14
MTT assay	0	3	3

Relative optical density unit (RODU) values were calculated by dividing the OD of the drug containing tubes with the OD of drug free control. A strain was defined as rifampicin susceptible when the relative optical density unit (RODU) value was below 0.2 and resistant when it was above 0.5. Out of the 115 isolates tested for rifampicin resistance, the MTT assay identified 6 (5.2%) as resistant and 109 (94.8%) as susceptible (Table 5).

Table 5: Susceptibility testing of *M. tuberculosis* by MTT assay and the proportion method (n= 115)

MTT assay	Standard proportion method		
	Susceptible	Resistant	**Total**
Susceptible	108	1	109
Resistant	0	6	6
Total	108	7	115

4.2.4. Test characters

Taking the proportion method as the gold standard, sensitivity, specificity and accuracy of MTT assay were calculated as shown in (Table 6). Of the seven isolates found to be rifampicin resistant by the proportion method, the MTT assay identified six as resistant

and one as susceptible. All isolates determined as susceptible by proportion method were also identified as susceptible by the MTT assay. The MTT assay thus showed specificity and sensitivity of 100% and 85.71% respectively, with a positive predictive value of 100% and negative predictive value of 99.08%. The overall concordance was 99%. This result showed that there was no statistically significant difference between MTT assay and standard proportion method in rifampicin susceptibility test ($p > 0.05$ [Wilcoxon's singed ranks test]).

Table 6: Efficiency of the MTT assay in detection of rifampicin susceptible/ resistant *M. tuberculosis* isolates in comparison with the standard proportion method

Test character	Efficiency of MTT assay
Specificity (%)	100
Sensitivity (%)	85.71
Positive predictive value (%)	100
Negative predictive value (%)	99.08
Accuracy of MTT assay*	0.99

* The probability of obtaining a true positive or true negative result

4.2.5. Relative Optical Density Unit (RODU)

The RODU, which is the ratio of drug containing tube to drug free tube, has been used to show possible isolate differences in the ability to reduce MTT and define resistance and susceptibility. Isolates with RODU value above 0.5 are considered as resistant and those with RODU value of below 0.2 as susceptible. The mean (± standard error) RODU value of resistant isolates was 0.926 ± 0.04 whereas the mean (± standard error) RODU value of susceptible isolates was 0.043 ± 0.003. For resistant isolates the first week MTT assay was not interpretable (OD value of control tubes <0.1). The RODU values of susceptible isolates remained below 0.2 and the RODU values of resistant isolates were above 0.5

(Fig. 4). The differences in the RODU of susceptible and resistant isolates were statistically highly significant ($p < 0.0001$ [Mann-Whitney U test]).

The resistant isolates varied in their growth pattern in rifampicin containing Middlebrook 7H9 broth range from an OD value of 0.32-1.16 at third week of incubation. The highest standard mean error was observed also at third week incubation (Fig. 4).

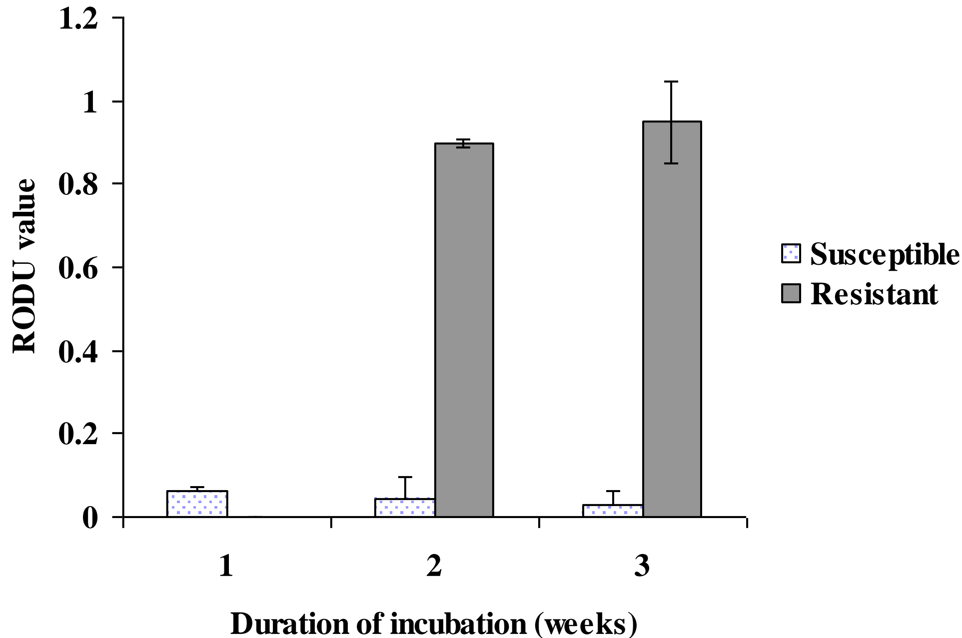


Figure 4: Relative Optical Density Unit (RODU) of susceptible and resistant *M. tuberculosis* isolates in 1st, 2nd, and 3rd week incubation: growth patterns of rifampicin susceptible isolates (n= 61 in the first week, 102 in the second week, and 109 in the third week) and rifampicin resistant isolates (n= 5 in the second week and 6 in the third week) measured by RODU values (means ± standard errors).

4.2.6. Drug susceptibility test by the proportion method

The proportion method was used as gold standard in this study. Drug sensitivity test was done on LJ medium containing rifampicin at a final concentration of 40 µg/ml. Out of 115 LJ culture positive isolates, 7 (6.1%) were found to be rifampicin resistant and 108

(93.9%) susceptible by the proportion method (Table 5). The DST result of proportion method was obtained in the range of 4-6 weeks (Table 4).

Drug susceptibility test by the proportion method was done both for RIF and INH on all 133 LJ culture positive isolates. The result showed that out of 133 isolates, 7 isolates were resistant to both RIF and INH and 14 isolates to INH alone. Those 14 isolates resistant to INH were found susceptible to RIF. In general out of 133 isolates, 21 of them were found to be INH resistant and 112 were susceptible. Of the same number, 126 isolates were RIF susceptible and 7 isolates resistant. Fig. 5 shows growth of RIF and INH resistant isolates on LJ medium.

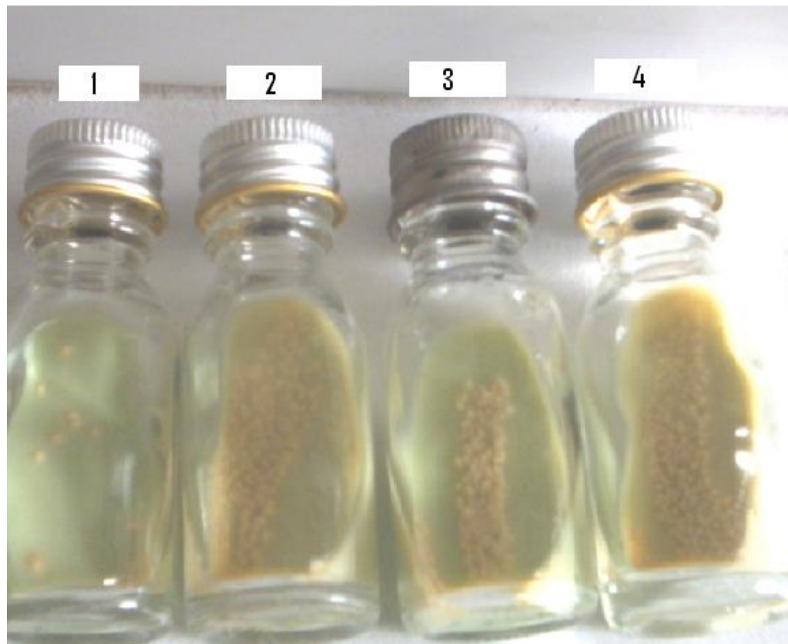


Figure 5: Growth of rifampicin and isoniazid resistant isolates on LJ medium: (1) Growth on 1: 10000 diluted drug free controls, (2) Growth on 1: 100 diluted drug free controls, (3) growth of rifampicin resistant isolates on LJ medium containing 40 µg/ml rifampicin (4) growth of isoniazid resistant isolates on LJ medium containing 0.2 µg/ml isoniazid.

4.3. Standardization of a direct MTT assay for detection of isoniazid resistant *M. tuberculosis*

Five known INH susceptible isolates (G5R207, 67SNW292, 6S594, G9S3 and 8S593) and five known resistant isolates (G10S6, G1R44, G2R549, G3R541, G4R4) from EHNRI were used to standardize the assay. Three different final concentrations of isoniazid (0.1µg/ml/, 0.2 µg/ml, and 0.4 µg/ml) were used to determine the critical concentration.

Growth pattern of *M. tuberculosis* at 0.1µg/ml isoniazid concentration indicated that out of the five tested susceptible isolates, three gave interpretable result beginning week one and the remaining isolates at week 2 and 3. Four of the resistant isolates gave interpretable result beginning from the 1st week and the remaining in the succeeding weeks (Fig. 6). The RODU of susceptible isolates remained below 0.2 in all the 3 weeks except one isolate with RODU above 0.2. The resistant isolate showed RODU < 0.5 in first week and RODU > 0.5 in the subsequent weeks. The mean RODU value of resistant isolates were 0.82 ± 0.12 and the susceptible isolates were 0.1 ± 0.03 .The mean RODU value of resistant and susceptible isolates was not statistically significant at week one ($p > 0.05$) but they showed a significant difference at week two and three ($p < 0.001$).

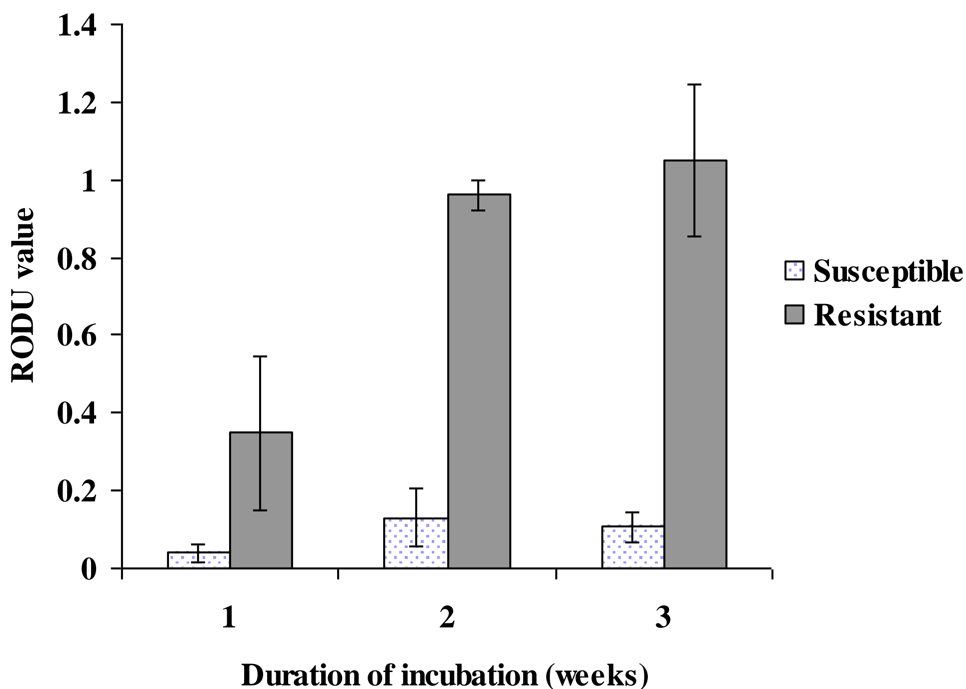


Figure 6: Growth pattern of *M. tuberculosis* isolates at INH concentration of 0.1µg/ml: growth of isoniazid susceptible isolates (n= 3 in the first and n= 5 in the second and third weeks) and isoniazid resistant isolates (n= 4 in the first and n= 5 in the second and third weeks) measured by RODU values (means ± standard errors)

The above five resistant and five susceptible isolates were tested against 0.2 µg/ml isoniazid concentration. Four resistant and three susceptible isolates gave interpretable result beginning from week one and the remaining isolates at week 2 and 3. As indicated in Fig. 7 the growth pattern of susceptible isolates showed a RODU < 0.2 and the resistant isolates above 0.5 in the three weeks. The mean RODU value of resistant and susceptible isolates was 0.94±0.11 and 0.08±0.02, respectively. There was significant difference among the mean RODU of susceptible and resistant strains at each 3 weeks ($p < 0.05$, $p<0.001$ and $p<0.001$, respectively).

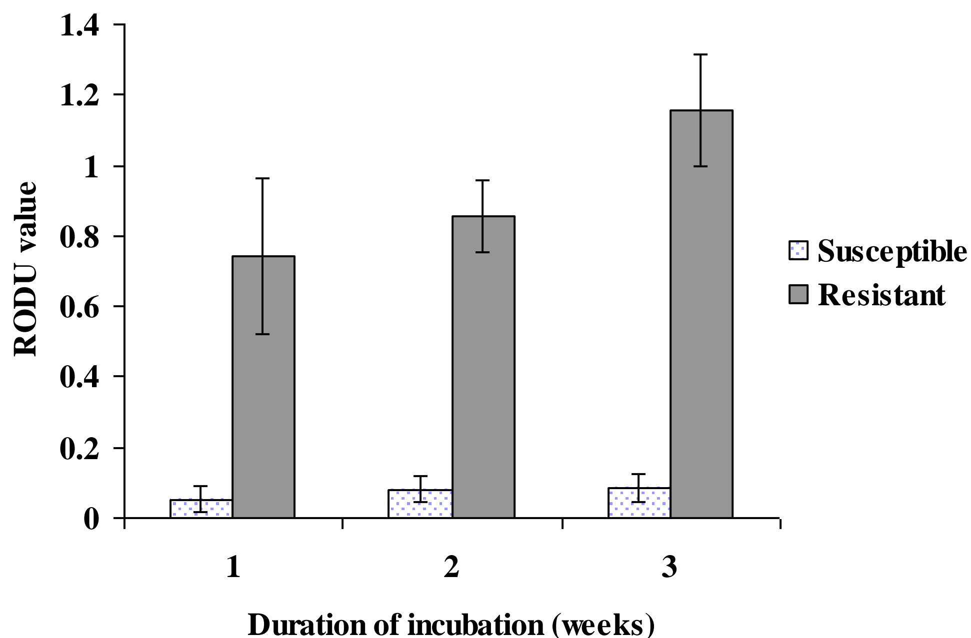


Figure 7: Growth pattern of *M. tuberculosis* isolates at INH concentration of 0.2 µg/ml: growth of isoniazid susceptible isolates (n= 3 in the first and n= 5 in the second and third weeks) and resistant isolates (n= 4 in the first and n= 5 in the second and third weeks) measured by RODU values (means ± standard errors)

The above isolates were also tested against 0.4 µg/ml isoniazid final concentration. Three susceptible and four resistant isolates gave interpretable result beginning from week one and the remaining 5 resistant and 5 susceptible isolates at week 2 and 3. The RODU of susceptible strains remained below 0.2 for all weeks. The resistant isolates showed mean RODU < 0.5 in first assay week and mean RODU > 0.5 in the subsequent weeks (Fig. 8). The mean RODU of resistant isolates was 0.51 ± 0.1. Except one strain with the RODU value of 0.2 all had RODU above 0.5. The mean RODU value of susceptible isolates was 0.07 ± 0.02. The mean RODU value of resistant and susceptible isolates were not statistically significant for first week ($p > 0.05$). There was a significant difference between the mean RODU of resistant and susceptible isolates at week two and three ($p < 0.01$).

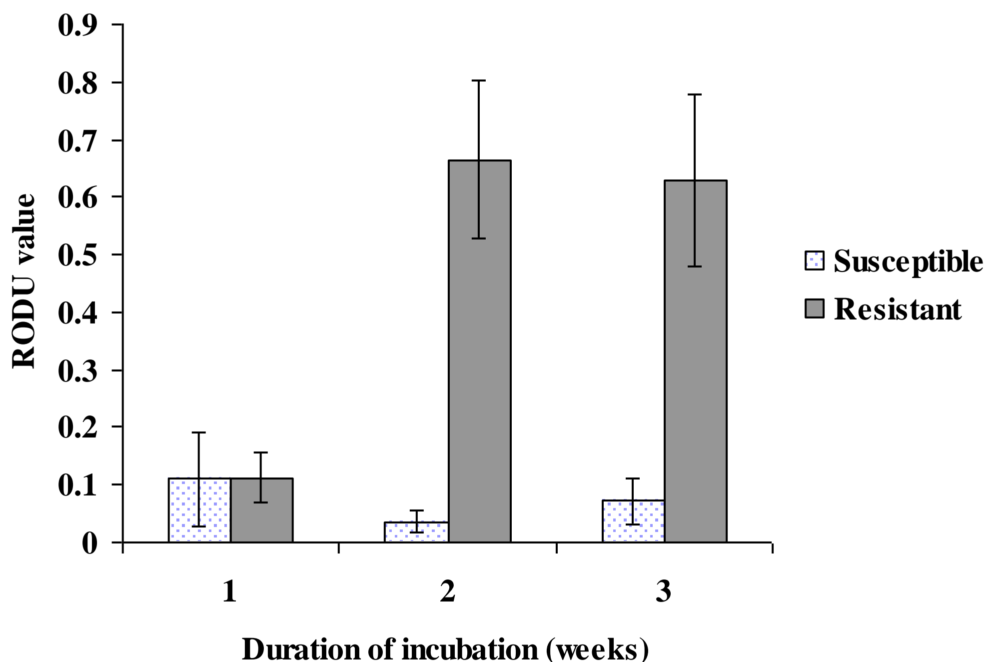


Figure 8: Growth pattern of *M. tuberculosis isolates* at INH concentration of 0.4 µg/ml: growth of isoniazid susceptible isolates (n= 3 in the first and n= 5 in the second and third weeks) and isoniazid resistant isolates (n= 4 in the first and n= 5 in the second and third weeks) measured by RODU values (means ± standard errors)

4.4. Evaluation of direct MTT assay for the detection of isoniazid resistance in *M. tuberculosis*

Out of 23 specimens used to evaluate direct MTT assay for the detection of isoniazid resistance in *M. tuberculosis*, 18 (78.26%) were culture positive and 5 (21.74) were culture negative on LJ medium. In Middlebrook 7H9 broth medium used in the MTT assay a similar result was also obtained (Table 7).

Table 7: Culture result on LJ medium and Middlebrook 7H9 broth used in MTT assay for detection of INH resistance in *M. tuberculosis* (n= 23)

LJ medium	Middlebrook 7H9 broth medium		
	Positive	Negative	**Total**
Positive	18	0	**18**
Negative	0	5	**5**
Total	**18**	**5**	**23**

The OD value that gives interpretable result was determined by transferring 100 μl of the bacterial suspension from the growth medium (7H9) into LJ medium to observe colony-forming units (CFU). The OD value was correlated with the CFU to determine the cut off OD value that gave interpretable value. In this study, the OD value less than 0.1 did not show colony formation on LJ medium. However OD value greater than 0.1 showed colony on LJ medium. Hence the cut off OD value to determine the result as interpretable was 0.1.

By taking a final concentration of 0.2 μg/ml isoniazid, a direct MTT assay for detection of INH resistance was done in Middlebrook 7H9 broth. Of the 18 culture positive isolates, the MTT assay identified 2 (11.1%) isolates as resistant and 16 (88.9%) as susceptible (Table 8). Both resistant isolates were obtained from re-treatment cases.

Drug susceptibility test for INH was done on LJ medium at a final concentration of 0.2μg/ml. Out of 18 isolate tested by the proportion method 2 (11.1%) isolates were resistant and 16 (88.9%) susceptible (Table 8). The growths of INH resistant isolates were shown in fig. 5.

Table 8: Susceptibility testing of INH resistance in *M. tuberculosis* by MTT assay and the proportion method (n= 18)

MTT assay	Standard proportion method		
	susceptible	Resistant	**Total**
Susceptible	16	0	**16**
Resistant	0	2	**2**
Total	**16**	**2**	**18**

The MTT assay correctly identified resistant and susceptible isolates that were conformed by proportion method as resistant ant susceptible, respectively. Thus, the MTT assay showed a sensitivity and specificity of 100% in detecting isoniazid resistant *M. tuberculosis* (Table 9*)*.

Table 9: Efficiency of the MTT assay in detection of isoniazid susceptible/ resistant *M. tuberculosis* in comparison with the standard proportion method

Test character	Efficiency of MTT assay
Specificity (%)	100
Sensitivity (%)	100
Positive predictive value (%)	100
Negative predictive value (%)	100
Accuracy of MTT assay*	100

* The probability of obtaining a true positive or true negative result

The interpretation time for INH was also rapid. Out of 18 isolates tested for INH resistance, 13 (72.22%) of isolates gave interpretable result at week one and 18 (100%) at the subsequent weeks. The assay fully identified the isoniazid resistant and susceptible isolates as of week two (Table 10). All isoniazid resistant and susceptible isolates were correctly identified with the naked eye (Fig. 3 L and K).

Table 10: Time of interpretation of a direct MTT assay for detection of isoniazid resistance in *M. tuberculosis*

week	Interpretable isolates (%)	isolates giving indicated MTT assay*	
		S	R
1	72.22	13	0
2	100	16	2
3	100	16	2
Total	**100**	**16**	**2**

* S, susceptible; R, resist

The difference in the growth pattern of isoniazid resistant and susceptible *M. tuberculosis* isolates were determined by RODU. The differences in RODU define the resistant and susceptible isolates. Isoniazid resistant isolates had RODU above 0.5 whereas the susceptible isolates below 0.2. As shown in fig. 9 the mean RODU value of isoniazid resistant isolates was 0.829 ± 0.11 whereas the mean RODU value of susceptible isolates was 0.053 ± 0.009. For resistant isolates the first week MTT assay was not interpretable (OD value of control tubes <0.1). All resistant isolates had RODU value above 0.5 and the susceptible isolates below 0.2. The differences in the RODU of susceptible and resistant isolates were statistically highly significant ($p < 0.0001$ [Mann-Whitney U test)

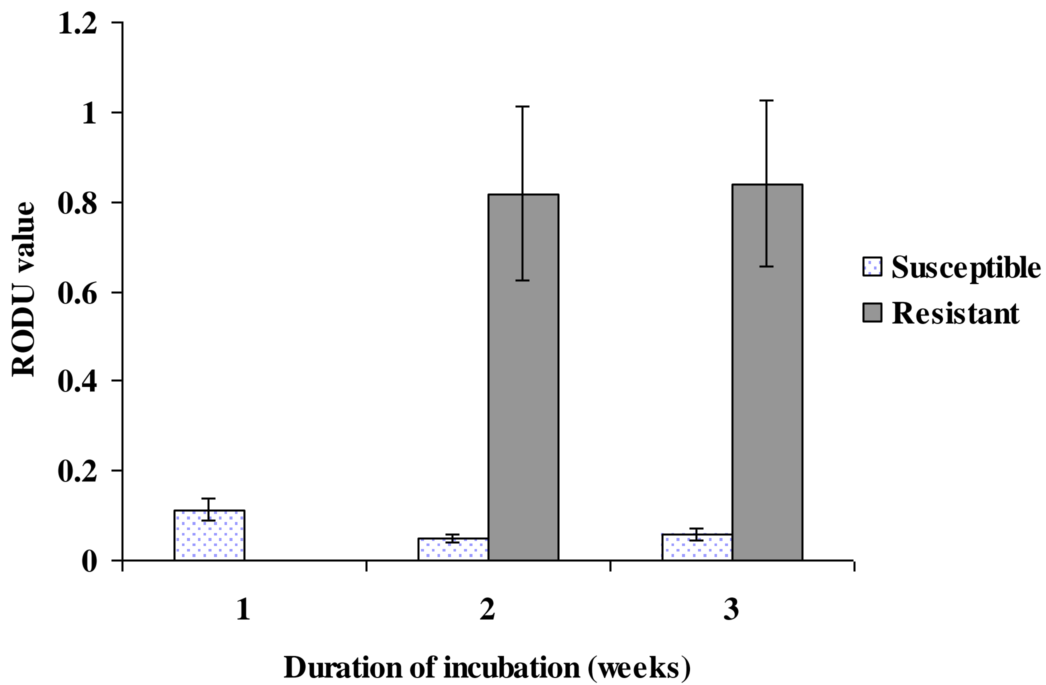


Figure 9: Relative Optical Density Unit (RODU) of susceptible and resistant *M. tuberculosis* isolates in 1st, 2nd, and 3rd week incubation: growth patterns of rifampicin susceptible isolates (n= 13 in the first week and n= 18 in the second and third weeks) and rifampicin resistant isolates (n= 2 in the second and the third weeks) measured by RODU values (means ± standard errors)

4.5. Species identification using polymerase chain reaction (PCR)

For all culture positive clinical isolates PCR-based species identification was done using RD10 primer pairs. All our clinical isolates and *M. tuberculosis* H37Rv strain produced identical bands (308bp) whereas *M. bovis* produced 202bp band size (Fig.10). Hence, all our clinical isolates confirmed to be *M. tuberculosis*.

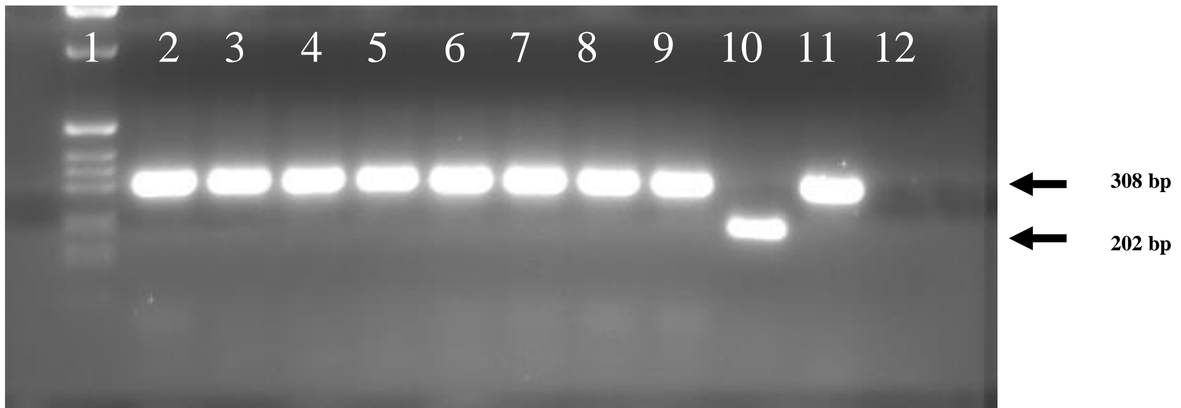


Figure 10: Images of PCR product amplified with RD10 primer; 1= 1kb ladder, 2-9= clinical isolates, 10= *M. bovis,* 11= *M. tuberculosis*, 12 = negative control (TE).

5. Discussion

In this study a total of 153 sputum specimens were collected. From the total number of patients who gave the sputum specimens, 88% belong to the economically productive age group, 15-54, with a mean and median age of 30.45 and 25.5, respectively. This shows that, in addition to being major public health problems, TB poses serious socio economic challenge to high TB burden countries. Therefore, there is a need for rapid diagnosis and effective treatment of TB. In the last few decades many drugs for the treatment of TB have been developed. However, in recent years drug resistant TB especially MDR-TB is becoming a serious challenge to treatment. As a result it is becoming mandatory that the drug sensitivity pattern of a particular *M. tuberculosis* isolate be known for physicians to prescribe the right combination of drugs.

The most widely used conventional method to test drug susceptibility of *M. tuberculosis* strain is time consuming. It requires 3-8 weeks for initial isolation and 4 to 6 weeks for the actual drug susceptibility test. Thus overall the conventional method takes between 7-14 weeks. During this lag waiting period, the patient could suffer and the time for treatment is prolonged. In addition, during this period, the chance of infecting other healthy individuals increases. Therefore, a rapid, reliable, accurate and affordable method for the detection of drug resistant *M. tuberculosis* is highly required.

Many methods such as BACTEC 460 system, BACTEC MGIT and molecular have been developed for rapid detection of drug resistance in *M. tuberculosis* (Martin *et al*., 2005). However, most of these methods are either expensive or require heavy machine and disposal of radioactive wastes (Heifets and Cangelosi, 1999; Guillerm *et al*., 2006). Recently a method based on reduction of MTT tetrazolium salt has been developed as cheap, rapid and reliable technique for the detection of rifampicin resistant TB (Getahun Abate *et al*., 2004) Hence this study was conducted with the objective of evaluating the reliability of a direct MTT assay for detection of *M. tuberculosis* resistant to first-line drugs, rifampicin and isoniazid (MDR-TB). The work was performed under the same

conditions and in facilities available for drug susceptibility test (DST) that the national TB referral laboratory routinely provides as a service to the community.

A direct MTT assay was previously standardized and evaluated for detection of rifampicin resistance in *M. tuberculosis* (Abate *et al*., 2004). This study was a further step of evaluation of the method under program condition. Using the direct MTT assay all drug resistant and susceptible isolates were detected in 3 weeks. The conventional proportion method used as gold standard took up to 7-14 weeks to get drug susceptibility results. It is also interesting to note that 94% of the isolate gave interpretable result in just two weeks. Getahun Abate *et al.* (2004), also reported a 98.5% interpretable result at week two of the MTT assay. This shows that in 90% of the cases, physician can get results of drug resistance/ susceptibility and the patient can start taking the right drugs, up to 10 weeks earlier than is normally done.

For a proper patient management and control of tuberculosis rapid detection methods are essential. The BACTEC 460 system is rapid and yield result on the average in 10 days. But it is expensive, requires heavy machine and disposal of radioactive wastes (Lemus *et al*., 2005), and consequently is not feasible in resource-poor countries such as Ethiopia. The BACTEC MGIT is rapid but the machine is extremely expensive and thus impractical to be implemented in recourse limited countries (Guillerm *et al*., 2006). Most of the patients in Ethiopia reside in rural areas but currently DST is done centrally. The turnaround time is thus important in order for the patient to receive an appropriate treatment on time. However, due to having low economic background, patients are not able to come to the center and wait for a long time to receive their DST result. As a result most patients are forced to take treatment in regional health centers where there is no DST. This greatly increases the chance of spreading drug resistant TB in the community. Our findings on the use of the direct MTT assay for the detection of rifampicin resistance shows that, if used routinely, it can shorten the waiting time by 75-85%. This method has 99% overall agreement with the standard proportion method. The method does not require hazardous reagents, sophisticated equipment or heavy machine. Thus it can easily be applied as routine drug detection method if used under program condition.

MTT is a yellow tetrazolium salt that is converted into purple formazan by dehydrogenases of live cells. The amount of purple formazan formation is directly proportional to the number of live mycobacteria in a given sample (Mosmann, 1983). As observed in this study, OD value result obtained from spectrophotometer readings closely match with color change detected visually with the naked eye. Culture tubes read as yellow with naked eye had OD values < 0.1. Cultures that gave interpretable OD value >0.1 had a visual color change ranging from faint to dark purple. This shows that the method can be carried out even in regional laboratories where there is no access for spectrophotometers, provided that there is facility to work aseptically and incubators. In this way, patients can stay close to their residence, physicians can prescribe the right combination of drugs, and all these could ultimately reduce the spread of drug resistance tuberculosis.

The MTT assay was evaluated against a gold standard proportion method for drug susceptibility test. An overall agreement between MTT assay and the proportion method was found in this study. The assay identified 5.2% isolates as rifampicin resistant and 94.8% as rifampicin susceptible. The results matched 99% with those obtained by the gold standard proportion method of susceptibility testing. In a similar study done by Getahun Abate *et al.* (2004), and Dawit Wolde Meskel *et al.* (2005), an overall concordance of drug susceptibility result between MTT assay and proportion method was reported as 100% and 97.3%, respectively. In an indirect MTT assay with a mixture of known sensitive, susceptible and unknown clinical isolates, Foongladda *et al.* (2002) has also reported a 98.9% correlation between MTT assay and the proportion method. A multicenter study conducted in seven Latin American countries on 30 clinical isolates of *M. tuberculosis* with a known drug susceptibility pattern also showed 98% overall agreement between the indirect MTT assay and the proportion method (Martin *et al*., 2005). Lemus *et al*. (2004) also reported a complete agreement between indirect MTT assay and proportion method in his study carried out on 20 panels of *M. tuberculosis* isolates with a known drug susceptibility pattern.

Relative optical density unit was employed to show the difference between resistant and susceptible isolates and their ability in reducing MTT. The difference in the ability to reduce MTT, which was directly proportional to the number of viable cells in the growth media, defines the resistant and susceptible isolates. A strain was defined as rifampicin susceptible when the relative optical density unit (RODU) value was below 0.2 and resistant when it was above 0.5 (Getahun Abate *et al*., 1998; Mshana *et al*., 1998). The mean RODU value of resistant isolates was 0.926 ± 0.04 and it was above 0.5. The mean RODU value of susceptible isolates was 0.043 ± 0.00 and it was below 0.2. This result was in match with the RODU for resistant and susceptible isolates reported earlier (Getahun Abate *et al.,* 2004; Dawit Wolde Meskel *et al.,* 2005). The differences in the RODU of the samples containing susceptible and resistant isolates were statistically highly significant ($p < 0.0001$). This confirms the homogeneity of the assay under research and diagnostic lab.

Multidrug-resistant tuberculosis defined as resistant to the two key anti-tuberculosis drugs, isoniazid and rifampicin remains a major public health problem. Isoniazid was one of the first line anti-tuberculosis drugs used in Ethiopia both in the initial and continuation phase of the treatment regimen. Similar to rifampicin the detection of isoniazid resistant *M. tuberculosis* isolates was also done with the conventional proportion method which is time consuming and laborious. Therefore, rapid and inexpensive method was also required for early detection of isoniazid resistance in *M. tuberculosis.* In this study, an attempt was made to standardize the MTT assay for the detection of isoniazid resistance in *M. tuberculosis* and evaluating it against the standard proportion method as a direct assay.

The MTT assay was previously used in 96-well plates as indirect detection of isoniazid resistance in *M. tuberculosis* (Martin *et al*., 2005; Montoro *et al*., 2005). However, this has disadvantage from the point of view of biosafety because manipulation of plates could generate aerosols. In this study, capped tube format was used. To standardize the assay five known INH susceptible isolates and five known resistant isolates were used. Reference strains, INH susceptible H37Rv and resistant ATTCC 35835 were also

included. Three different final concentrations of isoniazid (0.1 µg/ml/, 0.2 µg/ml, and 0.4 µg/ml) were taken to determine the critical concentration to be used in the direct MTT assay. These concentrations have been used normally in BACTEC 460, BACTEC MGIT and LJ proportion drug susceptibility tests.

The five resistant and five susceptible isolates were allowed to grow in Middlebrook 7H9 broth containing INH at a final concentration of 0.1 µg/ml, 0.2 µg/ml and 0.4 µg/ml. The RODU value was used to show the difference in growth among the isolates at these INH concentrations. The result shows that the mean RODU of resistant strains were above 0.5 and the susceptible strains were below 0.2 at all the three INH concentrations. However, there was variation among isolates in response to these drug concentrations. At 0.1µg/ml and 0.4 µg/ml INH concentration most resistant isolates had RODU value less than 0.5 at their 1st week incubation. However, the same isolates showed RODU value above 0.5 when grown at 0.2 µg/ml INH concentration. In the succeeding two weeks their mean RODU value remained above 0.5 at the three INH concentrations. One resistant isolate, G5R207, exhibited difference in growth at the three INH concentrations. This isolate was unable to grow well at 0.4 µg/ml INH concentration showing OD value of less than 2. But at 0.1 and 0.4 µg/ml INH concentration, it grows with an OD value of 0.14-0.62 and 0.2-0.9, respectively. This might indicate that G5R207 had low-level resistance to INH The susceptible isolates showed RODU value of less than 0.2 at the three INH concentrations except an isolate G6S594 which had RODU value above 0.2 at 0.1 µg/ml INH concentrations. This might show the presence of at least low-level INH resistance in G6S594 isolates that can tolerate 0.1 µg/ml INH concentration (NCCLS, 2003).

As it was observed in this test, the resistant and susceptible isolates showed clear demarcation of growth at 0.2 µg/ml INH concentrations than 0.1 and 0.4 µg/ml INH concentrations. A concentration of 0.2µg/ml INH inhibits the growth of the susceptible isolates but did not inhibit the resistant one. Moreover, 0.2 µg/ml INH was also used in the proportion method and BACTEC MGIT. Therefore, based on this result, 0.2µg/ml isoniazid concentration was chosen as critical concentration to be used in the direct MTT assay for the detection of isoniazid resistant *M. tuberculosis* in Middlebrook 7H9 broth.

This was the first test for INH using direct MTT assay. In this study, the MTT assay was employed for direct detection of isoniazid resistance on sputum specimens and was evaluated against proportion method as gold standard. The direct MTT assay correctly identified those isolates that were confirmed INH resistant by the proportion method as resistant and the susceptible isolates as susceptible. The result completely matched (100%) with the gold standard method. These finding showed that it was possible to use the assay directly on sputum samples with out the need of primary isolation on LJ medium as done in the indirect one. Therefore it reduces the time required for primary isolation and provides result in short period of time.

With the conventional method it took 7-14 weeks to know the DST result. On the other hand the direct MTT assay was relatively fast and it needed a maximum of 3 weeks to yield the result. The sensitivity and specificity of direct MTT assay for INH fully agreed with proportion method. This result is very important since isoniazid is one of the most important drugs used in the treatment of TB.

Even though the number of specimen tested was comparatively small, our result was promising. Therefore, the present test result could offer the potential for rapid indication of *M. tuberculosis* isolates resistant to INH. This has significant advantage from the patient's point of view. Since, in Ethiopia, INH is given in all three treatment regimen; both in the initial and the continuation phase, there is a large chance for INH resistance to develop. Different reports in Ethiopia also showed an increasing of INH resistance (Getahun Abate *et al.*, 1998). Our study on proportion method also indicated high level INH resistance (15.8%). If drug susceptibility is not tested before drug administration, INH resistant TB will be selected over the years and could ultimately make the drug ineffective. To avoid such problems a cheap and rapid method is required. The results of this study show that the MTT assay could also be effectively used for detection of INH resistance. Thus, the direct MTT assay could be used as an alternative method for the detection of MDR-TB owing to its promising result in detection of both INH and RIF resistant M. tuberculosis.

6. Conclusions and Recommendations

Multi-drug resistant *M. tuberculosis* is an increasing global problem. Therefore, there is a need for the development of rapid and inexpensive methods for timely detection and management of MDR-TB. Existing methods for DST are either time-consuming, as is the proportion method on solid media, or expensive, as is the BACTEC 460 method. The commercial MGIT system is reliable but requires heavy investments in equipment and running costs. The molecular methods are fast but far too expensive to be used in most resource-poor settings such as Ethiopia.

Colorimetric assays based on the reduction of dyes, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide have been tested with great success. The MTT assay is rapid, inexpensive, and easy to perform. It requires neither sophisticated equipment nor expensive substrates or reagents. The result is easily interpreted with a naked eye. It reduces the time to achieve results compared to the proportional method and could be implemented in laboratories with limited resources. Owing to its high level of agreement with the gold standard methods, it has the potential to provide rapid detection of rifampicin and ionized resistance.

Our result under program condition both for rifampicin and isoniazid was promising with high agreement to that of standard proportional method. Hence if the method is implemented as an alternative method it could reduce the drug susceptibility time and alleviate the problem of extra cost incurs on patients.

Hence in consideration of its good agreement with the conventional DST method, its short turnaround time, low cost and simple in application invites the direct MTT assay to be included under the TB control program. However, further studies are required in regional labs to substantiate our findings.

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APPENDICES

Appendix 1

Clinical information of the patient

Hospital: St. Peter's TB Specialized Hospital

SL No.		General information				Patient type		Remark
	Patient ID No.	Age	Sex	Address	Date	New case	Re-treatment case	

Appendix 2

Consent Form

St Peter's TB Specialized Hospital

No.________________

Addis Ababa University in collaboration with AHRI and EHNRI has planned to evaluate MTT assay for the detection of drug resistant *Mycobacterium tuberculosis*.

I am asked to participate in this study and informed that the study requires a total of 5 ml of sputum. I am also taught about tuberculosis, its effect on human and care that should be taken to prevent it. I am informed that I have the right to participate or not in the study and this will have no influence in the medical care I am supposed to get. I told that all data obtained from me will be kept confidential. Taking into consideration all points mentioned above, I agree to participate in this study.

Participant's Name_____________________________ Signature_____________________

Physician's Name______________________________ Signature_____________________

Witnesses' Name_______________________________ Signature_____________________

Appendix 3

¾eUU’ƒ pê

pÆe ä¨<KAe Jeú•M

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›Ç=e ›uv ¿”y`e+ Ÿ›`T¨` H”d” ¾U`U` }sU“ Ÿ›=ƒÄåÁ ¾Ö?““ e’UÓw U`U` }sU Ò` uS}vu` SÉ’>ƒ uT>ssS< dUv ui ›UÜ Iªe ¾SKÁ ²È LÃ Ø“ƒ KT"H@É ›pÇDM::

’@U u²=I Ø“ƒ ¨<eØ ”Éd}õ }ÖÃo›KG<:: KØ“~ ¾T>ÁeðMÑ¨< 5 T>K= K?ƒ` ›¡ SJ’< }’Óa—M:: u}ÚT]U eK dUv ui uc¨< LÃ eKT>ÁS×¨< Ñ<Çƒ“ ”Èƒ SŸLŸM ›”ÅT>‰M ƒUI`ƒ }cØ„—M:: T”—¨<U ¨<Ö?ƒ uT>eØ` ”ÅT>Á´ }’Óa—M::

u²=I Ø“ƒ ¾Sd}õ ¨ÃU ÁKSd}õ LÑ˜ ¾T>Ñv˜ ¾I¡U“ ›ÑMÓKAƒ ”ÅTÃÕÅMw˜ ›¨<o›KG<:: ŸLÃ u}Ökc¨< Sc[ƒ uØ“ƒ ¨<eØ KSd}õ }eTU‰KG<::

¾lSU}—¨< eU ___________________________________ò`T ________________________

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Appendix 4

Drug susceptibility test result of *Mycobacterium tuberculosis* isolates

Code	Drug susceptibility Test*	
	MTT assay	Proportion method
GB02	S	S
GB03	S	S
GB04	S	S
GB06	S	S
GB07	S	S
GB08	S	S
GB09	S	S
GB10	S	S
GB12	S	S
GB13	S	S
GB14	S	S
GB18	S	S
GB19	S	S
GB20	S	S
GB21	S	S
GB22	S	S
GB23	S	S
GB24	S	S
GB25	S	S
GB28	S	S
GB29	S	S
GB30	S	S
GB31	S	S
GB33	S	S
GB34	S	S
GB36	S	S
GB37	S	S
GB38	S	S
GB41	S	S
GB42	S	S
GB43	S	S
GB44	S	S
GB46	S	S
GB47	S	S
GB48	R	R
GB50	S	S
GB51	S	S
GB52	S	S

Code	Drug Susceptibility Test*	
	MTT assay	Proportion method
GB53	S	S
GB54	S	S
GB55	S	S
GB56	S	S
GB57	S	S
GB58	S	S
GB59	R	R
GB60	S	S
GB62	S	S
GB63	S	S
GB64	S	S
GB65	S	S
GB67	S	S
GB68	S	S
GB70	S	S
GB71	S	S
GB72	S	S
GB73	S	S
GB74	R	R
GB75	S	S
GB76	S	S
GB77	S	S
GB78	S	S
GB79	S	S
GB80	S	S
GB81	S	S
GB82	S	S
GB83	S	S
GB84	S	S
GB87	S	S
GB88	S	S
GB89	S	S
GB90	S	S
GB91	S	S
GB92	S	S
GB93	S	S
GB94	S	S
GB95	S	S
GB96	S	S
GB97	R	R
GB98	S	S
GB99	S	S

Code	Drug Susceptibility Test*	
	MTT assay	Proportion method
GB100	S	S
GB101	S	S
GB102	S	S
GB103	S	S
GB104	S	S
GB105	S	S
GB106	S	S
GB107	S	S
GB108	S	S
GB110	S	S
GB111	S	S
GB112	S	S
GB113	S	S
GB114	S	R
GB115	S	S
GB116	S	S
GB117	S	S
GB118	S	S
GB119	S	S
GB120	S	S
GB121	S	S
GB122	S	S
GB123	S	S
GB124	S	S
GB125	S	S
GB126	S	S
GB127	S	S
GB128	S	S
GB129	S	S
GB130	S	S
GB131	S	S
GB132	S	S
GB134	S	S
GB143	R	R
GB158	R	R

Code	Drug Susceptibility Test**	
	MTT assay	**Proportion method**
GB140	S	S
GB141	S	S
GB142	S	S
GB143	S	S
GB144	S	S
GB145	S	S
GB146	S	S
GB147	S	S
GB148	S	S
GB149	S	S
GB150	S	S
GB151	S	S
GB152	S	S
GB154	S	R
GB155	S	S
GB156	S	S
GB158	S	S
GB162	S	S

S= Susceptible R= Resistant

*** Drug susceptibility test for Rifampicin**

**** Drug susceptibility test for Isonaizid**

DECLARATION

I the undersigned declare that this thesis is my original work. It has not been presented for a degree in this or any university and all the source materials used for this thesis have been duly acknowledged.

Name of the candidate Gadissa Bedada

Signature -------------------------- Date --------------------------

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

Name of the advisors Dr. Amare Gesesse

Signature ------------------------- Date --------------------------

Ato Dawit Wolde Meskel

Signature………………………Date---------------------------