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**Drug Susceptibility Testing and Molecular Characterization
of *Mycobacterium tuberculosis* Isolates from Pulmonary TB
Patients at the End of Two Month Intensive Therapy in
Addis Ababa, Ethiopia**

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LIST OF ABBREVIATIONS

AAU	Addis Ababa University
AHRI	Armauer Hansen Research Institute
AIDS	Acquired Immuno Deficiency Syndrome
ALERT	All Africa Leprosy, Tuberculosis Rehabilitation and Training Center
AMK	Amikacin
BCG	Bacille Calmette-Guérin
BMI	Body Mass Index
CAM	Central Asian-Mediterranean
CAP	Capreomycin
CAS	Central Asian
CDC	Center for Disease Control and Prevention
CFU	Colony Forming Units
CIP	Ciprofloxacin
CS	Cycloserine
CTAB	Cetyltrimethyl Ammonium Bromide
DMSO	Dimethyl Sulphoxide
DOTS	Directly Observed Treatment, Short Course
DR	Direct Repeat/ Drug Resistance
DST	Drug Susceptibility Test
ECL	Enhanced Chemiluminescence
EDTA	Ethylenediamine Tetraacetic Acid
EPTB	Extra Pulmonary Tuberculosis
ETH	Ethionamide
FDC	Fixed Dose Combination
FW	Family Welfare
GAT	Gatifloxacin
H	Haarlem
HBC	High Burden Countries
INH	Isoniazid

IUATLD	International Union against Tuberculosis and Lung Diseases
KAN	Kanamycin
LEV	Levofloxacin
LJ	Lowenstein-Jensen
MAS	Multiplex Allele Specific
MDR	Multidrug Resistance
MIC	Minimum Inhibitory Concentration
MGIT`	Mycobacterial Growth Indicator Tube
MIRU	Mycobacterial Interspersed Repetitive Unit
MoH	Ministry of Health
MTB	<i>Mycobacterium tuberculosis</i>
MTBDR+	<i>Mycobacterium tuberculosis</i> Drug Resistance <i>Plus</i>
MTC	<i>Mycobacterium tuberculosis</i> Complex
ND	Not Done
NAID	National Institute of Allergy and Infectious Disease
OADC	Oleic Acid-Dextrose-Catalase
ORF	Open Reading Frame
PAS	<i>p</i> -aminosalicylic acid
PBS	Phosphate Buffer Saline
PRE	PCR Restriction Fragment Analysis
PTB	Pulmonary Tuberculosis
PTH	Prothionamide
PZA	Pyrazinamide
RD	Region of Difference
RFLP	Restriction Fragment Length Polymorphism
RIF	Rifampicin
S	Svedberg
SDS	Sodium Dodecyl Sulphate
SLD	Second Line Drug
SMI	Swedish Institute of Infectious Disease Control
SNP	Single Nucleotide Polymorphism

SpolDB4	Spoligotype Data Base Four
SPSS	Statistical Package for the Social Sciences
SSCP	Single Strand Confirmation Polymorphism
SSPE	Sodium Chloride-Sodium Phosphate-EDTA
ST	Shared Type
STM	Streptomycin
TE	Tris-EDTA
VNTR	Variable Number of Tandem Repeats
XDR	Extensive Drug Resistance

ABSTRACT

Tuberculosis is one of the major health concerns worldwide which continue to exert a heavy toll on public health. Now a days, the AIDS epidemic and the growing problem of resistance to anti-tuberculosis drugs have further complicated management of the disease. Thus, early detection of drug resistance constitutes one of the priorities of TB control programs. This project was designed to assess the drug susceptibility pattern of *Mycobacterium tuberculosis* isolates among patients who remained culture positive at the end of the intensive phase treatment in order to investigate the correlation between being culture positive at the second month of treatment in relation to final treatment outcome. Another aim was to establish a quality controlled method for the detection of anti-TB drug resistance including second line drugs used for MDR-TB. In total, 80 consecutive clinical samples that were on treatment with first line anti-TB drugs for the intensive phase were included in the study. Susceptibility testing for first- and second line drugs was done based on a proportion method adopted for 24-well plates containing 7H10 medium. The result showed that 49 (61.2%) isolates were resistant to one or more of the first line drugs tested. MDR-TB was identified in 20% of the study participants. It was also observed that all isolates resistant to rifampicin were also isoniazid resistant supporting that rifampicin resistance could be used as surrogate marker for MDR-TB. Out of the 80 patients, 51 (63.8%) were cured and 28.7% were defined as treatment failure. A majority of MDR-TB cases (87.5%) detected at the second month were found to be treatment failures which serves as an external validation of the DST method. Moreover, it was also found that all isolates were susceptible to the major second line drugs except for ethionamide (13.8 % resistant) as expected where a cross resistance to isoniazid has been described. The quality of the 24-well 7H10 agar based method was assessed using intra- and inter laboratory controls which showed that the method was highly reproducible. Spoligotyping analysis revealed that the T3_ETH (ST 149, n=23) and the CAS1_KILLI (ST 21, n=9) subfamilies were the predominant genotypes observed. One Beijing strain was also reported. MDR-TB isolates exhibited more clustering compared to drug susceptible and other drug resistant cases. Based on the above finding, culture and drug susceptibility testing for first and second line drugs using the newly developed DST method is advised for patients who remained culture or smear positive at the end of the intensive phase. The 24-well DST method was found to be easy to perform with relatively low cost and with minimal training necessary. It offers the production of hundreds of plates a day. In particular, it seems suitable for suspected MDR cases where both first line and second line drugs are necessary for providing the best therapy for the patient. This method is found to be advantageous in that it can test as many drugs as per the number of wells in a 24-well, single plate. It can also be used in determining the exact minimum inhibitory concentrations (MICs) of the drugs in addition to discriminating between resistance and susceptibility. Thus, in view of these advantages we suggest that the method should be further validated so that it could be used for routine drug susceptibility testing.

Key words: Culture positive, First line drugs, Intensive phase, *Mycobacterium tuberculosis*, MDR-TB, Proportion method, Second line drugs, Susceptibility testing, Spoligotyping.

1. INTRODUCTION

Tuberculosis (TB) is a fatal infectious disease that can affect most parts of the body mainly affecting the lungs. TB is due to infection by bacilli belonging to the *Mycobacterium tuberculosis* complex with majority of the cases caused by *Mycobacterium tuberculosis* (Koch's bacillus) (Plorde, 2004; Varaine *et al.*, 2010). The disease is one of the major public health problems worldwide. The number of tuberculosis cases has risen since 1985, reversing the decrease in the incidence of the disease that had occurred since 1970s (Montoro and Rodriguez, 2007). According to WHO (2009a), eight to ten million new cases and about two million deaths are reported annually worldwide. TB remains to be a major health concern particularly in developing nations due to factors such as poverty, HIV infection, malnutrition and lack of access to health services (WHO, 2005a).

Despite the widespread availability of tuberculosis treatment, the disease continues to impose severe illness and death. The long duration of TB treatment is one of the barriers to global TB control. This prolonged treatment frequently results in poor compliance increasing the risk of relapse after treatment which is associated with the growing problem of anti-TB drug resistance (Anuwatnonthakate *et al.*, 2008).

Drug resistant TB is a common problem in countries with poor national TB control programs (MOH and FW, 2010). A most serious aspect of the problem is the emergence of strains resistant to multiple drugs which is more difficult and expensive to deal with (WHO, 2008a). Management of patient with multiple drug resistance requires prolonged treatment which needs early detection and prompt treatment (Thomas *et al.*, 2007). Thus, drug resistance detection using reliable, cost-effective and rapid drug susceptibility testing (DST) techniques is necessary to guide clinicians in the management of the disease (Sharma and Mohan, 2006). Therefore, in line with the above issues we designed this project to develop a quality controlled method for the detection of anti-TB drug resistance. It was also planned to assess the drug resistance patterns and to characterize the clinical isolates using molecular techniques.

2. LITERATURE REVIEW

2.1. General Features of *Mycobacterium tuberculosis*

Mycobacterium tuberculosis is a large non-motile and non-spore forming rod shaped bacillus grouped under the order actinomycetales. The bacteria in the group are known for the high lipid content in their cell wall. The cell wall is the most distinctive anatomical feature of the bacteria. It is constituted by an inner peptidoglycan layer which seems to be responsible for the shape-forming property and the structural integrity of the bacterium. The lipid coat confers the distinctive characteristics of the group: acid fastness, extreme hydrophobicity (Ryan *et al.*, 2004), resistance to weak disinfectants (Murray *et al.*, 2009). It probably also contributes to the slow growth rate of some species by restricting the uptake of nutrients (Draper, 1998).

The bacterium has a slow growth rate of 12-16 hours compared to that of most bacteria that tend to have multiplicity measured in minutes. The slow growth rate might be partially determined by the cell wall impermeability that limits nutrient uptake. However, only a minimal stimulus to bacterial multiplication is achieved when the permeability is increased through treatment with some compounds that interact with the cell envelope. The rate of RNA synthesis is rather the major factor associated with the long generation time of the tubercle bacillus (Harshey and Ramakrishnan, 1977) which is resulted due to retarded protein synthesis (Verma *et al.*, 1999).

Another clinically important feature of *Mycobacterium tuberculosis* is its impermeability by certain dyes and stains due to high lipid content in its wall. *Mycobacteria* are structurally more closely related to Gram-positive bacteria. However, they do not fit into the Gram-positive category, as the molecules attached to the cell wall are distinctively lipids rather than proteins or polysaccharides. The ability of Mycobacterial organisms to resist decolorization with acid-fast solution after Ziehl-Neelsen staining has utmost practical importance in identifying the tubercle bacillus in clinical specimens (Plorde, 2004).

2.2. Epidemiology of Tuberculosis

2.2.1. Global Epidemiology

The incidence of the disease increases every year since WHO started its annual report in 1997. It has been estimated that one-third of the world's population are infected with latent tuberculosis. The disease continues to exert a huge impact since WHO has declared tuberculosis as a "global emergency" in 1993. Based on surveillance and survey data WHO estimates that about 9 million new cases of TB occurred in 2007 (139 per 100 000 population) which is about the same number of new cases (140 per 100 000 population) as in 2006. Of these 9 million new cases, an estimated 44% or 4.1 million (61 per 100 000 population) were new smear positive cases (WHO, 2009a).

The majority of the global cases of tuberculosis are reported from Asia and Africa, accounting for 55% and 31% of the global incidence, respectively. Consequently, the Americas, European and Eastern Mediterranean regions contribute small fractions of the global cases. Nigeria, South Africa and Ethiopia rank first to third with respect to the number of incident cases in Africa (WHO, 2009a). In regions with high incidence of TB, HIV is the most important factor contributing to the increase in incidence of TB. In 2007, the African Region accounted for 79% of the global HIV-positive TB cases, followed by the South-East Asia Region with 11% of total cases (WHO, 2009a).

Seventy five percent of the people with TB are within the economically productive age group of 15-54 years as HIV disproportionately affects the most productive group. The disease has significant impact on the livelihood of poor families because of diagnosis and treatment related costs. However, the greatest economic loss occurs as a result of loss of productivity from illness and premature death (WHO, 2005b). Directly observed therapy short-course (DOTS) is considered as one of the principal components of WHO's global TB control program (WHO, 2008). This internationally recommended approach to TB control works by combining the five elements: political commitment, quality-assured case detection, effective drug supply and management, standardized treatment and impact measurement (Alli *et al.*, 2003). The 22 high-burden countries (HBC) which account for about 80% of the estimated

global number of new TB cases arising each year, are the main focus of the DOTS program (Montoro and Rodriguez, 2007).

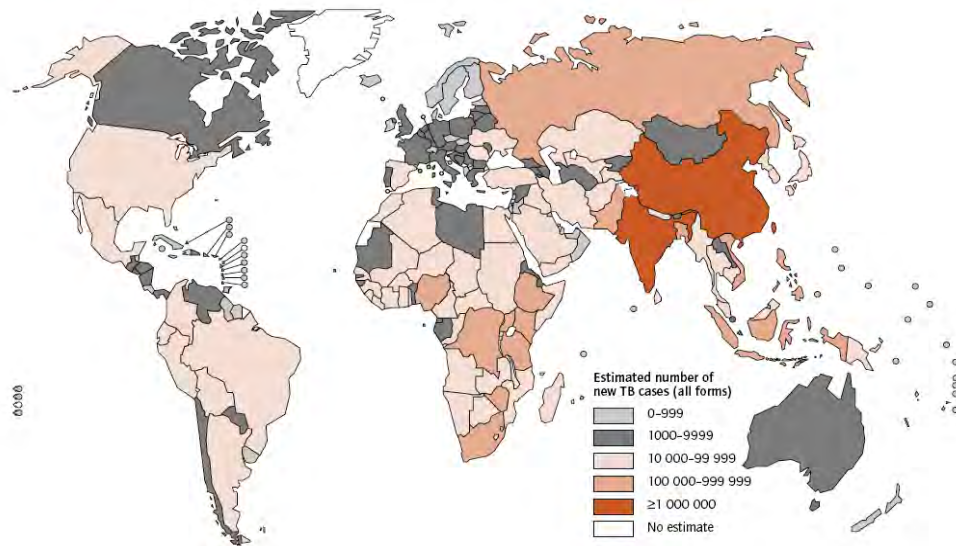


Figure 1. Estimated number of new TB cases, by country, 2007 (WHO, 2009a)

There is a small improvement in case detection rate under DOTS in 2007 as compared with that of 2006. In 2007, over 99% of all notified cases of smear-positive TB were from DOTS programs and the case detection rate under the program was 63%. In the African region, the case detection rate was the lowest, accounting for about 47% of the cases and highest in the Western Pacific Region with a rate of 77% (WHO, 2009a).

2.2.2. The Tuberculosis Situation in Ethiopia

Tuberculosis continues to be one of the major public health concerns in Ethiopia fueled by the expansion of the HIV epidemic since 1990s. Control efforts have been initiated by implementation of DOTS strategy in 1990s. However, tuberculosis, driven by HIV/AIDS epidemic, remains a challenging threat in Ethiopia. According to the Ministry of Health Hospital statistics data, tuberculosis is one of the leading cause of morbidity, the fourth cause of hospital admission, and the second cause of hospital death (WHO, 2008c).

According to the WHO global TB report 2009, Ethiopia ranks seventh among the world's 22 high burden tuberculosis countries. The country had an estimated incidence rate of 378 cases per 100,000 populations in 2007. DOTS coverage reached 95 percent of the population in 2007. Despite the wide coverage of DOTS only approximately 60% to 70% of the population has access to DOTS services. The DOTS detection rate remains low (28%) compared with WHO's target of 70% detection rate. The limited diagnostic capacity for TB in the country remains a challenge to improving case detection rates. The treatment success rate rose to 70% in 2003 after falling from 80% in 2000. In 2007, 84% detection rate was achieved which is closer to the DOTS achievement target (WHO, 2009a).

2.3. Tuberculosis Chemotherapy and Drug Resistance

Chemotherapy for tuberculosis began in the late 1940s with the discovery of streptomycin (Tempel *et al.*, 1951). To achieve TB control worldwide, WHO considers implementation of sound TB control programs following the DOTS strategy as a top priority for action. The choice of the individual drugs in the treatment regimen in short-course chemotherapy is crucial. For purposes of treating TB, anti-TB drugs are grouped into two classes: first line and second line. First line drugs have increased activity against TB with limited toxicity. However second line drugs are much less active, have a much higher toxicity, and are reserved for use only in case of multi drug resistance (Martin and Portaels, 2007).

2.3.1. First Line Anti-TB Drugs

Four first line anti-TB drugs are recommended for the initial treatment of tuberculosis. These include Isoniazid (INH), Rifampicin (RIF), Ethambutol (EMB) and Pyrazinamide (PZA) (Zhang, 2005).

Isoniazid (INH): Isoniazid is one of the most bactericidal drugs against TB. Following administration, the drug enters the cell as a pro-drug that requires processing by the bacterial catalase-peroxidase enzyme encoded by *katG* gene. Once in the cell, the drug is activated to a toxic form which affects intracellular targets such as mycolic acid biosynthesis (Barry *et al.*, 1998). Genetic studies demonstrate that INH-susceptible strains are characterized by the

presence of a functional *katG* gene whereas deletion of *KatG* gene results in the transformation of INH-susceptible strains to INH-resistant ones (Zhang *et al.*, 1993). Studies showed that mutation in *KatG* gene is associated with high level of INH-resistance (Bostanabad *et al.*, 2007; Zakerbostanabad *et al.*, 2009; Zhang *et al.*, 2005). INH drug resistance is also commonly controlled by mutations in the promoter or structural region of *inhA* gene that codes for an enzyme involved in mycolic acid biosynthesis. Resistance due to mutation in this gene is also responsible for resistance to the structurally related second line drug, ethionamide. Studies indicated that *inhA* promoter mutations display low or intermediate level of INH resistance which correlates with acquisition of Ethionamide resistance (Guo *et al.*, 2006; Schaaf *et al.*, 2009).

Rifampicin (RIF): Rifampicin has been used as an effective therapeutic agent since its introduction as an anti-TB drug in 1972 (Ramaswamy and Musser, 1998; Rattan *et al.*, 1999). RIF forms the backbone of tuberculosis chemotherapy along with INH. RIF interferes with transcription by the DNA-dependent RNA polymerase. The drug binds to the β -subunit of the enzyme hindering transcription and thereby killing the organism (Herrera *et al.*, 2003). Resistance to RIF develops when a missense mutation occurs in the region of *rpoB* gene that codes for the β sub unit of the RNA polymerase (Riska *et al.*, 2000). Now-a-days, detection of mutation that causes RIF resistance plays an important role in rapid screening of multi drug resistant TB (MDR-TB) (Heidi *et al.*, 2010; Morgan *et al.*, 2005) as RIF-resistance is highly correlated with MDR (Somoskovi *et al.*, 2001).

Ethambutol (EMB): Ethambutol is an oral chemotherapeutic agent which has a bacteriostatic effect against actively growing *Mycobacteria*. According to the WHO's recommendation, the drug is administered during the first two months of TB treatment together with other first line anti-TB drugs (WHO, 2010b). Following oral administration EMB inhibit the synthesis of mycobacterial cell wall through inactivation of arbinosyl transferase (Telenti *et al.*, 1997), thus causing impairment of cell metabolism and subsequent cell death. *In-vitro* susceptibility testing indicates that when EMB is used alone for treatment of tuberculosis, tubercle bacilli from these patients usually develop resistance to the drug. However, no cross-resistance

between EMB and other anti-TB drugs has been reported (Zhang *et al.*, 1993). Genetic and molecular studies suggest arabinosyl transferase enzyme as the target for EMB (Goude *et al.*, 2009; Sreevatsan *et al.*, 1997). The arabinosyl transferases are encoded by homologous genes that have been identified as *embC*, *embA* and *embB* genes (Telenti *et al.*, 1997). Mutation at codon 306 of *embB* gene is one of the important molecular indicators of EMB-resistance although it has been identified to explain only a minor part of resistance to the drug (Li *et al.*, 2010; Sreevatsan *et al.*, 1997; Starks *et al.*, 2009; Sugawara *et al.*, 2005).

Pyrazinamide (PZA): Pyrazinamide is one of the potent anti-TB drugs administered in combination with INH, RIF and EMB in the short course treatment regimens. It is a pro drug which becomes active upon conversion to pyrazinoic (POA) acid by bacterial pyrazinamidase (PZase). PZA is more active in acidic condition (pH 5.5) and kills bacilli that can survive in acidic medium. The requirement of acidic condition for the activity of the drug makes *in-vitro* DST more difficult since the growth of the bacilli is impaired in acidic pH (McDermott *et al.*, 1954; Cited in Prabhu *et al.*, 2009). The mechanism of action of POA is not well defined despite several studies since its introduction as an anti-TB agent. One suggestion is that pyrazinoic acid acts on the bacilli by inhibiting the protein and RNA synthesis and serine uptake as well as disruption of membrane potential at acid pH (Zhang *et al.*, 2003). Mutation in the *pncA* gene coding for PZase is known to be the molecular basis of PZA resistance (Portugal *et al.*, 2004).

Streptomycin (STM): Streptomycin is the first antibiotic to be developed and used against tuberculosis. It has been an important drug for the treatment of tuberculosis since its discovery. However, the increased frequency of STM-resistance limits the therapeutic efficacy of the drug in the treatment of tuberculosis. STM could be used interchangeably with EMB when the bacteria is known to be susceptible to it (Brzostek *et al.*, 2004). The drug is no more used as first line treatment option except when it is difficult to get the more expensive treatments in medically underserved populations. STM acts as inhibitor of protein synthesis by inducing structural changes in the bacterial ribosomes causing misreading of the

bacterial mRNA and subsequent inhibition of protein synthesis (Abbadi *et al.*, 2001; Sreevatsan *et al.*, 1996).

2.3.2. Second Line Anti-TB Drugs

Management of patients with MDR-TB involves the use of second-line drugs which are inherently more toxic and less effective than first-line drugs. The following drugs are classified as second line drugs for the management of multi-drug resistant tuberculosis (WHO, 2008e).

Aminoglycosides: Amikacin (AMK) and Kanamycin (KAN) are two closely related injectable second-line drugs that are used for treating patients infected with MDR isolates that are susceptible to the agents. These drugs have similar mechanism of action which is based on the interactions with ribosomal RNA. The drugs inhibit protein synthesis and thus cannot be used against dormant *Mycobacterium tuberculosis*. Aminoglycosides bind to bacterial ribosomes and disturb the elongation of the peptide chain in the bacteria. Resistance to AMK and KAN is associated with mutations in the *rrs* gene encoding for 16s rRNA (Suzuki *et al.*, 1998). Studies showed the presence of cross resistance between aminoglycosides (Allen *et al.*, 1983; Jugheli *et al.*, 2009) and some others reported discordant resistance to kanamycin and amikacin in drug resistant *Mycobacterium tuberculosis* (Kruuner *et al.*, 2003).

Fluoroquinolones: Fluoroquinolones are a family of synthetic broad-spectrum antibiotics with bactericidal activity. Among the fluoroquinolones used today are Ciprofloxacin (CIP), Ofloxacin (OFL), Levofloxacin (LEV), and more recently the Gatifloxacin (GAT) and Moxifloxacin (MOX) (WHO, 2001). CIP is no longer recommended for treatment of TB because of its weak efficacy compared with other fluoroquinolones whereas MOX and LEV are regarded as the most potent drugs with least side effects such as liver toxicity (WHO, 2001). Fluoroquinolones prevent DNA replication by inhibiting bacterial DNA gyrase and topoisomerase IV which are important targets of anti-bacterial agents (Chen and Lo, 2003; Cynamon and Sklaney, 2003; Mdluli and Ma, 2007; Piton *et al.*, 2010). Mutations in the

gyrase coding genes of the bacteria are known to confer fluoroquinolone resistance in *Mycobacterium tuberculosis* (Ginsburg *et al.*, 2003; Matrat *et al.*, 2006).

Capreomycin (CAP): Capreomycin is a polypeptide antibiotic which is given in combination with other anti-TB drugs for the treatment of MDR-TB. Studies indicate complete cross-resistance between capreomycin and viomycin in *Mycobacterium tuberculosis* owing to their structural similarities (Tsukamura and Mizuno, 1975). The mechanism of action or molecular basis of resistance for Capreomycin is not well defined (Ramaswamy and Musser, 1998). Both Capreomycin and Viomycin are thought to interfere with protein synthesis by binding to the 30S subunit of the ribosomal complex. In one study, it was reported that disruption of the *tlyA* open reading frame (ORF) can confer capreomycin resistance in laboratory-generated spontaneous capreomycin-resistant mutants and this was also observed in four capreomycin-resistant clinical isolates (Maus *et al.*, 2005).

P-aminosalicylic acid (PAS): PAS is a bacteriostatic drug which has been extensively used for many decades since its introduction as an anti-TB drug at the end of 1944. The drug is still in use in low-income countries due to its low cost. This anti-tubercular agent is often administered in association with Isoniazid (da Silva and Aínsa, 2007). The mechanism of action of this drug is not well defined. But, it is shown that the drug remains active in the presence of thymidylate synthase, a key enzyme in folate metabolism (Mathys *et al.*, 2009). Thus, it is proposed that PAS-resistance is associated with mutation in the *thyA* gene which codes for thymidylate synthase (Mathys *et al.*, 2009; Rengarajan *et al.*, 2004).

Thioamides: Ethionamide (ETH) and Prothionamide (PTH) are the two main drugs from the thioamide family. Thioamides are frequently used drugs for the treatment of drug-resistant tuberculosis and, therefore, are increasingly becoming relevant second line drugs (Wright *et al.*, 2006). ETH is a structural analogue of INH and similar mode of actions have been suggested for the two drugs (da Silva and Aínsa, 2007; Johnsson *et al.*, 1995). As in INH, mutation in the promoter and coding region of *inhA* is suggested to be the source for the molecular mechanism of ETH resistance (Wang *et al.*, 2007; Wright *et al.*, 2006).

2.3.3. Standard Tuberculosis Treatment Regimens

WHO has continually emphasized the DOTS strategy as a framework of tuberculosis prevention and control programs. One of the essential components of the DOTS strategy is the use of recommended treatment regimes (WHO, 2006, 2010b). The current standard treatment regimen for active TB requires the supervised administration of a multi-drug combination for a minimum period of 6 months. In addition to saving the lives of tuberculosis patients, effective multi-drug chemotherapy reduces the number of tuberculosis patients in the community which otherwise could be the potential sources of transmission of the disease (Reichman, 2000).

Ideally, WHO recommends drug susceptibility testing (DST) for all patients at the start of treatment, so that the most appropriate therapy for each individual can be determined. New patients with pulmonary TB should receive a regimen containing of 6 months. In intensive phase INH, RIF, PZA, and EMB are administered for a two-month period followed by a 4-month period of continuation phase consisting of INH and RIF (Table 1). Wherever feasible, patients take the optimal dosing frequency daily throughout the course of therapy. They may also receive a daily intensive phase followed by three times weekly continuation phase provided that each dose is directly observed (WHO, 2010b).

Table 1. Regimen options for the treatment of tuberculosis (FMoH, 2009; WHO, 2010b)

Patient Category	Indications	Intensive phase		Continuation phase	
		Drugs	Interval and duration	Drugs	Interval and duration
Category I	New Smear Positive, Smear negative with severe HIV & Severe EPTB	2HRZE	Daily for 8 Weeks	4HR	Daily or 3 times a Week for 16 Weeks
Category II	Relapses & defaulters of Category I	2HRZES/1HRZE	Daily for 12 Weeks	5HRE	Daily or 3 times a Week for 20 Weeks
Category III	Less severe form of EPTB New smear negative PTB (Not Category I)	2HRZE	Daily for 8 Weeks	4HR or 6HE	Daily for 16 or 24 Weeks
Category IV (Ethiopian Case)	MDR-TB but no DST/ MDR-TB susceptible to AMK & Quinolone	EMB-PZA-KAN(AMK)-LEV-ETH-CS			
	MDR-TB susceptible to KAN & resistant to Quinolone	EMB-PZA-KAN(AMK)-MOX-ETH-CS-PAS			
	MDR-TB susceptible to Quinolone & resistant to KAN	EMB-PZA-CAP-LEV-ETH-CS			
	MDR-TB Resistant to Quinolone & to KAN	EMB-PZA-CAP-MOX-ETH-CS-PAS			

H, isoniazid; R, rifampicin; Z, Pyrazinamide; E, Ethambutol

- Each regimen in category IV should be standardized or individualized based on DST data. Total duration will be at least 18 months past culture conversion with a minimum of 6 month intensive phase and a 12 month continuation phase.

TB patients returning after *defaulting* or *relapsing* from their first treatment course may receive Category II retreatment regimen (Table 1). In the cases of treatment failures i.e. patients whose sputum culture remains positive after 5 or more months of treatment, it is recommended that drug susceptibility testing should be performed for at least INH and RIF

before getting access to MDR-TB treatment. MDR-TB regimen should be embarked based on country specific DST data from similar groups of patients. WHO has endorsed the use of empirical MDR regimen by including high risk patients when countries have limited capacity to perform DST and when it takes too long to get DST results (WHO, 2010b). The suggested standard MDR-TB regimen in Ethiopia is 6EMB-PZA-KAN(AMK)-LEV-ETH-CS/12EMB-PZA-LEV-ETH-CS (FMoH, 2009).

Treatment of patients with MDR-TB is much more difficult and costly than other drug resistant cases. Although there are many review papers on the treatment of MDR-TB, the strategies are a subject of recurrent controversy (Caminero and Torres, 2005). Some authorities suggest that therapy of individuals with MDR strains should be based on DST results of both first-line and second-line drugs (SLD). As with other antimicrobial agents, the use of SLD can generate resistant mutants. *In vitro* second line drug DST often shows poor reproducibility and lack of correlation with clinical response. Nevertheless, many laboratories perform DST (Kim *et al.*, 2004), and molecular methods (Giannoni *et al.*, 2005) allowing rapid diagnosis of resistance.

2.3.4. Drug Resistant and Multi-drug Resistant Tuberculosis

Drug resistance in tuberculosis causes the decrease in the susceptibility to anti-TB drugs of *Mycobacterium* strains of human type that have never come in contact with the drugs (Mitchison, 1984). The emergence of drug resistance has posed a major challenge in the control of tuberculosis. Drug resistance in tuberculosis is the result of spontaneous mutation as well as poor programmatic and individual care performance (Gillespie, 2002). Patients who have never been previously treated for the disease could be found to develop resistance due to transmission of a drug-resistant strain. Such resistance is defined as “primary resistance”. Resistance developed during the course of treatment due to poor treatment adherence is said to be “acquired resistance” or “secondary resistance” (WHO, 2008d).

The effect of anti-TB drug resistance has started since the first anti-tuberculosis agent, streptomycin, appeared on the market as a monotherapy (Youmans *et al.*, 1946, Cited in

Espinal and Salfinger, 2005). The continual usage of the drug gradually brought an acquired resistance and hence it was no longer effective for most of the cases. Since then, it was found that the emergence of such resistance could be reduced by the combined use of streptomycin with other anti-TB drugs. This seemed to be promising in showing good clinical outcomes. However, the success of preventing tuberculosis by the use of combined chemotherapy has faced challenges due to the emergence of multi-drug resistant tuberculosis.

MDR-TB is defined as TB that is resistant at least to INH and RIF with or without resistance to other first line drugs. Among the different patterns of drug resistance, multidrug resistance has been clearly identified as a severe form of TB that requires prolonged duration of treatment and high cost. MDR-TB has a strong impact on morbidity and mortality. It is a challenge not only for the public health but also in the context of global economic burden, especially in the absence of proper national level programs in the developing nations (WHO, 2009a).

In 2008, globally there were an estimated 390 000–510 000 primary and acquired cases of MDR-TB with the best estimate at 440 000 cases. The highest numbers of MDR-TB were found in India, China and Russian federation. In some areas of the former Soviet Union, more than 20% of new cases were MDR-TB (WHO, 2010a). As compared to the 2007 WHO report of 0.5 million cases (WHO, 2009a), in the 2008 report relatively less MDR cases were detected. This decrement was not regarded as a true decline as the later report reflects the reporting of new drug resistance data, changes in TB incidence and the use of updated methods. The burden of MDR-TB in Africa remains largely unexplored, mainly due to lack of quality controlled second line testing. The proportion of MDR-TB, as has been shown by some African countries, seems relatively low with a frequency ranging from 0.5% to 3.9% among new TB cases and 0.0% to 16.7% among previously treated TB patients. Inadequate laboratory capacity to perform diagnostic testing among TB patients and barriers to conducting drug resistance surveys could be one of the reasons for the absence of representative data (WHO, 2010a).

Previous studies on anti-TB drug resistance indicated the existence of the problem of MDR-TB. According to the study of Abate *et al.*, (1998), the rate of MDR-TB among the retreatment groups was 12%. Later on, according to a study conducted on retreatment cases at St Peter's TB Specialized Hospital (SPTBSH), the rate of MDR-TB was found to be 26% (Woldemeskel *et al.*, 2005). According to WHO 2008 report, in Ethiopia 5825 (4964 among newly diagnosed and 861 among previously treated cases) MDR-TB cases were estimated to have occurred in 2006. In the WHO (2009a) global tuberculosis control report, the prevalence of MDR-TB in Ethiopia was estimated to be 1.6% among newly diagnosed cases and 12% among retreatment cases. In one recent study conducted at St Peter's TB Specialized Hospital and Ethiopian Health and Nutrition Research Institute, the prevalence of MDR-TB was found to be 43% (Agonafir *et al.*, 2010).

Multiple factors such as, under developed laboratory capacity, limited access to second line drugs and poor treatment have resulted in the emergence of a newer form of drug resistance known as an extensively drug resistant (XDR) TB (Pillay and Sturm, 2007). Isolates of bacilli are said to be XDR when MDR strains are resistant to at least any fluoroquinolone and any of the second line injectable agents (CDC, 2006b). In 2000-2004, the WHO and CDC surveyed a total of 17690 TB isolates from 49 countries and showed that 20% of those isolates were MDR and 2% were XDR-TB strains (CDC, 2006a). The total number and proportion of XDR-TB isolates observed in this study increased from 5% in 2000 to 7% in 2004 (WHO, 2007). Despite limitations in the quality assurance of laboratory testing, data from this report indicate that XDR-TB is widespread with 49 countries having reported at least one case. However, the report may not represent the global magnitude as the majority of the participated countries were low TB burden countries (WHO, 2008a).

Although there is an important report of high mortality in HIV co-infected TB patients with XDR-TB from rural Kwazulu Natal, South Africa (Gandhi *et al.*, 2006), little information is available regarding the prevalence and epidemiology of the outbreak in regions with high incidence of TB and HIV co-infection. Drug resistant TB is expected to exist in countries like Ethiopia where there is high risk of infection with HIV, as drug resistance has been

significantly associated with HIV infection (Mitike *et al.*, 1997). The presence of XDR-TB has not been thoroughly investigated in Ethiopia. Recently two XDR-TB strains were reported from a total of 45 MDR-TB cases in a study conducted at St. Peter's TB Specialized Hospital and Ethiopian Health and Nutrition Research Institute (Agonafir *et al.*, 2010).

XDR-TB is more expensive and difficult to treat than other forms and outcomes for patients are much worse (Jeon *et al.*, 2008), therefore understanding the magnitude and distribution of the case is important. To confront such type of TB, it is also necessary to improve strategies focusing on more rapid diagnosis, better access to drugs, adherence to treatment and decentralization of primary care level services (Goemaere *et al.*, 2007). Overall, XDR-TB is associated with increased mortality especially in high HIV prevalent settings (Gandhi *et al.*, 2006; Gandhi *et al.*, 2010) and is currently considered as one of the major challenging threats for public health (Alexander and De, 2007).

2.3.5. Detection of Drug Resistant TB

Early detection of drug resistance constitutes one of the priorities of TB control programs. The need for reliable DST increases with the emergence of anti-TB drug resistance (Martin and Portaels, 2007). Drug susceptibility of *Mycobacterium tuberculosis* can be determined either by observation of growth or metabolic inhibition in a medium containing anti-tuberculosis drug or by detection of mutations in the genes conferring drug resistance (Kim, 2005). Most of the conventional methods are laborious and require long period to obtain results. In recent years, new rapid technologies and approaches have been proposed but high cost hampers widespread implementation of these techniques (Martin and Portaels, 2007; Palomino, 2009).

2.3.5.1. Phenotypic Methods

Phenotypic methods using egg or agar based media are still the most utilized methods in many countries. The standard methods using Lowenstein-Jensen (LJ) medium include the proportion method, the absolute concentration method and the resistant ratio method which are fairly well standardized with clinical samples, at least for the major anti-TB drugs (Canetti *et al.*, 1969). Among conventional methods, the proportion method is the most

preferred choice. The method is based on the estimation of the proportion of mutant strains resistant to a given anti-TB drug. This is determined by comparing bacterial growth in the drug containing and the drug free control media. When performed in LJ media, the test is first read after 28 days of incubation or at 5-6 weeks if there is not enough growth. If the proportion of resistant bacteria is higher than 1%, the strain is considered resistant (Canetti *et al.*, 1969; Heifets, 2000). When Middlebrook 7H10/11 media is used, results are interpreted after 21 days of incubation or even earlier if there is clear growth difference between the drug containing and the drug free media (Kent and Kubica, 1985).

The absolute concentration method involves determination of the minimum inhibitory concentration (MIC) using serial two fold dilutions of the test drugs where a standard inoculum of the test strain is grown. The resistance of a strain is expressed in terms of the lowest concentration of the drug that inhibits 99% of the bacteria (Heifets, 2000; WHO, 2008b). MIC distribution results can also be used in defining clinical break points along with data related to pharmacokinetics, pharmacodynamics and clinical outcomes (Kahlmeter *et al.*, 2006), in adjusting anti-TB drug doses and in identifying mutations associated with high and low level of drug resistant phenotype (Ohno *et al.*, 1996; Schon *et al.*, 2009).

In the resistance ratio method, resistance is expressed based on the ratio of the MICs of a test strain and that of the drug-susceptible reference strain, H37Rv in the same set of test. This test was first introduced to prevent variation in MICs of a given strain of *Mycobacterium tuberculosis* when tested on different batches of the absolute concentration method. The method compares the resistance of an unknown clinical strain with that of a reference strain. Reading of the test is performed after 4 weeks of incubation at 37°C. An isolate with a resistance ratio value of two or less is considered susceptible, while a resistance ratio of eight or more defines the isolate as resistant (Heifets, 2000; Kent and Kubica, 1985; WHO, 2008a).

More recent phenotypic methods, including the BACTEC MGIT-960 TB system, are based on liquid media. These automated methods are designed for the rapid detection of Mycobacterial growth and drug susceptibility testing of *Mycobacterium tuberculosis*. MGIT

utilizes a modified 7H9 Middlebrook broth base with 0.25% glycerol and MGIT OADC enrichment media. The MGIT tubes contain an oxygen quenched fluorescent sensor embedded in silicon at the bottom. The consumption of oxygen by the growing bacterial causes the oxygen quenched fluorochrome to fluoresce under UV light. During DST, the bacterial suspension is inoculated into two MGIT tubes, one of them containing the test drug. If the anti-TB drug inhibits growth, the fluorochrome will remain quenched in the drug containing tube, while the growth control will grow uninhibited and will have increasing fluorescence. The degree of fluorescence is monitored by the instrument which automatically interprets results as susceptible or resistant (Tortoli *et al.*, 2002).

2.3.5.2. Genotypic Methods

In genotypic method, anti-TB drug resistance is detected by analyzing the genetic determinants of resistance rather than looking for the resistance phenotype. There are many molecular techniques available for studying antibiotic susceptibility. Most of them are based on amplifying a specific target zone of an antibiotic resistance determining gene, followed by the analysis of an amplified product to identify the gene mutation associated with the resistance to this antibiotic (Eisenach, 1999; Maus *et al.*, 2005). They offer several advantages over the conventional method such as faster turnaround times. However, by far not all molecular mechanisms of drug resistance are known. Even the more established ones are rather difficult and expensive to routinely implement for the detection of mutations for several drugs (Hazbon, 2004).

Solid-phase hybridization techniques are one of the potential tools for the genotypic resistance testing. The line probe assay and the HAIN GenoType MTBDR+ assay are two commercially available solid phase hybridization techniques used for the rapid detection of anti-TB drug resistance. The line-probe assay is used for the detection of resistance to RIF (Ozkutuk *et al.*, 2007) while the GenoType MTBDR+ assay detects resistance to INH and RIF simultaneously (Ling *et al.*, 2008). In solid-phase hybridization, amplified products of the resistance-determining regions are allowed to hybridize to membrane bound probes covering the wild-type sequences and specific mutated sequences. The banding patterns of

the hybridized oligonucleotides are visually detected after an enzyme mediated color reaction. The absence of a wild-type band or the appearances of bands representing specific mutations marks the presence of drug resistance (Richter *et al.*, 2009).

The other genotypic method for detection of drug resistance is the automated DNA sequencing approach. The method looks for any mutation by analyzing the nucleotide sequence of the resistance coding gene. It is the most accurate method among the molecular techniques available to detect *Mycobacterium tuberculosis* drug resistance. Because of its accuracy and reliability, the automated DNA sequencing approach has become a gold standard for mutation detection (Victor and van Helden, 2001). Several other genotypic methods have also been developed and are in use, such as PCR-single strand conformation polymorphism (SSCP) analysis, multiplex allele-specific (MAS) PCR hetero-duplex formation, dot blot hybridization assays, DNA microarrays or high-density oligonucleotide arrays, PCR-restriction fragment length polymorphism analysis (PRA) and real-time PCR techniques (Richter *et al.*, 2009). Although the genotypic methods have the advantage of being rapid, accurate and specific, such techniques will not exclude the need for screening of resistance which will investigate new resistance mutations. None of the established molecular tests target all possible genes or mechanisms involved in resistance and the price and technology of some of the commercial assays are not feasible to be largely implemented in high TB endemic countries (Caws and Drobniewski, 2001).

2.4. Molecular Characterization

Various molecular techniques have been used worldwide quite successfully to characterize *Mycobacterium tuberculosis* strains. Molecular Genotyping of *Mycobacterium tuberculosis* is useful for population dynamics analysis as well as for the identification of outbreaks (van Soolingen *et al.*, 1991). Genotyping methods currently rely upon analysis of restriction profiles including restriction fragment length polymorphisms (RFLP) using *IS6110* probing (van Embden *et al.*, 1993), amplification of selected regions of variable number tandem repeats (VNTR) (Frothingham and Meeker-O'Connell, 1998), mycobacterial interspersed repetitive units (MIRU) (Mazars *et al.*, 2001a), spoligotyping (Kamerbeek *et al.*, 1997) and

deletion and insertion site mapping (de la Salmoniere *et al.*, 2004). The genetic relationship between strains of *Mycobacterium tuberculosis* can also be determined based on single nucleotide polymorphism (SNP) analysis located in inter-genic spacers (Gutacker *et al.*, 2006).

2.4.1. Species Typing

Because of the slow growth rate of *Mycobacterium tuberculosis*, identification by using conventional biochemical tests may require several weeks (Kent and Kubica, 1985). The ability to differentiate between members of *Mycobacterium tuberculosis* complex (MTC) through the analysis of species-specific deletions could however, provide rapid epidemiological data that contribute to proper patient treatment and public health measures. Region of difference (RD) typing is among these rapid PCR based techniques used for the identification of *Mycobacterium* species. From close inspection of the flanking sequences, it is apparent that RD9 deletions occurred in MTC bacteria other than *Mycobacterium tuberculosis* and *Mycobacterium canettii*. Thus, RD9-typing can be performed to differentiate between *Mycobacterium tuberculosis* and other MTC members such as *Mycobacterium bovis* species especially when it is important to identify sources of infection (Parsons *et al.*, 2002).

2.4.2. Strain Typing

IS6110 RFLP: DNA fingerprinting by using the mobile element *IS6110* as a probe is considered as “a gold standard” to differentiate *Mycobacterium tuberculosis* complex strains (van Embden *et al.*, 1993). It has higher level of differentiation compared to other genotypic methods (Kremer *et al.*, 2002). However, *IS6110* RFLP is labor intensive especially during large-scale genotyping and hence can be susceptible to errors. The method has less discrimination power in isolates that have fewer than six *IS6110* elements. Besides these draw backs, *IS6110* RFLP typing is reported to have less inter- laboratory reproducibility hindering global based genotyping of *Mycobacterium tuberculosis* strains using this typing technique (Cowan *et al.*, 2002). When used in combination with MIRU-VNTR,

spoligotyping is rather a faster and more robust genotyping technique than *IS6110*-RFLP fingerprinting (Sola *et al.*, 2001; Sola *et al.*, 2003).

Spoligotyping: Spoligotyping is a rapid PCR based method for simultaneous detection and typing of *Mycobacterium tuberculosis* strains. The typing method is based on the strain dependent DNA polymorphism present at the direct repeat locus (DR) within the genome (Mostowy and Behr, 2005). The DR locus consists of identical 36bp DRs interspersed with 35 to 41 bp non-repetitive spacer sequences (Groenen *et al.*, 1993). The presence or absence in the DR region of spacers of known sequence can be detected by hybridization of amplified spacer DNA to a set of immobilized oligonucleotides, representing each of the unique spacers (Kremer *et al.*, 2002).

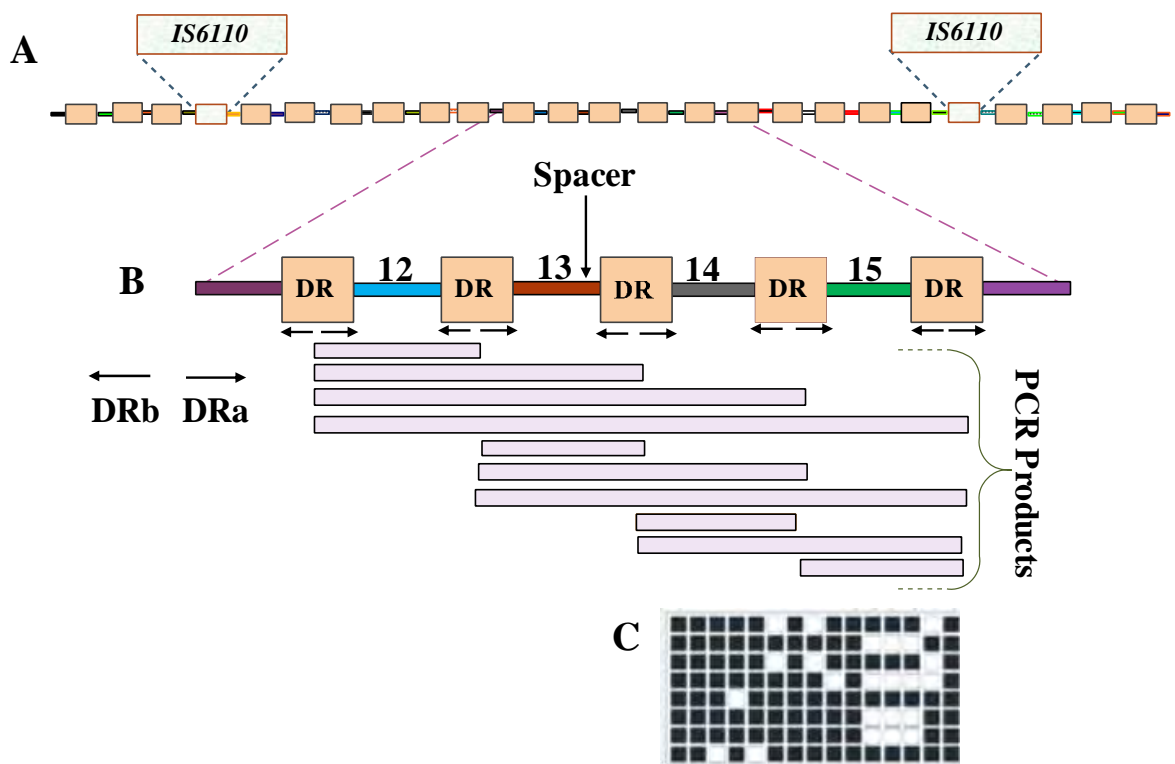


Figure 2. Structure, amplification and hybridization patterns of spacers of the DR locus in Mycobacterial genome. A) Structure, B) Principle of *in vitro* amplification and C. Hybridization patterns of amplified spacers (Kamerbeek *et al.*, 1997).

Spoligotyping has been used to type a large number of strains (Goguet de la Salmoniere *et al.*, 1997). The method enables classification of isolates into distinct strains, and thus allows characterization of genetic diversity of *Mycobacterium tuberculosis* species in clinical samples. It has gained widespread use for studying tuberculosis outbreaks and identifying sources and chains of infection (Luciani *et al.*, 2008). Another advantage is its ability to measure prevalence of endemic strains (Filliol *et al.*, 2002). The technique is useful not only for studies of tuberculosis epidemiology, but also for deciding about possible cross-contamination in mycobacteriology laboratories (Mazurek *et al.*, 1991).

MIRU-VNTR: Another PCR based method for the identification of various types of *Mycobacterium tuberculosis* strains is MIRU-VNTR typing. This genotyping method is based on the variability in copy number of the mycobacterial interspersed repetitive units (MIRU) (Magdalena *et al.*, 1998; Mazars *et al.*, 2001a; Supply *et al.*, 2001). MIRU-VNTR typing has been increasingly used to type MTC strains. Originally the method was employed using 12 MIRU loci. More recently, a 24 loci based MIRU-VNTR typing with higher level of discriminatory power has been proposed (Supply *et al.*, 2006). The discriminatory power of the 24 loci MIRU-VNTR may exceed that of the gold standard which is *IS6110* RFLP method when used in combination with spoligotyping (Christianson *et al.*, 2010). Thus, currently molecular typing based on MIRU-VNTR has been proposed, in combination with spoligotyping, as a preferred technique for large-scale, high-throughput genotyping of *Mycobacterium tuberculosis* strains (Supply *et al.*, 2006).

3. STATEMENT OF THE PROBLEM

Emergence of drug resistant strains in tuberculosis is becoming one of the growing challenges of TB control programs. Factors such as inadequate and incomplete treatment in tuberculosis have resulted in the emergence of strains resistant to combined anti-TB drugs. Reports showed the gradual increase of drug resistant TB among newly diagnosed and re-treatment cases in Ethiopia. In a recent report, relatively high proportion of MDR-TB (2.3% from new cases and 71.4% from retreatment cases and 4.4% XDR-TB cases were observed among pulmonary TB patients in Addis Ababa (Agonafir *et al.*, 2010). The TB control program in Ethiopia has already started Pilot of MDR-TB treatment after identifying MDR-TB as one of priority public health problem and is committed to initiate comprehensive treatment regionally (MoH, 2009). In line with anti-TB treatment, reliable and rapid drug susceptibility testing methods are required for guidance of treatment and surveillance of drug resistance. Early detection and prompt treatment of multidrug-resistant tuberculosis is crucial to avoid further spread of the disease and to prevent amplification of drug resistance to sever forms due to inappropriate treatment. Thus, early detection of drug resistant TB and establishment of quality controlled DST method for first line and second line drugs need to be addressed in this health facility based survey of drug resistance and molecular characterization.

4. HYPOTHESIS

- Failure of culture conversion at the end of the intensive phase chemotherapy among new pulmonary TB patients who are under category I treatment is due to MDR-TB strains with higher rate of genetic clustering

5. OBJECTIVES OF THE STUDY

5.1. General Objective

- To investigate the drug resistance pattern of *Mycobacterium tuberculosis* isolates from pulmonary TB patients who remained culture positive at the end of the intensive phase chemotherapy

5.2. Specific Objectives

- To establish quality controlled method for the detection of *Mycobacterium tuberculosis* isolates resistant to first line and second line drugs
- To look into the drug susceptibility pattern of *Mycobacterium tuberculosis* isolates among patients who become culture positive at the end of the intensive phase chemotherapy
- To early detect MDR-TB cases from patients who fail to respond to the two-month intensive chemotherapy so that such patients could be referred to appropriate MDR regimen
- To provide DST data on second line anti-TB drugs required to make a definitive diagnosis of MDR-TB or XDR-TB
- To assess correlation of culture positivity at the end of intensive phase and final treatment outcomes in patients receiving first line anti-TB drug treatment
- To characterize *Mycobacterium tuberculosis* isolates using genotypic techniques to identify the circulating strains within the clinical samples

6. MATERIALS AND METHODS

6.1. Study Participants

Eighty consecutive clinical samples were included in the study. All the participants were selected from 500 pulmonary TB patients using convenience sampling technique that is based on sputum culture positivity at the second month of category I treatment. The 500 patients were newly diagnosed cases who came to St Peter's TB Specialized Hospital (SPTBSH) for a WHO sponsored 4FDC clinical trial from April 2007 to October 2009.

6.2. Sample Collection

The sputum specimens that were positive for smear microscopy at the eighth week of treatment were collected from each patient by the laboratory technicians at the diagnostic center. After collection, the specimens were transported in a cold box (at +4°C) from the diagnostic centers to the core laboratory at AHRI for further processing.

6.3. Bacterial Isolation

The sputum samples were decontaminated and homogenized by the modified Petroff's method (Narvaiz *et al.*, 1998). An equal volume of 4% NaOH was mixed with the sputum in a screw-capped tube, digested for 15 minutes with occasional shaking, and then centrifuged at 3000g for 15 minutes. After decanting the supernatant, few drops of Phosphate buffer saline (PBS) or distilled water were added to reconstitute the pellet. Then phenol red was added as an indicator before neutralization. After neutralization with HCl, 3ml PBS was added to the neutralized solution and then cultured on two LJ media for primary isolation. After inoculation, LJ slants were held for 8 weeks at 37°C and were examined visually for growth every day for the first week and then twice per week thereafter for the total of 8 weeks. Primary isolates grown within 2-3 weeks were used for susceptibility testing on 7H10 media before they were stored at -20°C.

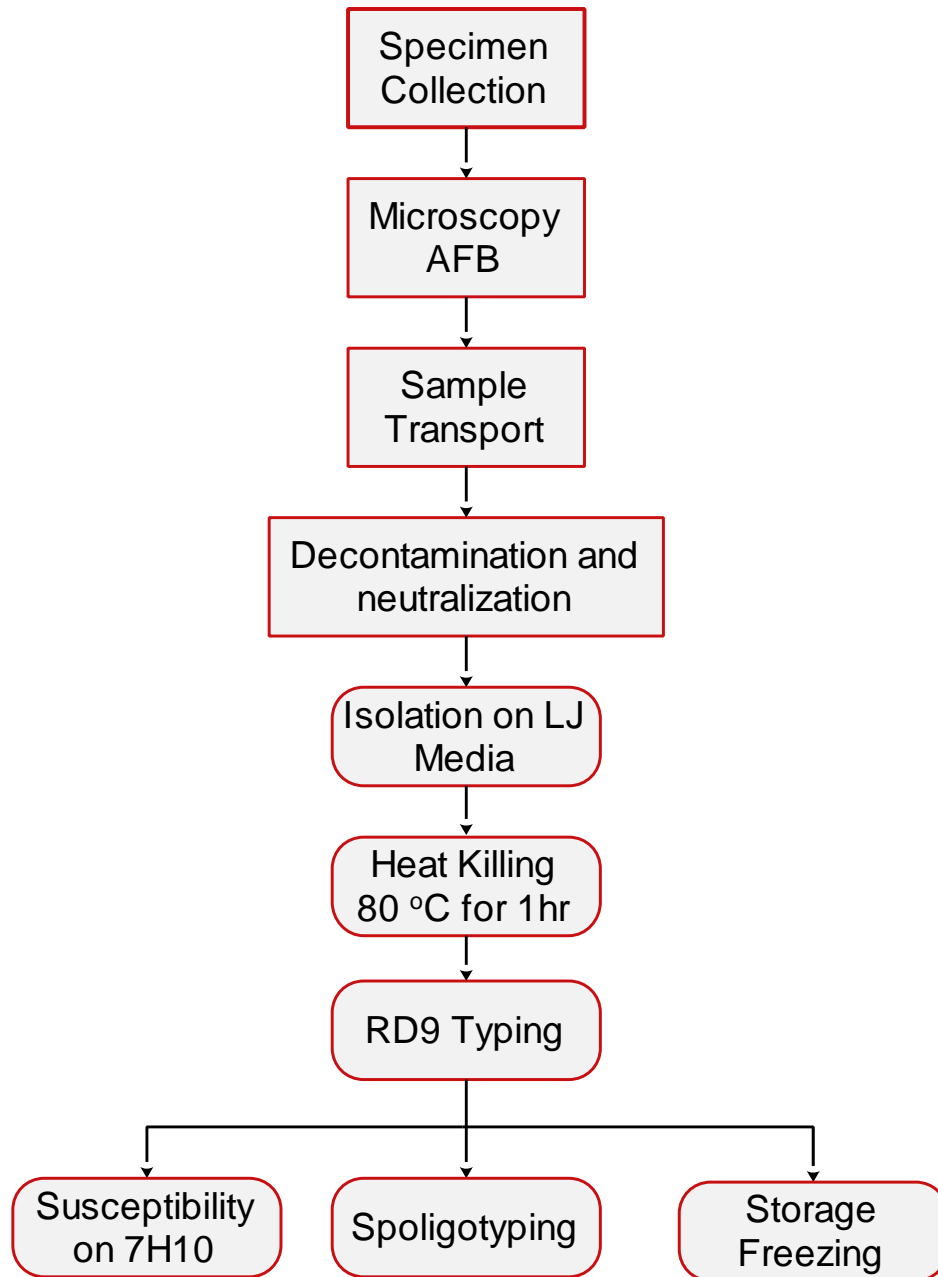


Figure 3. Flow chart showing some of the laboratory procedures

6.4. Drug Susceptibility Testing

Indirect drug susceptibility testing was performed to determine the resistance pattern of isolates to anti-TB drugs. The test was done based on a proportion method using Middlebrook 7H10 media on 24-well tissue culture plates (Becton Dickinson and Company, USA) (van Klingeren *et al.*, 2007).

6.4.1. Preparation of the Antibiotic Solutions

Stock solutions of the test drugs (Sigma, St. Louis, USA) were prepared in their respective solvents (Table 2). After filter sterilization using 0.2 µm filter, aliquots of the drug solutions were kept in cryotubes (Nunc, Denmark) and stored at -20°C. When required, these stock solutions were thawed and added to the 7H10 media to get the correct concentration of each antibiotic.

Table 2. Anti-tuberculosis drugs and their respective solvents and stock concentrations

Anti-TB drugs	Abbreviations	Product number (Sigma)	Solvent(s)	Stock (µg/ml)
First line drugs				
Isoniazid	INH	I-3377	dH ₂ O	1000
Rifampicin	RIF	R-3501	DMSO	1000
Etambutol	EMB	E-4630	dH ₂ O	1000
Streptomycin	STM	S-6501	dH ₂ O	1000
Second line drugs				
Amikacin	AMK	K-1876	dH ₂ O	1000
Kanamycin	KAM	A-1774	dH ₂ O	1000
Capreomycin	CAP	C-4142	dH ₂ O	1000
Ofloxacin	OFL	O-8757	dH ₂ O	1000
Moxifloxacin	MOX	M-8039	NaOH	1000
<i>P</i> -amino salsaylic acid	PAS	A-3504	dH ₂ O	1000
Ethionamide	ETH	E-6005	dH ₂ O	1000

6.4.2. Preparation of Middlebrook 7H10 Media

For all the test drugs Middlebrook 7H10 medium supplemented with glycerol and 10% oleic acid albumin dextrose catalase (OADC; Becton Dickinson and Company, Sparks, MD) was used. According to the supplier, 17.25g of 7H10 agar was dissolved in 810 ml distilled water containing 4.5 ml of glycerol to prepare 900 ml Middlebrook 7H10 medium used for one batch test. The agar-glycerol mixture was put in a water bath to dissolve the mixture and the

solution was then sterilized in the autoclave for 10 minutes at 121°C. After cooling to 50°C, OADC enrichment preheated to the same temperature was added to make up to 10% of the total constituents (v/v). One milliliter of agar was then solidified to check the final pH of the medium, with the target of pH 6.6 ± 0.2.

6.4.3. Preparation of Drug Containing Agar Series in 24-Well Plates

In every batch of the experiment, 10 DST plates were prepared in the preceding day of inoculation. Dilutions of the antibiotics were prepared to get the required antibiotic concentrations (Table 3). A volume of each antibiotic was mixed with 7H10 medium in 50ml Falcon tube in a water bath to get the required drug concentration in the agar mixture. The drug containing and the drug free media were then transferred in 2.5 ml amounts into the respective wells of the 24-well tissue culture plates. The media containing plates were then left in the safety hood until the agar was completely solidified. After writing the code and the production date, the plates were then sealed with Parafilm and stored at +4°C until used the next day.

Table 3. The different serial concentrations of each anti-TB drug used for the test (Jureen *et al.*, 2010; van Klingerren *et al.*, 2007; WHO, 2008e)

First line anti-TB drugs		Second line anti-TB drugs	
Drugs	Concentrations (µg/ml)	Drugs	Concentrations (µg/ml)
INH	0.064, 0.125, 0.2 and 1.0	CAP	10.0
RIF	1.0	AMK	0.25, 0.5, 1.0 and 2.0
EMB	4.0, 5.0 and 8.0	KAN	5.0
STM	2.0	ETH	2.0 and 5.0
		PAS	2.0
		OFL	0.125, 0.25, 0.5, 1.0 and 2.0
		MOX	0.5

6.4.4. Inoculation and Incubation of the DST Plates

Bacterial suspensions were prepared from a primary culture or a sub-culture on LJ medium. A representative portion of the culture was obtained by sampling about one loopful of bacterial colonies within 2 or 3 weeks after appearance of growth. Suspensions were then prepared in a 15ml plastic tube containing 3ml sterile distilled water and glass beads 3.0 mm in diameter. The mixture was homogenized on a Vortex mixer for up to a minute. To allow sedimentation of coarse-grained particles, the suspensions were allowed to settle for 15 min. The supernatant was then collected and its turbidity was adjusted with a McFarland standard of 1.0 to obtain a density of approximately 3×10^8 CFU/ml. The opacity of the suspension was adjusted by the addition of sterile, distilled water.

Then, the DST plates were inoculated by adding 10 μ l of the suspension which was adjusted with McFarland suspension. One of the control wells containing medium without any drug was inoculated with a 1:100 diluted suspension, allowing the comparison at the 1 % level of the proportion methods of the inocula after incubation. After inoculation, the plates were securely sealed with parafilm for reasons of safety and contamination and incubated at 35°C in a 5% CO₂ incubator with a water reservoir to prevent evaporation from the plates.

6.4.5. Reading and Interpretation of DST Results

After 2 or 3 days, the plates were read to see if there is any contamination and mixtures with other bacteria. The plates were checked for mycobacterial growth at 6 (used as early check as well as the plate can sometimes be read already here), 12 and 19 days. The 1:100 control well has to show growth in order for the plate to be read according to the proportion method. The experiment was repeated for those plates with no growth on either of the control wells (1:1 or 1:100). Drug susceptibility was read by visual comparison of the drug containing media (1:1 bacterial suspensions) with the drug free control on which 1:100 bacterial suspensions was inoculated. Equal growth on the two media means that 1% of the isolates are resistant to that particular drug. However, for reason of accuracy, the test was repeated if the growth was equal or nearly equal for both. According to the recommendation from the WHO (2009b), the critical concentration determines whether a strain is sensitive or resistant to a certain drug.

The strain was reported S if there was clearly more growth in the 1:100 diluted control than in the drug containing well with the critical concentration and R if there was more growth in the drug containing well than in the 1:100 control. S and R were also used for all the other concentrations tested besides the critical concentration.

The inability to get optimum bacterial growth on the 1:100 drug free control well in resistant strains and the occurrence of contaminants were the major problems faced during our work. Absence of growth on the control well was encountered for few isolates and they were subjected to repetition. Contamination was common at the time of optimization but it was further controlled during the actual test by performing media preparation, inoculation, incubation and reading procedures in an extremely sterile condition.

6.4.6. Quality Control

To check the quality of the suspension, all inoculated bacteria were also cultured on blood agar and checked on day 2-3 for any contamination. Two drug free wells were set up for each strain tested to ensure if the media was able to grow the test strains. Parallel to each set of test batch the standard susceptible strain, H37Rv (ATCC 27249), where MIC levels have previously been shown (Angeby *et al.*, 2010; Jureen *et al.*, 2010; Schön *et al.*, 2009), were inoculated on separate plates for all drugs as internal quality control. A clinical MDR-TB strain from EHNRI which was confirmed to be resistant to INH and RIF during proficiency testing was also included as positive control.

As external quality controls, five clinical isolates which were deposited both at AHRI, Ethiopia and the Swedish Institute for Infectious Disease Control (SMI), were tested in blind fashion to check the inter laboratory reproducibility of the drug susceptibility test results. DST was performed at SMI using BACTEC MGIT-960 except for PAS where we used LJ media. Susceptibility to first line drugs was checked for all the five isolates. However, only two of the clinical isolates were considered to check susceptibility to the major second line drugs. We also compared the performance of the 24-well plate method with that of the Geno Type MTBDR*plus* test in detecting MDR isolates.

6.5. Molecular Characterization

6.5.1. Species Identification

RD9 deletion typing (Warren *et al.*, 2006) was used to distinguish *Mycobacterium tuberculosis* from other members of the MTC. The method was applied to heat-killed mycobacterial suspensions. A multiplex PCR was designed to amplify the non-deleted RD9 region. Two external primers (RD9_FlankFW: 5'-AACACGGTCACGTTGTCGTG-2' and RD9_FlankRev: 5'-CAAACCAGCAGCTGTCGTTG-3') and one internal reverse primer (RD9_InternalR: 5'-TTGCTTCCCCGGTTCGTCTG-3') were used per locus.

The PCR mixture used for RD9 typing was prepared in a total volume of 20 µl consisting of 10 µl HotStar Taq Master Mix (Qiagen, United Kingdom), 7.1 µl H₂O Qiagen, 0.3 µl of each oligonucleotide primer (100 mM) and 2 µl DNA template from heat killed bacterial cells. After initial denaturation at 96°C for 15 min, the reactions underwent 30 cycles of template denaturation at 96°C for 30s, primer annealing at 55°C for 1 min, and chain extension at 72°C for 30 s. This was followed by a final extension step at 72°C for 10 min. Amplicon size was then determined by electrophoresis on 1.5% agarose gel which was pre-stained with 10µg/ml Ethidium Bromide. The banding patterns were visualized and photographed under an UV trans-illuminator. A 100bp DNA ladder was used to estimate the band size of the clinical samples.

Reaction mixtures with *Mycobacterium tuberculosis* (H37Rv) and clinical *Mycobacterium bovis* DNA and without template DNA were also run simultaneously with samples every time as positive controls and a negative control, respectively to evaluate the multiplex PCR system and to compare the amplicon size of the unknown samples. After identification by RD9 typing, spoligotyping was performed to further distinguish the circulating strains of *Mycobacterium tuberculosis*.

6.5.2. Spoligotyping

Spoligotyping was carried out as described by Kamerbeek (1997). *Mycobacterium tuberculosis* strains were typed based on the presence of DNA polymorphism on the direct

repeat region (DR) of their chromosome. The procedure involved PCR amplification of the direct repeat region, hybridization of the amplicons with the pre-blotted spacers and detection of hybridization by autoradiography.

6.5.2.1. PCR Amplification of Spacers

Amplification of the target region was performed by PCR on heat-killed cells, as only small amount of DNA is required for the method. About one loopful bacterial colonies were taken from LJ slants and were heat killed in a screw capped eppendorf tube containing about 0.5ml dH₂O. The Rnase free water, the primers DRa (5'-GGT TTTGGGTCTGACGAC-3') and DRb (5'-CCG AGA GGGGACGGAAAC-3') and the Qiagen master mix were carefully mixed in a sterile eppendorf tube. Then 20µl of the mixture was dispensed into each sterile PCR tube. The unknown samples and the positive controls were mixed by a vortex shortly, spun for 30 seconds and 5µl supernatant from each sample was transferred to the respective PCR tubes which contained the required PCR reaction mixtures. After initial denaturation at 96°C for 15 min, the reactions underwent 30 cycles of template denaturation at 96°C for 1 min, primer annealing at 55°C for 1 min, and chain extension at 72°C for 30 s. This was followed by a final extension step at 72°C for 10 min.

6.5.2.2. Hybridization and Detection

The working concentrations of the spoligotyping buffers were prepared from the stock solutions and allowed to equilibrate at the required temperature (at 45°C or 60°C) in water bath. Using P200 micropipette, 150µl of 2X Sodium Chloride-Sodium Sulphate-EDTA (SSPE)/0.1% Sodium Dodecyl Sulphate (SDS) was dispensed into each amplification product and was put on the thermo-cycler for 10 minutes at 96°C for denaturing the PCR products. The denatured PCR samples were removed from the thermo-cycler and immediately placed on ice. In the mean time, the biodyne C-membrane was washed for 10 minutes on the shaking platform in the 60°C hybridization oven in 250ml 2XSSPE/0.1%SDS buffer which was pre-warmed in water bath of 60°C. The membrane was then sandwiched between the two plates of the mini-blotter so that the two outer lanes cover or are outside the holes on the lid. The denatured PCR products were then loaded in 150µl amount to the lanes

of the mini-blotter and left for one hour hybridization at 60°C. Lanes 1 and 45 were filled with 2XSSPE/0.1%SDS buffer from the diluting tray.

After hybridization, the samples were removed from the mini blotter by aspiration and the membrane was transferred to a thermo-resistant plastic container. After washing twice with 250ml 2X SSPE/0.1%SDS at 60°C, the membrane was incubated with streptavidin-peroxidase conjugate mixed with 12 ml 2XSSPE/0.1%SDS in a rolling bottle in the 42°C hybridization oven. After one-hour incubation, the membrane was washed twice for 10 minutes in shaking 42°C oven with 2X SSPE/0.1%SDS to remove any residual unbound PCR products and streptavidin peroxidase conjugate. This was followed by 5-minute double wash in 2X SSPE at room temperature and final 2 minute incubation in 30ml Enhanced Chemiluminescence (ECL) detection liquid.

The next step was detection of hybridization by autoradiography. Working in darkness, the membrane was exposed to an autorad film (Sigma-Aldrich, Germany) in an autorad “hyper cassette blue”. After 20 minutes exposure, the autorad was removed from the cassette and placed in the developer ensuring it is entirely submerged and tipped gently until an image of black squares was visible. Once satisfied with the autorad, PCR products were stripped from the membrane by washing two times for 30 minutes in 1% SDS at 80°C. The stripped membrane was then stored at +4°C in 20mM EDTA (PH = 8) after a 5 minute wash by the same buffer.

6.5.2.3. Database Comparison

Spoligotypes in binary format were entered in Excel spreadsheet and compared to an updated SpolDB4 spoligotyping database of the Pasteur Institute of Guadeloupe. An online version of this database is available at <http://www.pasteur-guadeloupe.fr:8081/SITVITDemo> (Brudey et al., 2006). Shared types (STs) in the SpolDB4 were used whenever a spoligotype pattern was found in the database. Spoligotype patterns of this study that did not share a pattern in the SpolDB4 were designated as “orphan”.

6.6. Statistical Analysis

Analyses were performed using SPSS (version 17.0) and Graph Pad prism 5 after importing the data from Microsoft office access 2007. Significant differences in proportions between different groups were assessed using Pearson's Chi² test with $p = 0.05$ considered significant. Spoligotype results were analyzed and interpreted based on SpoLDB4 and SPOTCLUST databases. Spoligotype patterns were compared with that found in the SpoLDB4 database and we used SPOLCLUST to assign families/subfamilies for orphan spoligotypes.

6.7. Ethical Consideration and Bio-safety

6.7.1. Ethical Consideration

This project was conducted after obtaining ethical clearance from AHRI/ALERT ethical clearance committee. Confidentiality was considered in every steps of the study. Analysis was done on data without revealing the identity of the study participants.

6.7.2. Bio-safety Issues

The transportation of TB specimens and cultures presents special risks in the event of accidents or container breakage. Hence, the exchange of strains between the diagnostic center and the laboratory was carried out according to standard regulations. Analysis of the specimen in the experimental site was performed with extreme care to avoid the possible risk of infection. Preparing samples for culture, identification and susceptibility testing were performed carefully to avoid the possible risk of aerosol formation and were carried out in a certified and well-maintained biosafety cabinet. Particular care was taken when tubes were being opened, closed or shaken and when materials were being centrifuged. All cultures, glass and plastic ware, and other potentially contaminated materials from the tuberculosis laboratory were decontaminated before disposal or reprocessing. Wastes were decontaminated as close to the point of use as possible, ideally before materials are removed from the laboratory area.

7. RESULT

7.1. Socio-demographic Characteristic

The data presented here are based on analysis of sputum samples from 80 consecutive clinical cases who remained culture positive at the end of the intensive phase chemotherapy comprising of category I regimen. Out of the 80 patients, 67.5% were males and a majority of them were HIV negative with only 2.5% being HIV positive. All the participants were residing in Addis Ababa.

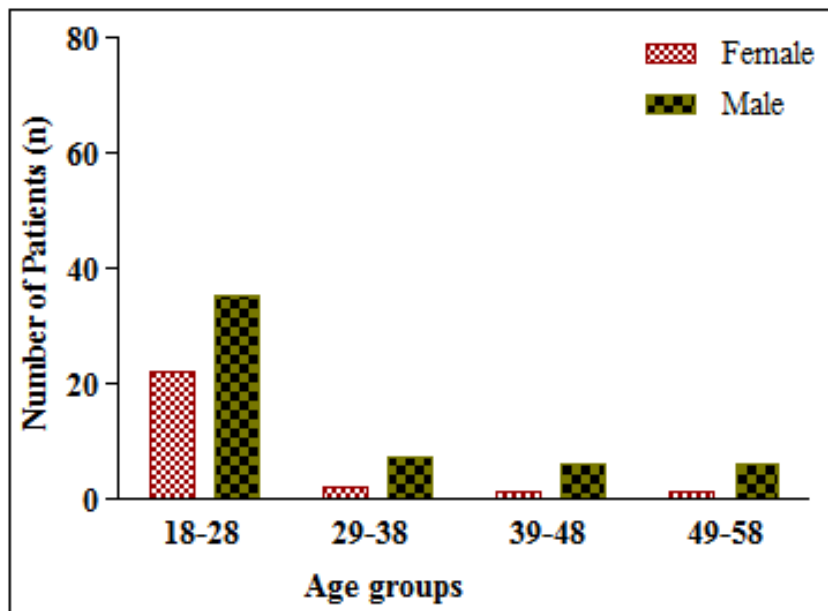


Figure 4. Age and gender distribution of the study participants

Study variables such as age, weight, height and body mass index (BMI) were measured. The age range of the patients was between 18 and 65, most of whom, 57 (71.3%), were from the 18-28 age group. The average age was 28.67 ± 9.54 . The body weights of the patients were distributed between 40 kg and 63 kg with an average weight of about 49.2 kg. The study participants had mean BMI of 17.39 ± 1.48 (range 13.6 to 21.1).

7.2. Level of Anti-TB Drug Resistance

Assessment of drug resistance among the 80 clinical samples showed that 38.8% of the TB patients were infected with *Mycobacterium tuberculosis* strains susceptible to INH, RIF, EMB and STM whereas 16 (20%) were found to be resistant to all of these drugs. Resistance to only one anti-TB drug was detected in 15 (18.8%) of the cases. The most notable mono-drug resistance was identified for STM in 12.5% of the isolates. In addition, assessment of overall resistance revealed that highest level of drug resistance (46.3%) was observed for STM followed by INH and EMB with a proportion of 43.8% and 37.5%, respectively. Overall, the prevalence of any type of drug resistance among the 80 clinical isolates was 61.2%. MDR-TB was detected in 20% of the 80 isolates. All TB Patient infected with strains mono resistant to RIF were found to be MDR-TB cases. Thus, there was 100% correlation of RIF-resistance to MDR-TB.

The relation of age and gender with level of drug resistance was also assessed. The highest rate of drug resistance was observed in patients with age between 29 and 38. As far as gender is concerned, almost equal proportion of males (61.5 %) and females (61.1%) were infected with any drug resistant TB. The pattern of overall drug resistance was shown in table 1 and resistance to first line drugs was not significantly related to age group (Chi-square = 1.662, $p = 0.646$) and gender (Chi-square = 2.054, $p = 0.152$).

Susceptibility of isolates to the major second line drugs was determined together with first line drugs on the 24 well plates. It was found that all isolates were susceptible to these drugs except to ethionamide where 13.8% of the cases were resistant. Out of the MDR-TB cases, 36.4% were detected to be ethionamide resistant. However, there was no any MDR strain which was resistant to the fluoroquinolones and aminoglycosides. Thus drug susceptibility testing on the major second line drugs revealed no XDR-TB among the study participants.

Table 4. Pattern of drug resistance to the major first line drugs among the clinical samples

Mono resistance	Male (%)	Female (%)	Total No (%)
INH	3 (3.8%)	0	3 (3.8%)
RIF	0	0	0
ETB	1 (1.3%)	1 (1.3%)	2 (2.6%)
STM	3 (3.8%)	7 (8.8%)	10 (12.5%)
INH and RIF			
INH and RIF	0	0	0
INH + RIF + EMB	0	0	0
INH + RIF + STM	0	0	0
INH + RIF + EMB + STM	13 (16.2%)	3 (3.8%)	16 (20%)
INH and Others			
INH + EMB	4 (5%)	3 (3.8%)	7 (8.8%)
INH + STM	5 (6.2%)	1 (1.3%)	6 (7.5%)
INH + EMB + STM	2 (2.5%)	1 (1.3%)	3 (3.8%)
RIF and Others			
RIF + EMB	0	0	0
RIF + STM	0	0	0
RIF + EMB + STM	0	0	0
STM and EMB	2 (2.5%)	0	2 (2.5%)
Any drug resistance			
Total INH	26 (32.5%)	9 (11.3%)	35 (43.8%)
Total RIF	13 (16.2%)	3 (3.8%)	16 (20%)
Total EMB	28 (27.5%)	8 (10%)	30 (37.5%)
Total STM	29 (36.3%)	8 (10%)	37 (46.3%)
Over all Resistance	33 (41.2%)	16 (20%)	49 (61.2%)

7.3. MIC Distributions

Most of the isolates grew at the lower concentrations of INH (92.5%; at 0.064 μ g/ml and 52.5%; at 0.125 μ g/ml). A higher proportion, i.e. 35 (43.8%) showed more growth at the critical concentration than the 1:100 control well. Twenty-six isolates (32.5%) were resistant at a concentration which is two dilution steps higher than the critical concentration. For AMK, 60 (75%) of the isolates were resistant at 0.25 μ g/ml and 5 (6.3%) of them grew at 0.5 μ g/ml. However, all isolates were susceptible to AMK at higher concentrations (1.0 μ g/ml and 2.0 μ g/ml). Regarding OFL, all the clinical samples tested were able to grow at 0.125 μ g/ml concentration. Only 10% were susceptible at 0.25 μ g/ml. The highest concentration of OFL where 28.7% isolates grew was 0.5 μ g/ml. Higher concentrations of OFL including the critical concentration inhibited the growth of any *Mycobacterium tuberculosis* isolate in the study.

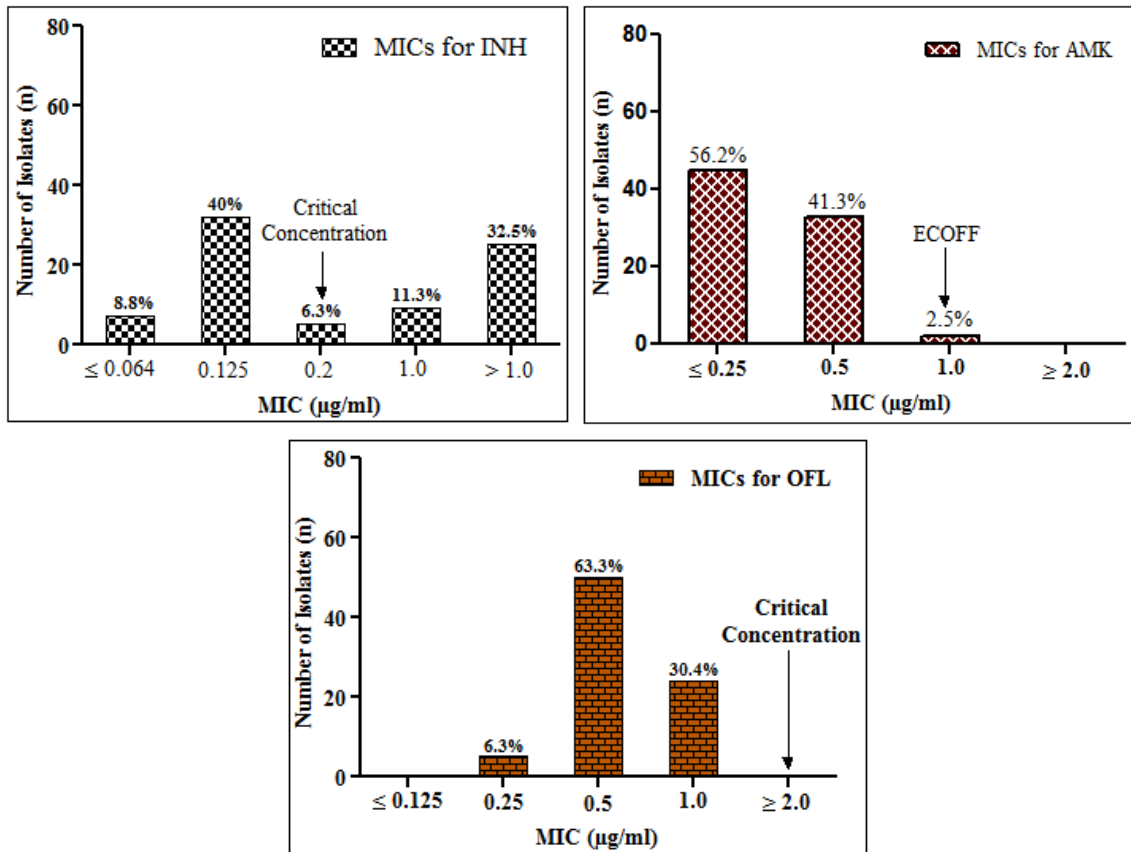
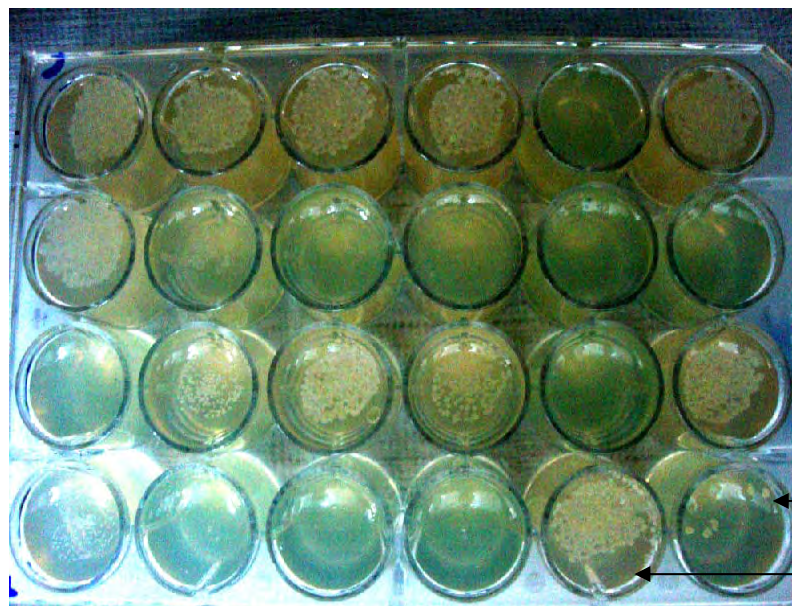


Figure 5. MIC distributions among the 79 *Mycobacterium tuberculosis* isolates

0.064 INH	0.125 INH	0.2 INH C	1.0 INH	1.0 RIF C	4.0 EMB
5.0 EMB C	8.0 EMB	10.0 CAP C	0.125 AMK	0.5 AMK	1.0 AMK C
5.0 KAN C	2.0 STM C	2.0 ETH	5.0 ETH C	2.0 PAS C	0.125 OFL
0.25 OFL	0.5 OFL	2.0 OFL C	0.5 MOX C	1:1 Cont-1	1:100 Cont-2



1:100
Control

1:1 Control

Figure 6. The upper panel shows the distribution of the drug free and the drug containing wells with anti-TB drugs in their respective concentrations ($\mu\text{g/ml}$). Wells containing the critical concentration of each test drug are labeled C. The critical concentrations for AMK and MOX were used as suggested by Angeby *et al.* (2010) and Jureen *et al.* (2010). The lower panel shows a typical DST result. This isolate is found to be resistant to INH, EMB, STM and ETH. The MIC for INH is $>1.0\mu\text{g/ml}$, for AMK $\leq 0.125\mu\text{g/ml}$ and for OFL = $0.25\mu\text{g/ml}$.

MIC was defined as the lowest concentration of an anti-microbial agent that inhibits the visible growth of about 99% of the mycobacterial culture. The MIC values of INH, AMK and OFL for most of the clinical strains of *Mycobacterium tuberculosis* isolates were evaluated to be 0.125µg/ml, ≤ 0.25µg/ml and 0.5µg/ml, respectively. Most of INH-resistant isolates (71.4%) had MIC of >1.0 µg/ml.

7.4. Treatment Outcomes

In this study treatment outcome was assessed after patients were treated with category I regimen containing INH, RIF, EMB and PZA for 2 months (intensive phase) and INH and RIF for another four months (continuation phase). Among the 500 pulmonary tuberculosis patients registered for the 4FDC clinical trial about 80 (16%) were found to be culture positive at the end of the two month intensive therapy. According to the WHO/IUATLD criteria about 51 (63.8%) of the 80 patients were cured whereas 23 (28.7%) were classified as treatment failures while two died and another two defaulted before completion of treatment and the remaining two lost follow up. Of the 16 MDR-TB cases 14 (87.5%) were treatment failure cases. A majority (61%) of treatment failures were found to be MDR-TB cases. About 72.2% of isolates resistant only to isoniazid were successfully completed their treatment while treatment outcome was poor for those resistant to rifampicin.

7.5. The 24-Well DST Method and Quality Control

The 25 well 7H10-agar DST method was first evaluated as a high throughput reproducible method by the Department of Microbiology at the National Institute of Public Health and the Environment (RIVM) in The Netherlands. The method has been used for routine susceptibility testing for more than 7000 clinical isolates in The Netherlands with a nearly 100% reproducibility (van Klingeren *et al.*, 2007). The sensitivity, specificity, efficiency, predictive value for resistance and sensitivity and reproducibility of the assay was assured as the laboratory has been taking part in the quality assurance program by the supranational laboratory network attached to the WHO/IUATLD by considering the results obtained by the majority of the participating laboratories as judicial results (“gold standard”) (Laszlo *et al.*, 2002).

The quality of the DST method was assured at two levels. The pan susceptible H37Rv (ATCC 27294) strain and one clinical MDR strain were included once in each test batch as internal controls. The MIC value of INH, AMK and OFL were evaluated for H37Rv tested in each round. Overall, there was low MIC variation between experiments for H37Rv with only one dilution step deviation at two and three experimental occasions for INH and OFL, respectively. Regarding the MDR clinical strain used as a control, there was almost full intra laboratory reproducibility of both the MIC and drug susceptibility results in each test round (Table 5).

Table 5. MIC distributions and intra laboratory variations for INH, AMK and OFL

Test Batch	MIC ($\mu\text{g/ml}$) for H37Rv			MIC ($\mu\text{g/ml}$) for MDR-644/09		
	INH	AMK	OFL	INH	AMK	OFL
01	0.125	0.5	0.5	ND	ND	ND
02	0.125	0.5	0.5	ND	ND	ND
03	0.125	0.5	0.5	1.0	1.0	1.0
04	0.125	0.5	0.5	>1.0	1.0	1.0
05	0.125	0.5	1.0	>1.0	1.0	1.0
06	0.125	0.5	1.0	>1.0	1.0	1.0
07	0.125	0.5	1.0	>1.0	1.0	1.0
08	0.125	0.5	0.5	>1.0	1.0	1.0
09	0.125	0.5	0.5	>1.0	1.0	1.0
10	0.2	0.5	0.5	>1.0	1.0	1.0
11	0.2	0.5	0.5	>1.0	1.0	1.0
12	0.125	0.5	0.5	>1.0	1.0	1.0
13	0.125	0.5	0.5	>1.0	1.0	1.0

ND, Not done

The performance of the 24-well DST assay in discriminating between resistant and susceptible strains was compared with the results obtained from SMI. There was an overall good agreement of the DST classification for most of the test drugs. We observed about 5 (14.7%) discrepancies out of the 35 DST results. The discrepancies were found only for drugs (EMB, SM, ETH) which have previously been reported as difficult in proficiency testing rounds from the WHO (2008a).

Table 6. Inter-laboratory variations of DST results for the first line drugs

Strain Code	INH		RIF		EMB		STM	
	7H10 0.2 µg/ml	BAC 0.1 µg/ml	7H10 1.0 µg/ml	BAC 0.5 µg/ml	7H10 5.0 µg/ml	BAC 0.1 µg/ml	7H10 2.0 µg/ml	BAC 1.0 µg/ml
BTB05-084	R	R	R	R	R	S	R	R
BTB05-087	R	R	S	S	R	S	R	R
BTB05-096	R	R	S	S	S	S	R	S
BTB05-109	S	S	S	S	S	S	S	S
BTB05-113	S	S	S	S	S	S	R	S

S, Susceptible; R, Resistant; BAC, BACTEC MGIT-960

Table 7. Inter-laboratory variations of DST results for the second line drugs

Strain Code	AMK		KAN		CAP		ETH		MOX		OFL		PAS	
	7H10 1.0 µg/ml	BAC 1.0 µg/ml	7H10 4.0 µg/ml	BAC 5.0 µg/ml	7H10 10.0 µg/ml	BAC 2.5 µg/ml	7H10 5.0 µg/ml	BAC 4.0 µg/ml	7H10 0.5 µg/ml	BAC 0.25 µg/ml	7H10 2.0 µg/ml	BAC 2.0 µg/ml	7H10 2.0 µg/ml	LJ 1.0 µg/ml
BTB05-084	S	S	S	S	S	S	S	S	S	S	S	S	S	S
BTB05-087	S	S	S	S	S	S	S	R	S	S	S	S	S	S
BTB05-096	S	ND	S	ND	S	ND	S	ND	S	ND	S	ND	S	ND
BTB05-109	S	ND	S	ND	S	ND	S	ND	S	ND	S	ND	S	ND
BTB05-113	S	ND	S	ND	S	ND	S	ND	S	ND	S	ND	S	ND

S, Susceptible; R, Resistant; ND, Not done; BAC, BACTEC MGIT-960

7.5.1. The 24-well plate method and the MTBDRplus test

The accuracy of the conventional 24-well plate method on 7H10 medium was found to be 100% comparable with the Geno Type MTBDRplus test in detecting RIF and INH resistance. All the 16 MDR-TB isolates that were screened by the conventional method revealed mutations on the *rpoB* and *KatG* genes by the Genotype MTBDRplus technique, confirming that the isolates were MDR cases.

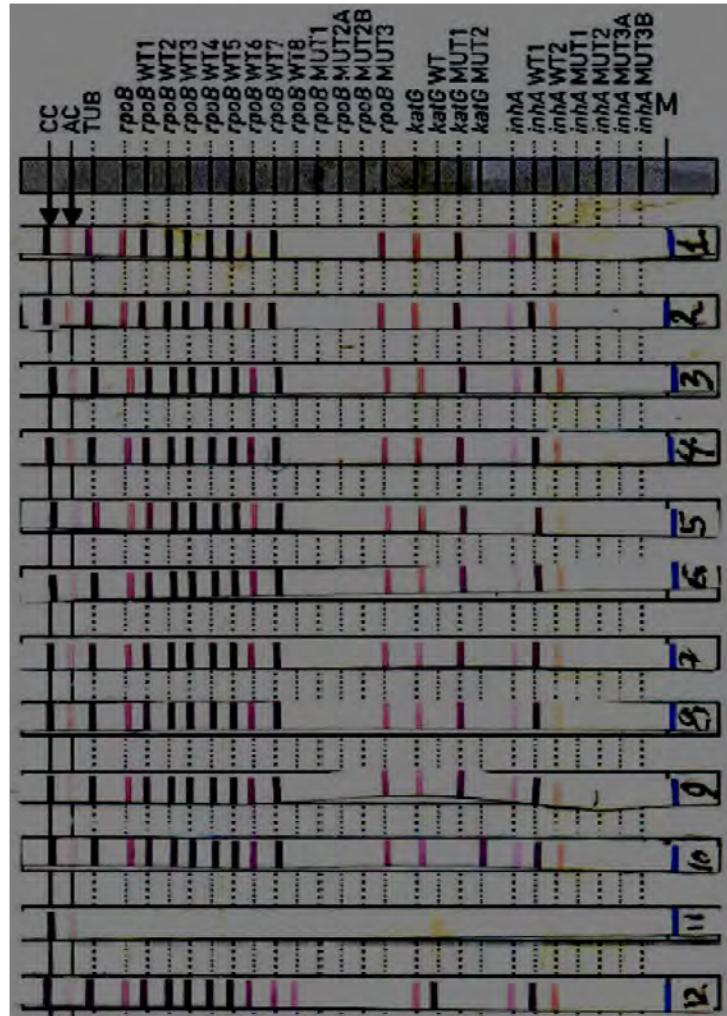


Figure 7. Representative banding patterns obtained by the GenoType MTBDRplus test for isolates with mutation on the *rpoB* and *KatG* genes conferring rifampicin and isoniazid resistance, respectively. **Lanes 1 to 10**, clinical samples; **Lane-11**, H₂O; **Lane-12**, H37Rv.

7.6. Molecular Characterization

7.6.1. Species Identification

Genomic analysis showed that except one isolate, the clinical specimens generated a PCR product of 396bp confirming the presence of the RD9 region and were accordingly identified as *Mycobacterium tuberculosis* species. The isolate with deleted RD9 region was further characterized based on two loci, one specific to the genus *Mycobacteria* and the other specific to the MTBC group. The genus specific primers produced a PCR product confirming that the isolate was from the *Mycobacterium* genus. However, no MTBC specific PCR product was detected suggesting the strain is not a member of the typical *Mycobacterium tuberculosis* complex.

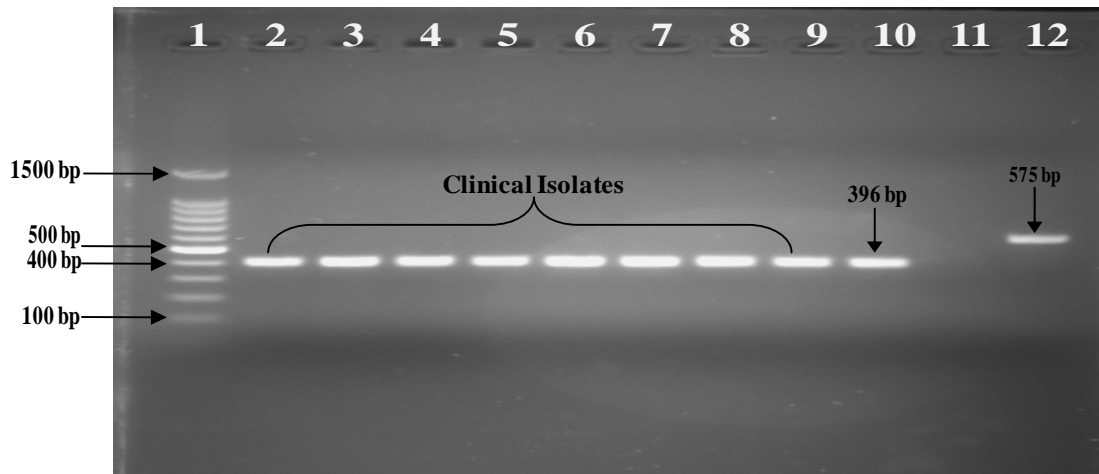


Figure 8. Typical PCR banding pattern for RD9 deletion typing: PCR products were analyzed on 1.5 % agarose gel electrophoresis following Ethidium bromide stain. **Lane 1**, 100bp Ladder, **Lanes 2 to 9**, clinical isolates, **Lane 10**, H37Rv (ATCC 27249), **Lane 11**, H₂O Qiagen and **Lane 12**, *Mycobacterium bovis*. **Lanes 10, 11 and 12** were controls.

7.6.2. Strain Typing

Spoligotyping of the 79 *Mycobacterium tuberculosis* isolates that were types as RD9 positive produced 30 different spoligotype patterns. Twenty one (70%) of these patterns were identified among single isolates whereas nine patterns represented clustered isolates with 58 (73.4%) strains.

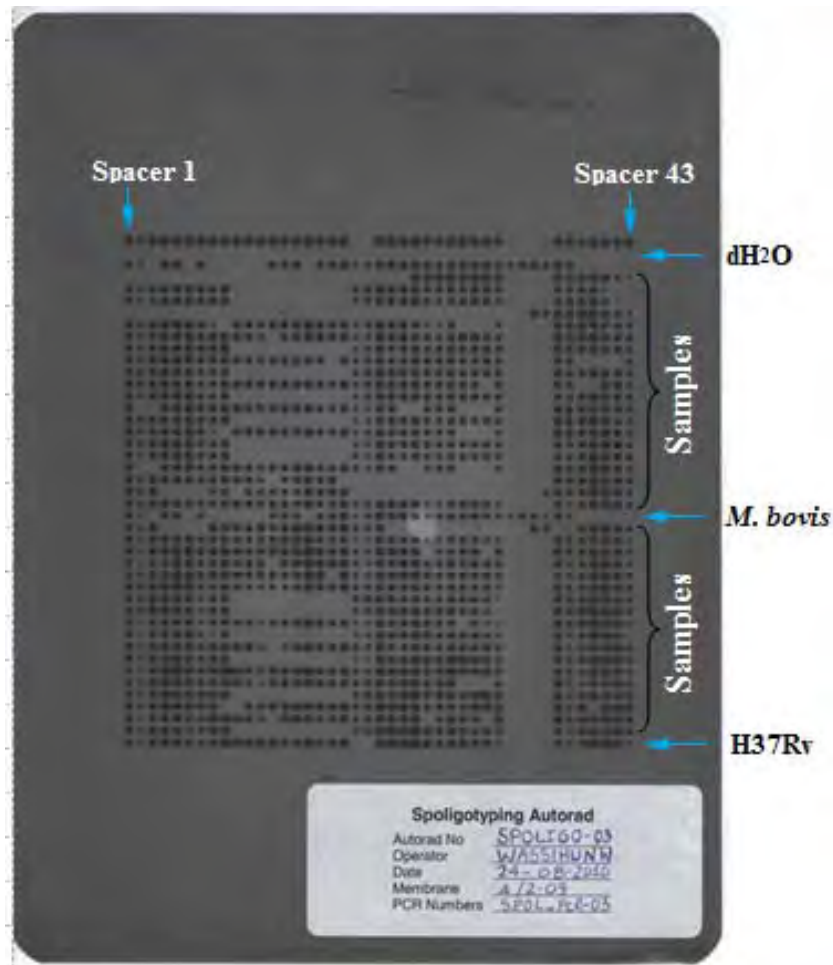


Figure 9. A spoligotyping result of 38 different clinical isolates. H37Rv and *Mycobacterium bovis* (Clinical Strain) were used as positive controls and ddH₂O as negative control. A membrane with 43 spacer oligonucleotides derived from BCG and H37Rv strains was used (vertical lines). The black dots show presence of spacers.

After comparing the binary outcomes with the updated version of the SpolDB4 database, nine patterns were recorded as orphans (clinical isolates showing unique spoligotypes) as no counterpart patterns were yet described in the database at the time of comparison. Major phylogenetic clades were assigned according to signatures provided in SpolDB4. The ST149, known as the T3_ETH sub family, was the predominant cluster detected in 22 (27.8%) of the isolates. ST21 was the second most common spoligotype which belongs to the CAS lineage particularly to the sub lineage CAS1_KILI with nine (11.3%) strains. ST53 of the T1 sub lineage, ST37 that belongs to the T3 subfamily and ST25 of the CAS1_DELHI subfamily were detected among seven (8.8%), five (6.3%) and another five (6.3%) of the total typed isolates, respectively. Three shared types (ST777, n=4, 5%; ST262, n=2, 2.5% and ST47, n=2, 2.5%) were found to belong to the Haarlem (H) family. The rest 21 (26.6%) of the spoligotype patterns occurred only once in our study. When the overall repartition of the strains was considered, the majority of them, i.e. 59 (74.7%) were of the Euro-American lineage. Sixteen (20%) of the patient isolates belonged to the Central Asian (CAS) lineage. Three single spoligotype patterns were assigned to three different lineages (ST1, Beijing lineage; ST54, MANU family; and ST343, U family). One strain with new spoligotype pattern did not fall to any of the major families described in the SpolDB4 database.

The relationship between patient characteristics and spoligotype results was also assessed. Twenty-one (80.8%) females and 38 (70.4%) males in the study were clustered. Statistical analysis revealed that there was no any association between isolate clustering and gender ($p=0.924$) as well as patient ages ($p=0.291$). Regarding drug resistance, 22 (71%) drug susceptible (DS), 37 (75.5%) drug resistant other than MDR (DR) and 14 (87.7%) MDR isolates had clustered spoligotype patterns. Statistical analysis showed that there were no significant difference between patients harboring MDR-TB isolates and those harboring other drug resistant cases. There was also no significant difference between patients infected with any type drug resistant isolates and those infected with drug susceptible isolates. However, higher proportion MDR-TB isolates were found to aggregate to three different spoligotype clusters ($n = 8/16$, ST21; $n = 3/16$, ST149 and $n = 3/16$, ST53) more frequently than other types of drug resistant or drug susceptible isolates.

8. DISCUSSION

8.1. Socio-demographic Characteristics

Tuberculosis (TB) has remained a challenge for the world and emerged as a serious public health problem particularly for developing nations due partly to HIV/AIDS. The emergence of drug resistant tuberculosis, especially multi-drug resistant tuberculosis, poses a major threat to the control and prevention of the diseases. Determining the antimicrobial susceptibility of *Mycobacterium tuberculosis* is of primary importance for both patient management and infection control. Thus, in this report we present drug resistance profile of 80 patients who failed to smear convert after two months of intensive therapy.

Among the study participants, the majority of them 54 (67.5%) were males. Most of the patients, 57 (71.3%) were from 18-28 age group. Tuberculosis affects both women and men in their economically and reproductively active years (Converse, 2004). In most low income countries more tuberculosis cases were reported among men than among women (Connolly and Nunn, 1996). In the study of Jimenez-Corona, *et al.* (2006), the incidence rate of pulmonary TB was 58% higher in men (31.79 cases per 100,000 person/year) than in women (20.13 cases per 100,000 person/year). The reason for higher TB notification rate in males is poorly understood. The difference could be attributed to biological and epidemiological characteristics as well as socioeconomical and cultural barriers in access to health care facilities (WHO, 2009a).

8.2. Drug Resistance Patterns

Our result shows that resistance to one or more anti-TB drugs was observed in 49 (61.2%) of the 80 consecutive clinical isolates. The overall resistance rate is markedly higher than some of the resistance rates found so far in Ethiopia (Asmamaw *et al.*, 2008; Demissie *et al.*, 2001; Eyob *et al.*, 2004; Lemma *et al.*, 1984; Woldemeskel *et al.*, 2005). This difference can be attributed to several features of the patients under study. The disparity could be due partly to the study setting in which our isolates were collected. All the 80 samples were taken from 500 cases in total based on culture positivity at two months after taking first line drug

treatment in fixed and loose formulations. Thus, there is a selection towards a high probability of drug resistance from such clinical cases.

The most notable drug resistance was identified for streptomycin in 46.3% of the isolates followed by isoniazid and ethambutol with a proportion of 43.8% and 37.5%, respectively. Streptomycin containing regimens have been used for the treatment of tuberculosis or other bacterial infections especially in resource limited countries since its discovery. Thus, a higher rate of STM resistance could be due to its long usage as an anti-TB drug. Isoniazid resistance was the second next to streptomycin. The fact that INH has been given in the continuation phase only with EMB, which is relatively weak, in previous treatment regimen, may possibly exacerbated the development of isoniazid resistance (Zhang, 2005).

It was also observed that all isolates resistant to RIF were also INH-resistant suggesting that majority of RIF-resistant strains are also resistant to INH. Thus, in our case there was complete correlation of RIF resistance to MDR-TB. In one study done in Vietnam, about 98% of the rifampicin resistant isolates were also found to be MDR-TB strains (Caws *et al.*, 2006). In our report, no isolate with mono-resistance to RIF was detected; however, mono resistance to INH was observed for three (3.8%) isolates. This is in concordance with the fact that mono resistance to INH is common whereas mono resistance to RIF is rare (Mitchison, 1998; Somoskovi *et al.*, 2001).

Resistance to two or more of the test drugs accounted for the highest rate of drug resistance. Resistance to INH, RIF, STM and EMB was the predominant pattern among the combined resistant cases. In general, the proportion of isolates resistant to more than one anti-TB drugs was significantly higher than those resistant to any single drug suggesting amplification of resistance. The low rate of mono-drug resistance, as compared to resistance to multiple drugs, could be due to amplification of mono-resistance through acquisition of additional resistance. The proportion of MDR-TB in this cohort was 20% among the 80 patients who failed to respond to the two-month intensive therapy. Many MDR strains were harboring

resistance to all the four major first line drugs which are the core components of the National Tuberculosis Control Program (FMoH, 2009).

Isolates were also subjected to susceptibility testing to the major second line anti-TB drugs. *In vitro* activities of the second line drugs were determined simultaneous to the first line drugs. Our finding revealed that all isolates were susceptible to the selected second line drugs except for ETH. Thus, we did not detect any XDR-TB cases within the study samples. The country is currently scaling up tuberculosis laboratory services for diagnosis of MDR-TB (FMoH, 2009). If the patients that were already screened as MDR-TB are properly diagnosed, there could be effective treatment success since all the MDR-TB isolates were pre-tested to be susceptible to the major second line anti-TB drugs.

However, there is relatively high level of ETH resistance among isolates where 13.8% of the tubercle bacilli were resistant to it. Higher rate of ETH resistance was also detected in some reports. In the study of Agonafir *et al.* (2010), ETH resistance was observed in 65.2% of 46 MDR-TB isolates. In one study, 21 out of 28 (75%) tuberculosis cases which were submitted to SLD treatment were ETH resistant (Isabel *et al.*, 2007). The drug, like isoniazid, is considered an inhibitor of mycolic acid synthesis (Mendoza *et al.*, 2004). Thus, the relatively high rate of drug resistance could be due to the occurrence of cross resistance between the two drugs. In our report about 45.5% of ETH resistant cases were also INH resistant. In a study done in South Africa, ETH co-resistance was observed in 19 of the 39 INH resistant *Mycobacterium tuberculosis* isolates (Schaaf *et al.*, 2009). The higher rate of ETH resistance could also be attributed to the property of the drug itself. Drug susceptibility testing in ETH is usually difficult to perform partly because the drug is thermo-labile during the test or at the time of storage (Mitchison, 2005). Thus, the distribution of isolates as resistant and susceptible may not be clearly separated. Over all, the findings on second line drug resistance may suggest that low level of SLD resistant isolates could be circulating in the area. Untreated or inadequately treated patients are at high risk of spreading drug resistant strains. However, there may be less chance for the emergence of SLD resistant isolates in the area

due to poor treatment adherence as treatment of MDR-TB patients has not broadly been started yet in the country.

8.3. Treatment Outcomes

The treatment outcomes of the study participants were also determined according to the WHO's classification. Out of the 80 patients, 51 (63.8%) were cured and 26.3% showed treatment failure while two patients died and another two defaulted before completion of treatment. Of the 16 MDR-TB cases which were detected among the culture positive isolates at the second month, 14 (87.5%) were found to be treatment failures, one patient died before completing treatment and only one could achieve quiescence after treatment. The result indicates that, unlike other reports (Alavi and Salami, 2009; Gandhi *et al.*, 2006), the rate of mortality among the identified MDR cases seems very low which might be due to the small number of HIV co-infected patients. The low proportion of HIV sero-positivity within the study samples was mainly because the inclusion criteria was in favor of HIV negative patients when the 500 participants were recruited for the trial.

About 72.2% of isolates resistant only to isoniazid were cured while treatment outcome was poor for those resistant to rifampicin. A retrospective study on INH-resistant TB done in the low-burden country, Denmark showed that there was successful short and long-term treatment out come in 80% and 95% of 111 mono and poly INH resistant cases, respectively (Bang *et al.*, 2010). However, as this report shows, there is an association between RIF-resistance and treatment failure. Similarly, Jain *et al.* (1992) reported that 20 (90.4%) out of 22 RIF and INH resistant isolates did not respond to treatment. Thus, a high rate of RIF resistance and hence MDR-TB in the area could impose a challenge in the control of tuberculosis. The definitive diagnosis of MDR-TB is difficult especially in resource limited low-income countries as treatment requires availability of reliable laboratory facilities and prolonged use of rather expensive second line drugs with a potential of toxicity.

One of the objectives of this research project was to detect MDR-TB cases from those patients who failed to respond to the intensive phase therapy. Out of the 21 patients who

failed category one treatment, 14 (66.7%) were early detected as MDR isolates. Similar results have also been reported by Chavez Pachas *et al.* (2004) where culture and susceptibility done at the second month captured about 74% of treatment failures and have recommended DST for patients with positive smears at two months or more. When compared to smear negative cases there is higher risk of treatment failure among patients who continue to be smear positive at the end of the intensive phase. It is experienced that an important cause of failure is MDR-TB (WHO, 2010b). This indicates the need for culture and susceptibility for those patients who fail to smear convert despite a two-month intensive therapy instead of the current recommendations of five month (Kanade *et al.*, 2010). This would enable early screening of drug resistance, particularly of MDR-TB cases, and referring them to appropriate treatment regimen. The drug susceptibility profiles of all the patients that were eventually proved to have MDR-TB were reported to St Peter's TB Specialized Hospital to be used as a guide for prompt initiation of second line drug therapy. Overall, the higher proportion of MDR-TB cases screened after two months of anti-TB treatment could strengthen the case finding strategies and procedures for MDR-TB set by the Ethiopian Federal Ministry of Health (FMOH, 2009).

8.4. MIC Distributions

This report also presented some indications on MIC distributions of some of the test drugs. The MIC value of INH for the majority of isolates was determined to be 0.125µg/ml. Most of INH susceptible isolates had MIC of 0.125µg/ml and of the 35 INH resistant isolates, 25 (71.4%) had MIC of $\geq 1.0\mu\text{g/ml}$. This result is in agreement with the findings of Nateche *et al.* (2006) where out of 136 isolates, 117 susceptible strains had MIC of 0.125µg/ml and 17 resistant isolates had MIC of $\geq 1.0\mu\text{g/ml}$. The lowest MIC value found among most of INH resistant isolates was 1.0µg/ml whereas the highest MIC reading for most of the susceptible isolates was 0.2µg/ml. For some anti-TB drugs such as INH the gap between the highest MIC for the susceptible isolates and the lowest MIC for the resistant strains is wide (Heifets and Cangelosi, 1999a). In our case, there was a two MIC step gap between the highest MIC of the susceptible strains and the lowest MIC of resistant strains. Thus, the critical concentration of INH seems quite reliable to *in-vitro* identify resistant and susceptible strains.

About 71.4% of the clinical isolates were resistant to a concentration above two dilution steps from the critical concentration for isoniazid. The presence of INH drug resistance at higher concentrations is usually linked with mutation at amino-acid position 315 of *katG* gene. High level INH resistance is also associated with additional resistance to streptomycin and rifampicin (Van Doorn *et al.*, 2006; van Soolingen *et al.*, 2000). In our report, 21 (84%) isolates resistant at higher INH concentration were also resistant to streptomycin and all rifampicin resistant cases were resistant to higher concentration of INH. Moreover, Isolates with high level of INH resistance, involving *katG* gene mutation, are usually characterized by frequent clustering (Van Doorn *et al.*, 2006). In this report, higher clustering rate (92%) was observed among high-level INH resistant strains.

MIC testing of AMK and OFL was performed as a class representative of aminoglycosides and fluoroquinolones, respectively. For most of the clinical isolates, the MICs of these drugs were determined to be $\leq 0.5\mu\text{g/ml}$ and $0.5\mu\text{g/ml}$, respectively (Figure 5). The MIC levels of OFL correlates with the work of Angeby *et al.*(2010) where MICs of OFL were distributed from 0.25 to 1.0 $\mu\text{g/ml}$. Regarding AMK our data showed that most isolates were resistant at lower concentrations of amikacin. However, no isolate was able to grow at higher concentrations (1.0 $\mu\text{g/ml}$ and 2.0 $\mu\text{g/ml}$). In our finding, the highest MIC of the wild type *Mycobacterium tuberculosis* strains for AMK, was determined to be 1.0 $\mu\text{g/ml}$ which is in agreement with the result of Jureen *et al.* (2010). Amikacin currently lacks critical concentration for 7H10-agar medium (WHO, 2008e). Thus, we used 1.0 $\mu\text{g/ml}$ as a critical concentration to determine susceptibility of isolates to AMK as suggested by Jureen *et al.* (2010). For OFL, relatively higher proportions (28.7%) of isolates were resistant at a concentration of 0.5 $\mu\text{g/ml}$ which is two-dilution step lower than the critical concentration. According to our MIC data, the gap between the MIC of the majority of the strains and the current critical concentration of OFL seemed wide although larger samples size could push the MIC upward towards the critical break point. In a recent study, based on PK/PD calculations and wild type MIC distributions, it was suggested to use the highest MIC of the wild type isolates (1.0 $\mu\text{g/ml}$) as critical break point for OFL rather than the currently recommended critical break point which is 2.0 $\mu\text{g/ml}$ (Angeby *et al.*, 2010).

8.5. The 24-Well 7H10 Agar Based DST Method

Drug susceptibility testing in tuberculosis is one of the challenges of TB control programs (Aziz *et al.*, 2007), particularly in resource-limited settings partly due to the longer time required to get results. Different levels of phenotypic and genotypic techniques have been introduced for *in vitro* drug susceptibility of *Mycobacterium tuberculosis* isolates. Alternative to the conventional methods which are rather slow, rapid molecular techniques could be the prerequisites in detecting anti-TB drug resistance. However, molecular approaches require highly expensive equipments and reagents which could not be widely applied in developing countries where the burden of the disease is quite high. Phenotypic methods on liquid media seem more efficient and rapid and are widely used methods in susceptibility testing. However, the high cost of the automated liquid media system may restrict the implementation of such techniques for low-income countries. Moreover, the inability to check the colony morphology and the sensitivity of the liquid media to contamination are the other disadvantages of liquid media based DST methods.

Conventional DST methods on egg based and agar-based media are relatively cheap and simple to perform but have the major disadvantage of being slow (Guillerm *et al.*, 2006). The conventional method on 7H10 agar is reported to be relatively fast in reporting DST results (Guillerm *et al.*, 2006; van Klingeren *et al.*, 2007). One of the objectives of this study was to develop a low-cost quality controlled DST method performed on 24-well tissue culture plates using 7H10 media. This DST method was first described by van Klingeren and his associates at the National Institute for Public Health and the Environment (RIVM), Bilthoven, The Netherlands (2007). The performance of the method was evaluated by WHO/IUATLD in 10 rounds of proficiency testing and sensitivity, specificity, efficiency, reproducibility, and predictive value for resistance and susceptibility was 100% for INH, 96 to 100% for RIF, 91 to 100% for STM and 85 to 100% for EMB (van Klingeren *et al.*, 2007).

With the above good qualities in mind, we planned to establish this well-plate DST method to our set up using quality control systems. The experiment was controlled using the pan susceptible H37Rv strain of known MIC distributions and a confirmed clinical MDR-TB

isolate. Both intra laboratory controls produced reproducible MIC levels within one MIC dilution range. As an inter-laboratory control, five clinical isolates present at both AHRI and a Swedish laboratory were tested. There was a close to 100% reproducibility for most of the test drugs despite the presence of some discrepancies in the DST results of STM, EMB and ETH. The problem with reading and interpreting DST results in STM and EMB was also encountered in the study of van Klingeren *et al.* (2007). These drugs have also been previously reported as difficult in proficiency testing rounds from the WHO (2008a).

The method is found to be highly suitable specially when processing large number of samples like in case of prevalence study and case detection where simultaneous testing of first line and second line drugs may be required (FMoH, 2009). It allows producing many plates a day and processing hundreds of isolates. The method is easily manageable during inoculation, incubation and reading as it is possible to test as many drugs as per the number of wells in a single plate. The method can be applicable in clinical laboratories with low-cost laboratory materials and reagents if not the standard laboratory equipments. It can also be performed with minimal training of laboratory technicians. The cost of the media can be further lowered if bovine serum (from sheep, calf or horse blood) is used instead of OADC supplement which otherwise is supplied from companies with relatively high cost (Heifets and Cangelosi, 1999a). The other important advantage of this 7H10 agar-based method is the relatively shorter turnaround time required to get DST results. In our report, test results were available, on average, after 12 days of incubation which is somehow lower than the reporting time in LJ media which is about 42 days (Heifets and Cangelosi, 1999a). Overall, this DST method could be an alternative for other DST methods that are either too expensive or too complicated to be employed in large number of specimens. However, it is emphasized that efforts should be exercised to further validate and standardize the method with a large number of samples against a reference method.

We also evaluated the performance of the 24-well plate method with the Geno Type MTBDR*plus* in detecting resistance to RIF and INH. The two DST methods had 100% concordance in identifying MDR isolates suggesting that the overall performance of the 24-

well plate method for detection of MDR-TB is acceptable in our setting. The Geno Type MTBDR*plus* technique could thus be used as a supplement to the low cost conventional 24-well plate method for MDR case detection owing to its specificity, short turnaround time and ease of use (Barnard *et al.*, 2008).

8.6. Strain Characterization

Identification and differentiation of the various strains of *Mycobacterium tuberculosis* could provide epidemiologically important information about endemic and ubiquitous strains. Different genotypic techniques have been used as important molecular tools for detecting strain variation in *Mycobacterium tuberculosis*. In the present study, we used spoligotyping to detect the different strains of *Mycobacterium tuberculosis* circulating in the study area. Among the 79 *Mycobacterium tuberculosis* isolates detected by RD9 deletion typing, 30 different spoligotype patterns were identified. Fifty-eight isolates were clustered into nine different spoligotypes while the remaining 21 isolates were found only once in this study. From the non-clustered cases, nine were not found in the SpolDB4 at the time of data base comparison and hence were unique patterns. When broadly partitioned, almost 75% of the isolates were of the Euro-American lineage which is characterized by the loss of spacers 33 to 36. The most prevalent shared type in the present study was ST149 detected in 22 (27.8%) of the isolates. This spoligotype which corresponds to the T3_ETH subfamily, is reported to be predominantly distributed in Ethiopia (Brudey *et al.*, 2006). The occurrence of the T3_ETH subfamily was also the highest in the study of Agonafir *et al.* (2010) detected in 43% of 45 MDR-TB cases isolated in and around Addis Ababa. It was also reported that ST149 was frequent in Ethiopian patients and in Ethiopian immigrants residing in Denmark. From the report, ST149 was detected in 36.2% of the cases isolated from Ethiopia (Hermans *et al.*, 1995). The consistent occurrence with high degree of clustering of this type of *Mycobacterium tuberculosis* in the area may suggest the epidemiological importance of this strain.

The Central Asian (CAS) lineage, characterized by the absence of spacers 4 to 7, is the second predominant spoligotype group detected in 20.2% of all the cases. ST21 which

constituted 56.2% of all the CAS strains, was the second most frequent genotype in this study. This shared type which is grouped in the CAS1_KILL sub lineage has been previously reported in Europe, the USA, eastern and southern Africa, Australia and the Middle East (Brudey *et al.*, 2006). Higher proportion (30%) of CAS1_KILLI was also reported in northern Tanzania and found to be one of the most predominant subfamilies in the area (Kibiki *et al.*, 2007). Higher proportion of this spoligotype has also been recently reported from Ethiopia in the work of Agonafir *et al.*(2010). CAS1_DELHI which is detected in 8.9% of all the cases, is the other significant spoligotype group in our report. ST25 and ST26 were found to be from the CAS1_DELHI subfamily each with five and one cases, respectively. The CAS1_DELHI genotype is commonly distributed in Asia particularly in India and in the Indian subcontinent (Bhanu *et al.*, 2002; Stavrum *et al.*, 2009). There are also reports on the distribution of CAS lineage in neighboring east African countries like Kenya with 35.6% (n=37) (Githui *et al.*, 2004), Uganda with 6.1% (n=344) (Asiimwe *et al.*, 2008) and Tanzania 37% (n=147) (Eldholm *et al.*, 2006). The Haarlem (H) family (n=8), the Latin American Mediterranean (LAM) lineage (n=1), the MANU family (n=1) and the Beijing lineage (n=1) were also the other spoligotype groups identified in this study.

As to our knowledge, this report is the first to detect Beijing strain from human sputum samples in the area. The lineage is commonly distributed in eastern Asian countries (Park *et al.*, 2000) though there have also been few reports from Europe, Africa and the United States (Glynn *et al.*, 2002). The prevalence of the Beijing strains has been documented in countries neighboring to Ethiopia where fourteen cases from Tanzania (Kibiki *et al.*, 2007), 6 cases from Kenya (Githui *et al.*, 2004) and 4 cases from Uganda (Asiimwe *et al.*, 2008) were detected. Reports revealed the association of Beijing strains and anti-TB drug resistance. Outbreaks of MDR-TB has also been associated with Beijing strains although this association is not consistent between different regions (Glynn *et al.*, 2002). Unlike other reports (Drobniewski *et al.*, 2005; Johnson *et al.*, 2006), in our case there was no association between MDR-TB and the Beijing strain except that the strain was resistant to a combination of INH, EMB and STM. The fact that the Beijing strains are associated with increased rate of transmission (Drobniewski *et al.*, 2002) and enhanced ability to acquire drug resistance and

cause disease (Hanekom *et al.*, 2007) may call up concerned bodies to consider this newly emerging strain.

The majority of MDR-TB isolates identified in the present study were found to aggregate in three-cluster groups namely in the CAS1_KILLI (ST21, n=8), T3_ETH (ST149, n=4) and in the T1 (ST53, n=2) sub families. There was higher rate of clustering in MDR-TB isolates than in non MDR ones suggesting possibility of recent (Niemann *et al.*, 2000) or ongoing transmissions (Durmaz *et al.*, 2003). Similarly, in one study, significantly higher rate of clustering was observed in MDR-TB cases than the non MDR isolates with proportion of 40.3% and 21%, respectively (Durmaz *et al.*, 2007) supporting the suggestion that drug resistant strains are more likely to cluster than drug susceptible ones (Feizabadi *et al.*, 2003). Generally, the fact that the predominant T3_ETH and CAS1 genotypes are associated with drug resistance in this study may indicate the need to target these individual genotypes in intervention programs to control the strains from continual dissemination.

9. CONCLUSIONS AND RECOMMENDATIONS

9.1. Conclusions

High level of drug resistance has been shown to be prevalent in patients that fail to smear convert despite the two month intensive therapy with highest level of drug resistance to streptomycin followed by isoniazid and ethambutol.

There was a high degree of correlation between drug resistance detected at the second month and final treatment outcomes. The majority of MDR-TB cases (87.5%) which were identified at the end of the intensive phase therapy were found to be treatment failures.

It was also found that co-resistance to isoniazid, rifampicin, streptomycin and ethambutol was predominant among all MDR-TB isolates. Thus, there was no rifampicin mono resistance suggesting the significant correlation between rifampicin resistance and MDR-TB.

No isolates were found to be resistant to any second line drug tested except to ethionamide where 13.8% isolates were resistant to it. Thus drug susceptibility testing on the major second line drugs revealed no XDR-TB among the study participants.

The 24-well 7H10 agar based method may be suitable in processing large number of specimens in relatively shorter turnaround time. The method was found to be reproducible and easy to perform with relatively low cost and with minimal training of laboratory experts.

The T3_ETH (ST149, n=23) and the CAS1_KILLI (ST21, n=9) were the predominant strains circulating in the study area as detected by spoligotyping. There was higher rate (87.5%) of clustering among MDR-TB isolates suggesting recent or ongoing transmissions.

One Beijing strain was detected as a newly emerging TB type in the region. However, unlike other reports there was no association between the Beijing strain and multi-drug resistance.

9.2. Recommendations

Culture and drug susceptibility testing may be necessary for patients that remained culture positive at the end of the intensive phase than the current recommendation of five month as this would enable earlier detection of drug resistant strains.

The significant correlation between rifampicin resistance and MDR-TB in this study could support the suggestion that rifampicin resistance could be used as a surrogate marker for rapid detection of MDR-TB.

Patients that were proved to harbor MDR-TB cases should be registered to appropriate treatment program for prompt initiation of second line drug regimen based on their drug resistance profiles.

Quality controlled cost effective culture and DST methods are required for the definitive management of drug resistant TB. Thus, it is highly emphasized that the method described in this study should be further validated against a reference method and be used for the routine surveillance of drug resistance. Further testing and confirmation might be needed for those drugs that showed discrepant results.

Extensive molecular studies are necessary for screening and controlling the circulating strains of *Mycobacterium tuberculosis*. Moreover, detailed epidemiological investigations in patients harboring clustered isolates are needed to control the strains from continual dissemination.

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ANNEX: INFORMATION SHEET

Study Title: Drug Susceptibility Testing and Molecular Characterization of *Mycobacterium tuberculosis* Isolates from Pulmonary TB Patients at the End of Two Month Intensive Therapy in Addis Ababa, Ethiopia

1. General Information

- 1.1. Patient initials /___/___/___/
- 1.2. Randomization Number /___/___/___/
- 1.3. Card Number /_____/
- 1.4. Sex /___/ 1. Female 2. Male
- 1.5. Age /___/___/ (Years)
- 1.6. Weight /___/___./___/ (kg)
- 1.7. Height /___/___/___/ (cm)
- 1.8. Address: Region _____ Woreda _____ Kebele _____
- 1.9. Date of Sputum collection ___/___/_____ G.C. (dd/mm/yyyy)
- 1.10. Multiple sputum samples taken from this patient /___/ 1. Yes 2. No
- 1.11. Week of treatment at which sputum is collected /___/ 1. Week **0** 2. Week **8**
3. Week **20** 4. Week **24** 5. Week **36** 6. Week **48**

2. Current Clinical Presentation

- 2.1. TB symptoms manifestation before treatment: /___/ 1. Yes 2. No
If yes, duration (in weeks) _____
- 2.2. X-ray: /___/ 1. Normal 2. Abnormal
If abnormal, please specify _____

2.3. HIV status: /____/ 1. Positive 2. Negative 3. Not Known

3. Treatment and Clinical Outcome

3.1. Treatment regimen _____

3.2. Time of initiation of treatment regimen ____/____/____ G.C. (dd/mm/yyyy)

3.3. Treatment outcome according to WHO /____/ 1. Cured 2. Treatment failure

3. Died 4. Transferred out or defaulter

3.4. Treatment regimen in case of treatment failure (name of substances) _____

4. Laboratory Data

4.1. Culture result /____/ 1. Positive 2. Negative

4.2. Anti-TB drug Susceptibility testing

Table 1: Drug susceptibility results

Roll No	Test drugs	Susceptible	Resistant	Not Available	MIC Value (INH, AMK & OFL)
01	Isoniazid	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	
02	Rifampicin	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	
03	Ethambutol	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	
04	Capreomycin	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	
05	Amikacin	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	
06	Kanamycin	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	
07	Streptomycin	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	
08	Ethionamide	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	
09	PAS	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	
10	Ofloxacin	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	
11	Moxifloxacin	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	

4.3. Molecular Characterization

4.3.1. RD 9 region /____/ 1. Present 2. Deleted

4.3.2. Spoligotyping Result: SIT Number _____ Lineage _____

DECLARATION

I hereby declare that this thesis is my own original work and that it has not been presented for a degree at this or any other university and all source materials used for the thesis have been duly acknowledged.

Declared By:

.....

Wassihun Wedajo Aragaw