MOLECULAR DETECTION OF MULTIDRUG RESISTANCE TUBERCULOSIS (MDR-TB) AND ASSOCIATED RISK-FACTORS AMONG SMEAR-POSITIVE PLUMONARY TUBERCULOSIS PATIENTS IN JIGJIGA TOWN, SOMALI REGION OF ETHIOPIA

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Molecular Detection of Multidrug Resistance Tuberculosis (MDR-TB) and Associated Risk-Factors among Smear-Positive Pulmonary Tuberculosis Patients in Jigjiga Town, Somali Region of Ethiopia

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As Thesis research advisors, we hereby testify that we have read this Thesis prepared under our guidance by Mussie Brhane entitled; "Molecular Detection of Multidrug Resistance Tuberculosis (MDR-TB) and Associated Risk factors among Smear-positive Pulmonary Tuberculosis Patients in Jigjiga Town, Somali Region of Ethiopia". Hence, we recommend that the Thesis be submitted as it fulfills the requirements.

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DEDICATION

This thesis is dedicated to my Mother, W/o Hiwot Mebrahtu for being my guardian angel and to all my friends for their patience and friendship.

STATEMENT OF THE AUTHOR

By my signature below, I declare and affirm that this Thesis is my own work, I have followed all ethical and technical principles of scholarship in the preparation, data collection, data analysis and compilation of this Thesis. Any scholarly matter that is included in the Thesis has been given recognition through citation.

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BIOGRAPHICAL SKETCH

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ABBREVIATIONS AND ACRONYMS

AFB	Acid Fast Bacilli
AOR	Adjusted Odds Ratio
BSL	Bio-safety Level
CON	Conjugate solution
COR	Crude Odds Ratio
DEN	Denaturing solution
DOTS	Directly Observed Treatment Short course
DR-TB	Drug Resistant Tuberculosis
DST	Drug Susceptibility Testing
EMB	Ethambutol
EPTB	Extra Pulmonary Tuberculosis
ETH	Ethionamide
FLDs	First Line Drugs
FMoH	Federal Ministry of Health
HYB	Hybridization buffer
INH	Isonicotinic acid hydrazide (Isoniazid)
LPA	Line probe assay
MDR-TB	Multidrug resistance tuberculosis
MTB	Mycobacterium tuberculosis
MTBC	Mycobacterium tuberculosis complex
MUT	Mutant
NTM	Non-Tuberculosis Mycobacterium
NALC-NaOH	N-Acetyl L-Cysteine _Sodium Hydroxide
NTP	National TB control Program
PCR	Polymerase Chain Reaction
PNM	Primer Nucleotide Mixture
PTB	Pulmonary Tuberculosis

PZA	Pyrazinamide
RIF	Rifampicin
RIN	Rinse solution
RRDR	Rifampicin Resistance Determining Region
RT-PCR	Real Time PCR
SLD	Second Line Drugs
STM	Streptomycin
STR	Stringent
SUB	Substrate solution
TB	Tuberculosis
WT	Wild Type
XDR-TB	Extensive drug resistant tuberculosis
ZN	Ziehl Neelsen

TABLE OF CONTENTS

STATEMENT OF THE AUTHOR **v**
BIOGRAPHICAL SKETCH **vi**
ACKNOWLEDGEMENTS **vii**
ABBREVIATIONS AND ACRONYMS **viii**
LIST OF TABLES **xii**
LIST OF FIGURES **xiii**
ABSTRACT **xiv**
1. INTRODUCTION **2**
2. LITERATURE REVIEW **5**
2.1. The Basics of Tuberculosis and Its Pathogenesis 5
2.2. Tuberculosis Chemotherapy 7
2.2.1. First Line Anti-TB Drugs (FLDs) 7
2.2.2. Second Line Anti-TB Drugs (SLDs) 8
2.2.3. Standard Tuberculosis Treatment Regimens 8
2.3. Drug Resistance in Tuberculosis 9
2.3.1. Multidrug Resistance in Tuberculosis 10
2.3.2. Mechanism of Drug Resistance 12
2.3.2.1. Isoniazid 12
2.3.2.2. Rifampicin (RIF) 14
2.4. Molecular Detection of Drug Resistant in TB 16
2.4.1. Line probe assays (LPAs) 17
2.4.2. GeneXpert (Xpert MTB/RIF) assay 19
3. MATERIALS AND METHODS **21**
3.1. Study Area 21
3.2. Study Design 21
3.3. Population 21
3.3.1. Target Population 21
3.3.2. Study Population 22
3.4. Eligibility Criteria 22
3.4.1. Inclusion Criteria 22
3.4.2. Exclusion Criteria 22
3.5. Sample Size Determination and Sampling Techniques 22
3.5.1. Sample Size Determination 22
3.5.2 Sampling Technique 23
3.6. Data Collection 23

3.6.1. Data Collection Instruments 23
3.6.2. Study Variables 24
3.6.3. Data Collection Procedures 24
3.6.3.1. Sample collection and processing 24
3.6.3.2.GenoType MTBDRplus assay 25
3.7. HIV Testing 31
3.8. Quality Control 31
3.9. Data Quality Management 31
3.10. Data Processing and Analysis 32
3.11. Ethical Clearance 32
3.12. Operational Definitions 32
4. RESULTS 34
4.1. Characteristics of the Study Participants 34
4.2. Genotype MTBDRplus Assay Result of the Study Participants 36
4.3. Association Between Risk-factors and Drug Resistance Patterns 37
4.4.Frequency of Gene Mutations Associated with Drug Resistant Specimens 39
5. DISCUSSION 41
6. SUMMARY, CONCLUSIONS AND RECOMMENDATIONS 45
6.1. Summary 45
6.2. Conclusions 46
6.2. Recommendations 47
7. REFERENCES 48
8. APPENDIX 55
Annex 1.Ethiopia Somali Regional State Map 55
Annex 2.Questionnaire 56
Annex 3. National Standard Operating Procedures for Biological Sample Transportation 59
Annex 4. Tuberculosis Laboratory Requesting and Reporting Form 61
Annex 4. Acid Fast Bacilli Smear Preparation and Staining Procedure 63

LIST OF TABLES

Table		Page
1.	The common mutations of *katG* and *inhA* probes…….	13
2.	The distribution of the study participants (n= 98) by socio-demographic characteristics, life style and HIV status, Karamara Hospital, Jigjiga town, September 2014 - May2015…….	34
3.	GenoType MTBDRplus assay result among new and previously treated cases (n=98) in karamara hospital, Jigjiga town…….	35
4.	Bivariate and Multivariate analysis for selected characteristics of study participants and their association with any resistance to RIF and/or INH and based on GenoType MTBDRplus…….	37
5.	Frequency of gene mutations associated with resistance to INH and RIF……	39

LIST OF FIGURES

	Figure	page
1.	Rifampicin resistance region of the *ropB* gene…….....	15
2.	DNA extraction with GenoLyse ® method……...	26
3.	Preparation of master mix using the MTBDRplus® Version 2 kit……...	26
4.	Work-flow diagram illustrating the different steps of the Hybridization and Detection procedure using the Twincubator……..	28
5.	Example of banding patterns and their evaluation with respect to RIF and/or INH resistance……...	29
6.	Examples of banding patterns and their evaluation with respect to RIF and /or INH resistance……...	39

MOLECULAR DETECTION OF MULTIDRUG RESISTANCE TUBERCULOSIS (MDR-TB) AND ASSOCIATED RISK-FACTORS AMONG SMEAR-POSITIVE PLUMONARY TUBERCULOSIS PATIENTS IN JIGJIGA TOWN, SOMALI REGION OF ETHIOPIA

ABSTRACT

Drug resistant tuberculosis has emerged as a serious threat to global health. Molecular methods that target drug resistance mutations are suitable approaches for rapid drug susceptibility testing to detect drug resistance. The primary aim of this study was to investigate gene mutations associated with rifampicin and/or isoniazid resistance of Mycobacterium tuberculosis and to determine the associated risk factors related to drug resistant tuberculosis among smear-positive pulmonary tuberculosis patients attending Karamara General Hospital, located in Jigjiga Town. Institutional based cross sectional study design was employed. Sputum specimens were collected from 105 consecutive smear-positive pulmonary tuberculosis patients and decontaminated by1% N-acetyl L-cysteine NaOH method. Of the total specimens, 98 (93.3 %) gave interpretable results; in which 67 (68.4 %) were new cases and 31 (31.6 %) were previously treated cases. Among the patients, 58(59.2 %) were males. The mean and median age was 35 and 33 years, respectively. Of the 98 valid specimens, 80 (81.6%) were sensitive to both drugs and 18 (18.4%) were resistant to rifampicin and/or isoniazid. The prevalence of multidrug resistance tuberculosis in all, new and previously treated cases were 10/98 (10.2%), 3/67 (4.5%) and 7/31 (22.6%), respectively. While there was no mono-resistance to rifampicin, the prevalence of mono-resistance to isoniazid was 8 (8.2%).In the present study, previous treatment history of tuberculosis, close contact with tuberculosis patients and smoking cigarette were significantly associated with the development of drug resistance (P< 0.05).Mutations conferring resistance to rifampicin and isoniazid were detected in 18(18.2 %) and 10 (10.2 %) of the specimens, respectively. Among the total 10 rifampicin resistant specimens, the distribution of mutant genes in various regions showed that 8 (80%)had resulted because of absence of rpoB WT8 presence of rpoB MUT3 and the amino acids changed were Ser531Lue. Whereas, of the remaining two, 1 (10%) was due to the presence rpoB MUT2B and 1 (10%) in rpoB MUT2A. Of the total 18 isoniazid resistant specimens, 15 (83.3%) had mutations in the katG (codon 315) gene, indicating high level resistance, while 3 (14.7 %) of had mutations in the inhA promoter gene, indicating low level resistance. In the present study, relatively high prevalence of multidrug resistance-TB was observed in the study. This situation might threaten efforts of Tuberculosis control activities and further aggravate development of multidrug resistance tuberculosis. So the study underscores the importance of establishing advanced diagnostic facilities for early detection of multidrug resistance tuberculosis.

Keywords: GenoType MTBDRplus assay, N-acetyl L-cystein NaOH method, isoniazid, rifampicin, pulmonary tuberculosis

1. INTRODUCTION

Tuberculosis (TB) is a chronic infectious disease caused by *Mycobacterium tuberculosis complex* (*MTBC*) with majority of the cases caused by *Mycobacterium tuberculosis (MTB)* (Varaine *et al.*, 2010). With an estimated 8.6 million new cases of TB (13% co-infected with HIV) and 1.3 million deaths in 2012, Tuberculosis remains a major cause of morbidity and mortality worldwide (WHO, 2013). Ethiopia is one of the 22 high-burden TB countries (FMOH**,** 2013). The national population based TB prevalence survey conducted in 2010/11 revealed that the prevalence of smear-positive individuals among adults and all age groups were found to be 108 and 63 per 100,000 populations, respectively and the prevalence of bacteriologically confirmed TB was found to be 156 per 100,000 (FMOH**,** 2013).

Efforts to control TB are hampered by the expansion of HIV infection and its association with active diseases and increasing resistance of *MTB* strains to the most effective anti-TB drugs (Harries and Dye, 2006). Drug resistant TB (DR-TB), including multidrug resistant TB [MDR-TB, that are resistant to at least isoniazid (INH) and rifampicin (RIF), the two most important first line anti-TB drugs (FLDs)], has emerged as a serious threat to global health (Gandhi *et al*., 2010). In 2012, 3.6% of newly diagnosed and 20% of previously treated cases was estimated to have MDR-TB globally with noticeable geographical variations in prevalence (WHO, 2013). Based on the WHO (2012) report, in Ethiopia, there were an estimated 1700 and 550 MDR-TB, among the new and previously treated Pulmonary Tuberculosis (PTB) cases, respectively, in 2011 (FMOH**,** 2013). However, the Drug Susceptibility Testing (DST) coverage among new and previously treated cases in the country in 2012 was 1% and 4%, respectively (WHO, 2013). On the second round drug resistance survey performed in the country (2011-13), MDR-TB prevalence was reported as 2.8% in new and 18.6% in previously treated TB patients (FMOH, 2014)

The lack of information on drug resistance is a result of inadequate laboratory capacity to perform diagnostic testing among TB patients and is a barrier to conducting drug resistance surveys. Estimated numbers of MDR-TB cases in many African countries are thus based on mathematical modeling rather than empirical studies (WHO, 2010). Accurate and rapid

detection of TB, including DR-TB, are critical for improving patient outcomes (increased cure and decreased mortality, detection of additional drug resistance, treatment failure, and relapse) and decreasing TB transmission (Steingart *et al*., 2013). The effectiveness of a standard anti-TB treatment regimen correlates well with the *in-vitro* drug susceptibility pattern of the infecting tubercle bacilli. The results of DST help select a proper treatment regimen or modify treatment regimen for a better management of patients and for surveillance and timely control of the spread of DR-TB in the community (Dawit *et al*., 2005).

Drug resistance develops due to random genetic mutations in particular genes responsible for resistance in *MTB* strains, usually because of inappropriate treatment of patients (Hillemann *et al*., 2007). For proper treatment and control of TB, WHO is recommending countries to expand their capacity for culture based DST and consider new, molecular-based assays for diagnosing drug resistance (WHO, 2008). Conventional acid fast bacilli (AFB) culture and DST requires significant laboratory infrastructure and has a slow turnaround time which can result in delayed initiation of proper therapy, increasing risk of disease transmission and amplification of drug resistance due to initiation of inadequate treatment regimens (Espinal *et al*., 2000; Raviglione *et al*., 2013).

Although conventional methods are still the main-stay in detection of drug resistance in most laboratories, several studies have been conducted to develop more rapid and specific methods of detection of MDR markers in *MTBC*. These assays are based on the detection of specific mutations in a variety of genes reported to confer resistance to several of the anti-TB drugs (Babady and Wengenack, 2012). Molecular methods that target drug resistance mutations are a suitable approach for a rapid DST (FMOH, 2013). Rapid tests can provide results within days (even without culture, i.e. directly on specimens) and thus enable prompt and appropriate treatment, decrease morbidity and mortality, and control transmission (Heifets and Cangelosi, 1999). Molecular DST of INH and RIF or of RIF alone is recommended over conventional testing at the time of diagnosis of TB (WHO, 2011). The study conducted by Ling *et al*. (2008) suggested that molecular DST for first line drugs is accurate for RIF and INH but less reliable for streptomycin (STM), ethambutol (EMB), and pyrazinamide (PZA)

In 2008, the WHO recommended the use of molecular line probe assays (LPAs) for the diagnosis of MDR-TB (WHO, 2008). The GenoType MTBDR*plus* (Hain Lifescience, Nehren, Germany) is a commercial LPA developed for the detection of INH and RIF resistance in*MTB* isolates and smear-positive specimens. This assay is a DNA strip assay, which uses Polymerase Chain Reaction (PCR) and reverse hybridization, that detects the most common mutations found in the *rpoB*, *katG* and *inhA* genes (Hillemann *et al*., 2007). It was reported that PCR, following reverse hybridization, gave very successful results in smear-positive direct clinical specimens (Johansen *et al*., 2003). The mutations that predominate in RIF resistant *MTB* strain are located in an 81-bp "core region" of the *rpoB* gene (95% of all RIF-resistant strains) (Riccardi, 2009; Maurya *et al*., 2011). Resistance to INH conferred by mutations in catalase-peroxidase enzyme gene (50-95% of INH-resistant specimens) is targeted in codon 315 of the *katG* gene and 8-43% contain mutations in the *inhA* regulatory region (Alcaide and Coll, 2011; Zhang and Yew, 2009). Mutations in *katG* gene and *inhA* gene were related to the high-level and low-level INH resistance, respectively (Vilcheze, and Jacobs, 2007).

A recent laboratory evaluation study from South Africa estimated the accuracy of the GenoType MTBDRplus assay performed directly on AFB smear-positive sputum specimens. Compared with conventional DST the assay were 98.9% sensitivity and 99.4% specificity for detection of RIF resistance and for the detection of INH resistance were 94.2% sensitivity and 99.7% specificity. For detection of MDR were also 98.8% sensitivity and 100% specificity (Barnard *et al*., 2008). Although the assay is limited to the detection of known mutations of RIF and INH, the high concordance rate with conventional methods and the rapid time to results makes the GenoType MTBDR*plus* assay a useful test for the management of MDR-TB (Barnard *et al*., 2008).

Ethiopia is working towards controlling transmission dynamics of TB, reducing morbidity and mortality, and preventing emergence and spread of drug resistance in the general population (FMOH, 2013).The eastern part of Ethiopia is known for its high TB case load and anti-TB drugs have been in use for a long time in the area before the implementation of Direct Observed Treatment Short course (DOTS) (Mitike *et al*., 1997). The prevalence of DR-TB in

Somali region of Ethiopia in general and Jigjiga area in particular, is not well documented. Based on the observation by the researcher of the study area, is expected that high prevalence of PTB cases with high proportion of transmission and high risk of DR-TB still exists amongst the population. So far, there is no report of the prevalence of MDR-TB among new and previously treated cases in the study area. Indeed, the LPAs test may be used to improve the management of TB and MDR-TB incidence in the area as in most of the industrialized countries (WHO, 2011). Thus, the primary aim of this study was to investigate gene mutations associated with RIF and INH resistance of *MTB* and to determine the associated risk factors related to MDR-TB among smear-positive PTB patients attending Karamara General Hospital in Jigjiga town.

Objective of the Study

- The general objective of this study was to rapidly identify MDR-TB and determine risk-factors related to DR-TB, among smear-positive PTB patients.

Specific objectives:

- To identify drug resistance pattern, including MDR-TB cases among smear-positive PTB patients in Jigjiga Town.
- To analyze the frequency of gene mutations associated with INH and RIF resistant specimens by GenoType MTBDR*plus* assay.
- To determine the association between risk-factors and any RIF and/or INH drug resistance patterns in the study area.

2. LITERATURE REVIEW

2.1. The Basics of Tuberculosis and Its Pathogenesis

Tuberculosis is a chronic infectious disease which affects both animals and humans and is caused by Mycobacteria recognized as the *MTBC* (Angela *et al*., 2006). The *Mycobacterium tuberculosis complex* comprises seven members which include *MTB*, the primary causative agent of human TB, *Mycobacterium bovis, Mycobacterium africanum, Mycobacterium microti, Mycobacterium canettii, Mycobacterium pinnipedii, and Mycobacterium caprae* (Kallenius *et al*., 1999). *MTB* and the regional variants or subtypes of "*Mycobacterium africanum* and *Mycobacterium canettii"* are primarily pathogenic in humans. *Mycobacterium bovis* and *Mycobacterium microti* are the causative agents of TB in animals, and can be transmitted to humans (Palomino *et al*., 2007).

Mycobacterium TB are obligate aerobes growing most successfully in tissues with high oxygen content, such as the lungs. They are facultative intracellular pathogens usually infecting mononuclear phagocytes (e.g. macrophages), slow-growing with a generation time of 12 to 18 hours, and hydrophobic with a high lipid content in the cell wall. Because the cells are hydrophobic and tend to clump together, they are impermeable to the usual stains, e.g. Gram's stain (Central TB division, 2009). Thus, special staining techniques like Ziehl-Neelsen (ZN) staining, which uses acid decolorizing agents, are widely used to stain them (Kent & Kubica, 1985). Some of the distinctive characteristics of *MTB* such as acid fastness (resistance to acid decolorizing), resistance to injury (even for many antibiotics) and extreme hydrophobicity are strongly attributed to the waxy coat of the bacteria. These, probably, contribute to the slow growth of the bacteria by hindering nutrient intake (Barrera, 2007).

The immune response against TB plays a fundamental role in the outcome of *MTB* infection. It is clear that the immune system reacts efficiently in the vast majority of infections. This is particularly evident in the case of TB, where most people infected by the tubercle bacillus (~ 90 %) do not develop the disease throughout their lifetimes. Nevertheless, the risk of

developing the disease increases considerably when TB infection co-exists with an alteration in the immune system, such as co-infection with HIV (Palomino *et al*., 2007).

Active TB disease is divided into PTB (80-90% of cases) and extra-pulmonary TB (EPTB) (10-20% of cases), and is further subdivided according to the presence (smear-positive) or absence (smear-negative) of stained *MTB* organisms in microscopically examined clinical specimens, i.e. sputum. Pulmonary tuberculosis is the most common type of TB and is a major cause of morbidity and mortality worldwide. Signs and symptoms of PTB include cough for at least two weeks, fever, chills, night sweats, weight loss, haemoptysis (coughing up blood), and fatigue. Signs and symptoms of EPTB depend on the site of disease (Steingart *et al*., 2013). A positive sputum smear is an indicator of infectiousness; thus, identifying these patients and promptly administering treatment is the primary focus of the internationally recommended TB control strategy known as DOTS (Directly Observed Treatment Short course). Microbiological detection of TB, such as via smear microscopy, is a critical element of the DOTS strategy (WHO, 2006).

Transmission of TB occurs when untreated sputum-positive PTB patient's expels small droplets containing tubercle bacilli into the air when they cough, sneeze, sing or speak and a susceptible person inhales the bacilli. These tiny droplet nuclei (1-5 mm in diameter) float in air; the fluid evaporates and the living tubercle bacilli may remain airborne for long periods until inhaled. The risk of infection depends on the extent of an individual's exposure to droplet nuclei and on his/her susceptibility to infection (FMOH, 2013).Tuberculosis affects individuals of all ages and both sexes. There are however groups, which are more vulnerable to develop the disease. Poverty, malnutrition and over-crowded living conditions have been known for decades to increase the risk of developing the disease. Human immune deficiency virus infection has been identified as a major risk factor for developing TB. The age group mainly affected is between 15 and 54 years, and this leads to grave socio-economic consequences in a country with a high prevalence of the disease (FMOH, 2013).

Whereas several bacterial species acquire resistance through mobile genetic elements (such as plasmids and transposons), resistance to anti-TB drugs is caused by spontaneous chromosomal

mutation (Caminero, 2013). The spread of DR-TB has been accelerated by several factors, including ineffective and interrupted treatment, co-infection with HIV, and inadequate infection control .There are two pathways for infection with DR-TB. Acquired or amplified resistance typically emerges in settings where TB treatment is inadequate, patients fail to adhere to proper treatment regimens, or incorrect or non-quality-assured drugs are used for treatment. Primary resistance results from the direct transmission of drug resistant strains from one person to another (Institute of Medicine, 2009).

2.2. Tuberculosis Chemotherapy

Chemotherapy is the most potent weapon available in the fight against TB. When used properly, available anti-TB drugs are able to reach cure rates above the 85% target recommended by the WHO (WHO, 2013). 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 anti-TB drugs (SLDs) are much less active, have a much higher toxicity, and are reserved for use only in the case of patients with MDR-TB (Martin and Portaels, 2007).

2.2.1. First Line Anti-TB Drugs (FLDs)

Worldwide, four FLDs are recommended for the initial treatment of TB. These include INH, RIF, EMB and PZA (Zhang *et al*., 2005). All four drugs are given to the patient in the first two months of treatment and this period is known as the ‘initial phase’. For the next set of four months known as the ‘continuation phase’ only RIF and INH are administered (Espinal *et al*., 2000). First line drugs for the treatment of TB in Ethiopia include: RIF, EMB, INH, PZA, and STM. The fixed dose combination drugs available for adults and adolescent are the following: RIF-INH-PZA-EMB 150/75/400/275 mg, RIF-INH-PZA 150/75/400 mg, RIF-INH 150/75 mg and EMB-INH 400/150 mg (FMOH, 2013).

2.2.2. Second Line Anti-TB Drugs (SLDs)

Previously all other anti-TB agents other than FLDs were placed in the grouping of SLDs. In a more recent WHO policy anti-TB drugs have been categorized into 5 groups. These groups are listed in order of efficacy in the treatment of TB with group one being the most efficacious. The first group is the FLDs listed above. Group two named as injectable anti-TB agents, contains STM, Amikacin, Kanamycin, Capreomycin and Viomycin. Group three is the Fluoroquinolones and contains antibiotics such as Ciprofloxacin, Ofloxacin, Levofloxacin, and Moxifloxacin. The fourth group known as the oral bacteriostatic SLDs is represented by Ethionamide (ETH), Protionamide, Cycloserine, Terizidone, P-aminosalicylic acidand Thioacetazone. Group five anti-TB agents are drugs with unclear efficacy and are recommended by the WHO not to be used in the treatment of MDR-TB patients. The drugs include Clofazamine, Amoxicillin/Clavulanate, Clarithromycin and Linezolid (International Union Against Tuberculosis and Lung Disease, 2007).

2.2.3. Standard Tuberculosis Treatment Regimens

WHO has continually emphasized the DOTS strategy as a framework of TB prevention and control programs. One of the essential components of the DOTS strategy is the use of recommended treatment regimens (WHO, 2010). 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 TB patients, effective multi-drug chemotherapy reduces the number of TB patients in the community which otherwise could be the potential sources of transmission of the disease (Reichman and Earl, 2000).

Ideally, WHO recommends DST for all patients at the start of treatment, so that the most appropriate therapy for each individual can be determined. New patients with PTB should receive a regimen continuing for six months (WHO, 2010). In Ethiopia, treatment regimen for new TB cases consists of two months treatment with RIF, INH, PZA and EMB during the intensive phase, followed by four months with RIF and INH: 2RIF-INH-PZA-EMB/4RIF-INH. Treatment regimen for previously treated TB cases consists of two months treatment

with STM, RIF, INH, PZA and EMB followed by one month treatment with RIF, INH, PZA and EMB during the intensive phase, followed by five months with RIF, INH and EMB: 2STM-RIF-INH-PZA-EMB/1RIF-INH-PZA-EMB/5(RIF-INH)EMB (FMOH, 2013).

Treatment of patients with MDR-TB is much more difficult and costly than other drug resistance 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). 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, 2010).The suggested standard MDR-TB regimen in Ethiopia, intensive phase: 8 PZA–Capreomycin 6–Levofloxacin–Prothionamide (ETH)–Cycloserine and continuation phase: 12 PZA–Levofloxacin–Prothionamide (ETH)–Cycloserine. Treatment should continue for a minimum of 20 months and at least 18 months after the patient becomes culture-negative. Chronic patients with extensive pulmonary disease may require MDR-TB treatment for 24 months or longer (FMOH, 2014).

2.3. Drug Resistance in Tuberculosis

The emergence of drug resistance has posed a major challenge in the control of TB. Drug resistance in TB is the result of spontaneous mutation as well as poor programmatic and individual care performance (Gillespie, 2002). Because genetic resistance to an anti-TB drug is due to spontaneous chromosomal mutations the MDR/XDR phenotype is caused by sequential accumulation of mutations in different genes involved in individual drug resistance. This drug resistance may be attributable to direct transmission of drug resistance strains (primary resistance) or to *de novo* acquisition of resistance during individual patient treatment (Acquired resistance), i.e. due to inappropriate treatment or poor adherence to treatment (Alcaide and Coll, 2011).

Drug resistance among new TB patients who have never been treated with anti-TB drugs before is due to transmission. Drug resistance among previously treated TB patients can come

from three potential sources, namely primary infection with resistant bacilli, acquisition of resistance during treatment and reinfection with resistant bacilli (FMOH, 2013). To date, there is no single chromosomal mutation that has been found to cause resistance to two or more anti-TB drugs (except for cross-resistance between some drugs). Resistance to two or more drugs is caused by sequential mutations in different genes. In appropriate regimens, use of lower-than-recommended dosage, poor drug quality and poor adherence to treatment are commonly associated with emergence of drug resistance in TB patients (Caminero, 2013).

There are different case definitions among the different drug resistance patterns. Primary drug resistance is a drug resistant strain from a patient without a history of previous treatment and acquired drug resistance is from a patient with a history of previous treatment. Any resistance refers to resistance to at least one anti-TB drug, while mono-resistance is a resistance to only one anti-TB drug. Poly-resistance is resistance to more than one first line anti-TB drugs, but not to both INH and RIF. MDR-TB is defined as resistance to both INH and RIF with or without resistance to other FLDs (FMOH, 2013). Whereas, XDR-TB defined as an MDR-TB that is resistant to any one of the three second line injectable drugs (amikacin, kanamycin or capreomycin) as well as any fluoroquinolone (FMOH, 2013).

2.3.1. Multidrug Resistance in Tuberculosis

Among the different patterns of drug resistance, MDR has been clearly identified as a severe form of TB that requires prolonged duration of treatment and high cost. Multidrug resistance 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, 2009). Multiple factors such as, under developed laboratory capacity, limited access to SLDs and poor treatment have resulted in the emergence of a newer form of drug resistance known as an extensively drug resistant-TB (XDR-TB) (Pillay and Sturm, 2007).

According to the WHO Global TB Report 2013, among patients with PTB notified, there were an estimated 300 000 MDR-TB cases in 2012. The highest numbers of MDR-TB were found

in India, China and the Russian federation. Globally, an estimated 3.6% of new cases and 20.2% of previously treated cases have MDR-TB. The highest level are in eastern Europe and central Asia where several countries, more than 20% of new cases and more than 50% of previously treated cases have MDR-TB (WHO, 2013). The burden of MDR-TB in Africa remains largely unexplored, largely as a result of the current weak laboratory infrastructure. 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, 2010).

In Ethiopia, previous studies on anti-TB drug resistance indicated the existence of MDR-TB. The countrywide anti-TB DR survey conducted in 2005 showed that the prevalence of MDR-TB was 1.6% and 11.8% among new and previously treated TB cases, respectively (FMOH, 2009). In study conducted among new and previously treated cases, the prevalence of MDR-TB was 6.5%. Drug resistance for one or more drugs in new cases and re-treated cases was 23.6% and 58.5% respectively (Ahmed *et al*., 2014). Moreover, Reports showed the gradual increase of DR-TB among newly diagnosed and re-treatment cases in Ethiopia. According to multiple studies conducted on different parts of the country, the rate of MDR-TB cases range from 0.5% to 2.3% among new cases and from 3.5% to 71% among previously treated cases (Dawit *et al*., 2005; Mulualem*et al*., 2010; Gemeda *et al*., 2012; Ahmed *et al*., 2014; Berhanu *et al*., 2014).

The occurrence of MDR-TB was not associated with age, sex, study town, previous history anti-TB treatment and HIV sero-status of the patient. Males are more commonly affected by TB than females in most countries and male or female TB patients could have different levels of risks for drug resistance due to differences in access to health-care services or exposure to other risk factors (Shao *et al*., 2011). Nonetheless, no association was observed in the study between the occurrence of MDR-TB and either the sex or the age of patients from which the isolates were recovered (Bruchfeld *et al*., 2002 and Shao *et al*., 2011). The association between age and the risk of MDR-TB is not established in the literatures as different studies

use different cut-off points for age groups although MDR-TB patients were more likely to be younger than 65 years (Shao *et al*., 2011).

There are controversial reports on the effect of infection with HIV on the occurrence of MDR TB, as some studies found increased risks of MDR-TB among patients co-infected with TB and HIV (Ramaswamy and Musser, 1998) while others reported no association between the two (Bruchfeld *et al*., 2002). As found in many other studies, the history of anti-TB treatment has been consistently associated with the risk of MDR-TB and a systematic review of 29 studies in Europe reported the pooled risk of MDR was up to 10 times higher in previously treated cases than in new cases (Shao *et al*., 2011). However, some studies showed no association between history of anti-TB treatment and risk of MDR-TB (Bruchfeld *et al*., 2002).

2.3.2. Mechanism of Drug Resistance

Genetic studies have confirmed that mutations in genes encoding drug targets or drug activating enzymes are responsible for resistance, and point mutations and / or deletions have been found for all first-line drugs (Zhang and Telenti, 2000). Thus, strains of MDR-TB obtained from worldwide sources are not the result of a single genetic event, but of successive events in different loci.

2.3.2.1. Isoniazid

Isoniazid (isonicotinic acid hydrazide, INH) is a potent drug and is the most commonly used in combating active TB (Shi *et al*., 2007). Isoniazid has a bacteriocidal effect only on the growing tubercle bacilli and not on dormant bacilli. Oxygen is required in the activity of INH and this is more than likely due to necessity of oxygen in *katG* mediated INH activation. Under anaerobic conditions, INH is not active against *MTB*, presumably because *katG* mediated activation cannot take place (Wei *et al*., 2003). The mode of action of INH is very complex as it has the ability to affect nearly all metabolic pathways of *MTB* (Shi *et al*., 2007). 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). Resistance to INH occurs as a result of different mutations in various genes namely *katG*, *inhA*, *kasA*, *ahpC* and *oxyR* (Aslan *et al*., 2008).

The *katG* gene normally encodes for a catalase-peroxidase, but when the gene contains mutations or deletions, the catalase-peroxidase loses normal functioning. Thus, INH cannot be activated leading to drug resistance. Deletions in the *katG* gene lead to strains with very high resistance to INH (Zhang *et al*., 1993). However, point mutations in the *katG* gene are much more common than deletions. Of the many mutations in this gene(Table 1.), the *Ser315Thr* (Serine-to- threonine substitution) mutation is by far the most common and is evident in 60%-90% of strains showing INH resistance (Vilcheze and Jacobs 2007; Barnard *et al*., 2008; Riccardi *et al*., 2009; Albert *et al*., 2010; Alcaide and Coll, 2011; Dhole *et al*., 2013). Studies showed that mutation in *KatG* gene is associated with high level of INH-resistance (Zhang *et al.*, 2005; Bostanabad *et al.*, 2007).

Isoniazid drug resistance is also commonly controlled by mutations in the promoter or structural region of *inhA* gene that encodes an *enoylacyl* carrier protein reductase specific to NADH. *katG* activated INH normally inhibits *inhA*, but a mutation in the *inhA* gene causes resistance to this inhibition (Basso *et al*., 1998). *inhA* is not only a target for INH, but also for ETH, a second line drug used in *MTB* treatment regimes. This was shown in the study where 14 out of 42 strains tested had mutations in the *inhA* gene, but no mutations in *katG* or *ethA* (the gene associated with ETH resistance). These 14 strains showed co-resistance to INH and ETH, with the main mutation present being *Ser94Ala* (Morlock *et al*., 2003). Studies have shown that 8% to 43% of INH resistances are defined as the low-level drug resistance mainly caused by the mutations in the promoter region of *inhA* gene (Zhang and Yew, 2009).

Table 1. The common mutations of *katG* and *inhA* probes (Barnard *et al.,* 2012)

Name of gene	Missing wild type probe	Analyzed nucleic-acid position	Mutation probe	Mutation
katG	*katG* WT	315	*katG* MUT1	*Ser315Thr1*
			katG MUT2	*Ser315Thr2*
inhA	*inhA* WT1	-15	*inhA* MUT1	*Cys15Thr*
		-16	*inhA* MUT2	*Ala16Gly*
	inhA WT1	-8	*inhA* MUT3A	*Thr8Cys*
			inhA MUT3B	*Thr8Ala*

According to the study conducted by Belay *et al*. (2012), among the 35 INH resistant strains, 94% of the INH resistances were attributed to *katG* mutations which confer high level resistance to INH. Of these, 100% were identified as *Ser315Thr* mutation. However, 6% of the strains had mutations in the *inhA* gene at *Cys15Thr*, which confer low level resistance to INH. Similarly, Khadka *et al*. (2013) reported that of the total 14 INH resistant cases, 92.8% of them were due to mutation in *katG* gene; the distribution of mutation showed all in MUT1 with amino acid change at*Ser315Thr*, and the remaining 7.1% of the mutations were in *inhA* gene, which were due to MUT1 mutation with an amino acid changeof*Cys15Thr*. However, some studies reported that all identified INH resistant cases were carrying a *Ser315Thr* substitution in *katG* gene (Miotto *et al*., 2009).

2.3.2.2. Rifampicin (RIF)

Rifampicin is a broad-spectrum antibiotic that interferes with RNA synthesis by binding to the *β subunit* of RNA polymerase, thereby blocking elongation of the RNA chain. Most bacteria develop resistance to RIF via a mutation in a defined region of the RNA polymerase subunit β (*rpoB*) (Caminero, 2013). Rifampicin is active against growing *MTB*, in addition to stationary phase MTB with decreased metabolism. This makes RIF a very important drug in the treatment of MTB infections (Mitchison, 1985).Rifampicin in combination with INH forms the backbone of short-course chemotherapy. It is interesting to note that mono resistance to INH is common but mono resistance to RIF is quite rare (Zhang *et al.,* 2005).

Various experimental studies have shown that more than 95% of RIF resistant strains have one or more mutations in the 81bp region of the *rpoB* gene (Gillespie, 2002; Aslan *et al*., 2008). Most cases of RIF resistance are caused by mutation in the " rifampicin resistance determining region" (RRDR) which is an 81-bp region of the *rpoB* gene that encodes amino acids 507 to 533 using E-coli nomenclature (Kocagoz *et al*., 2005, Telenti *et al*., 1993). Mutation in the RRDR reduces the affinity of RNA polymerase to the binding of RIF (Johansen *et al*., 2003). In previous studies (Riccardi *et al*., 2009; Telenti *et al*., 1993), about 95% of resistances to RIF are associated with the *rpoB* gene mutations which are found to cluster mainly in the region of codon 507-533 (Figure 1.).

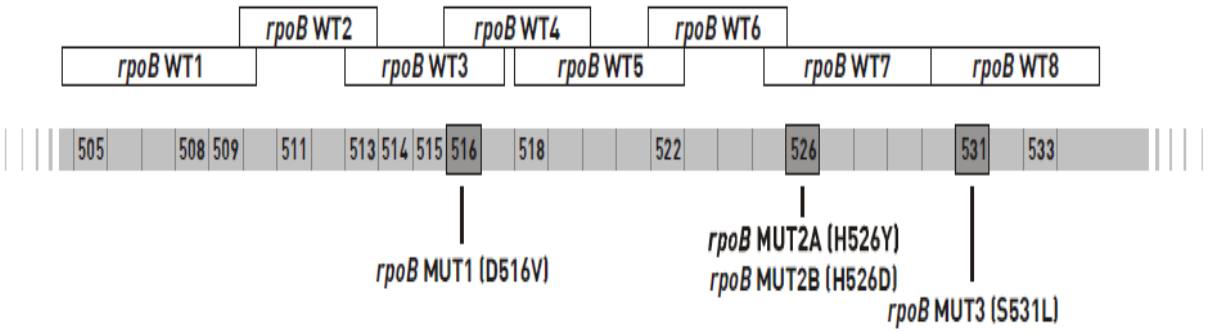

Figure 1. Rifampicin resistance region of the *rpoB* gene(Hain Lifescience, 2012)
rpoB WT1-8: *rpoB* wild type probes; *rpoB* MUT1-3: *rpoB* mutation probes. The numbers specify the positions of the aminoacids (codons) for all mutations. The codons for which mutation probes were designed are highlighted.

Point mutations at the codons 516, 526 and 531 are associated with high levels of RIF resistance (Bobadilla-del-Valle *et al*., 2001). Mutations at the codons 526 and 531 are the most commonly occurring and accounts for more than 80% of RIF resistance in *MTB* strains (Kocagoz *et al*., 2005; Yuen *et al*., 1999). The most common mutation seen at codon 531 is *Ser531Leu* (Bobadilla-del-Valle *et al*., 2001; Dhole *et al*., 2013). Now-a-days, detection of mutation that causes RIF resistance plays an important role in rapid screening of MDR-TB as RIF resistance is highly correlated with MDR (Heidi *et al.*, 2010).

In the study of analysis of gene mutations associated with drug resistance among *MTB* isolated from Ethiopia, the distribution of gene mutations among 15 RIF resistant strains were 73% at position *Ser531Leu* and 7% at *His526Asp*. In 20% of the resistant strains, mutation was detected only at the WT probes (Belay *et al*., 2012), which is different from the previously reported gene mutation distribution in China where 37% were at *Ser531Leu*, 3% at *His526Asp* and 60% at the WT probes (Zhang *et al*., 2011), reflecting the difference in the distribution of gene mutations associated with RIF resistance in different geographical locations (Belay *et al*., 2012). In a study conducted in Nepal, of the 14 RIF resistance cases, the distribution of mutation genes were 64.2% in *rpoB* MUT3 and amino acid changed were *Ser531Lue*; 28.5% in *rpoB* MUT1, *Asp516Val* and 16.6% in MUT2A, *His526Tyr* (Khadka *et al*., 2013).

In another study, frequency of *rpoB* mutations were: 81.6% *Ser531Leu*, 10.2% *His526Tyr*, 4.1% *Asp516Val*, 2.04% *His526Asp*, and 4 with no mutations but WT is missing (Abebaw, 2012). Similar findings were also reported from South Africa and Uganda (Barnard *et al*., 2008; Albert *et al*., 2010). While Miotto *et al*. (2009) reported, amongst the 31 RIF resistance cases detected, 13 (41.9%) were Asp516Val substitutions. Other mutations recognized were *His526Asp* (16.1%), *His526Tyr* (16.1%), and *Ser531Leu* (9.7%), respectively. In 16.1% of cases, resistance to RIF was identified by the lack of hybridization for WT probes in the analyzed region of *rpoB* gene.

2.4. Molecular Detection of Drug Resistant in TB

Major advances in molecular biology and the availability of new information generated after deciphering the complete genome sequence of *MTB*, stimulated the development of new tools for the rapid diagnosis of TB, differentiation of *MTB* from non-tuberculous mycobacteria, and for the rapid detection of drug resistance (Pai *et al*., 2006). The need for reliable DST increases with the emergence of anti-TB drug resistance (Martin and Portaels, 2007).

Drug susceptibility of *MTB* can be determined either by observation of growth or metabolic inhibition in a medium containing anti-TB drug (phenotypic method) or by detection of mutations in the genes conferring drug resistance (Molecular method) (Kim, 2004).

Phenotypic methods, culturing and DST, are commonly used for detecting drug resistance (Miotto *et al*., 2009). However, due to the slow and cumbersome process of culture and DST, new rapid molecular diagnostics should be prioritized in low resource countries with a high TB burden and MDR strains (Mokrousov *et al*., 2002).

A delay in diagnosing MDR-TB associated with standard DST methods is likely to contribute to the acquisition of further drug resistance, as well as to the dissemination of drug resistance strains through person-to-person transmission. By contrast, the rapid detection of drug resistance strains facilitates early access to the appropriate therapy, reduces transmission rates and improves treatment outcomes. The long turnaround time and laboriousness of DST methods has therefore stimulated the search for alternative and faster techniques. New molecular methods search for the genetic determinants of resistance rather than the resistant phenotypes. In this regard, the WHO has recommended the worldwide use of rapid genotypic assays for the rapid diagnosis of MDR-TB. Those genotypic assays should be able to detect the mutations responsible for INH and RIF resistance (Alcaide and coll, 2011).

Several molecular methods have been proposed to detect the specific mutations correlating with resistance in the amplified products: DNA sequencing, PCR-single-strand conformation polymorphism, PCR-heteroduplex formation, Real Time PCR (RT-PCR) or solid-phase hybridization assays (LPAs and GeneXpert assay) (Nahid *et al*., 2006). Two commercially available molecular assays using different methodologies have been implemented in Ethiopia: Molecular LPAs and the Xpert MTB/RIF (‘Xpert,’ Cepheid, Sunnyvale, CA).

2.4.1. Line probe assays (LPAs)

LPAs are a family of novel DNA strip tests that use both PCR and reverse hybridization methods. In these assays, a specific target sequence is amplified, and applied on nitrocellulose membranes. Specific DNA probes on the membrane hybridize with the amplified sequence applied on it. Color conjugates make the amplified target sequences appear as colored bands. These tests have been designed to identify *MTB* and simultaneously detect genetic mutations related to drug resistance both from clinical samples as well as culture isolates (Dash, 2012;

Miotto *et al*., 2009). Line probe assays are recommended by WHO for rapid screening of patients at risk of MDR-TB (WHO, 2008). Commercially available LPA kits include the INNOLiPA Rif.TB (Innogenetics, Belgium), the GenoType MTBDR, MTBDRplus, MTBDRsl assay (Hain Lifescience, Germany).

INNO-LiPA Rif.TB test is able to identify *MTBC* and simultaneously detect genetic mutations in the *rpoB* gene region related to RIF resistance. The GenoType MTBDR assay, introduced in 2004, identifies *MTBC* and simultaneously detects mutations in the *rpoB* gene as well as mutations in the *katG* gene for high-level INH resistance. The second generation MTBDRplus and MTBDRsl assays also detect mutations in the *inhA* gene (for low-level INH resistance) and mutations in the *gyrA*, *rrs* and *embB* genes (Dash, 2012). LPA shows more than 97% sensitivity and 99% specificity for the detection of RIF resistance alone or 90% sensitivity and 99% specificity in combination with INH (WHO, 2008). Currently, in Ethiopia, five regional laboratories are being rebuilt and equipped to perform culture and DST using methods including GenoType MTBDRplus assay (Belay*et al*., 2012).

GenoType MTBDRplus ('MTBDRplus,' Hain Lifescience, Nehren, Germany) assay: Is a strip test that detects MTB DNA and genetic mutations associated with drug resistance from smear-positive sputum specimens or culture isolates after DNA extraction and PCR amplification (Stop TB Partnership and WHO, 2008). This assay detects the most common mutations found in the *rpoB*, *katG* and *inhA* genes, an improvement from other LPAs such as the INNO-LiPA Rif (Innogenetics) and the GenoType MTBDR (Hain Lifescience). The GenoType MTBDR*plus* assay is highly sensitive with short turnaround times and a rapid test for the detection of the most common mutations conferring resistance in MDR-TB strains that can readily be included in a routine laboratory workflow (Dhole *et al*., 2013). Studies have shown such assays perform well when used directly on smear-positive sputum specimens (with sensitivity exceeding 97% and specificity exceeding 98%), confirming their value in rapid screening of patients suspected of MDR-TB (Stop TB Partnership and WHO, 2008).

Evaluation of the GenoType MTBDR*plus* assay by Hillemann *et al*. (2007) on clinical strains and smear-positive sputa revealed a detection rate of 98.7% and 96.8% of RIF resistance in

clinical isolates and sputa specimens respectively. Isoniazid resistance was detected in 92% and 90% of clinical isolates and sputa specimens respectively. Similar detection rates were obtained by Barnard *et al*. (2008) who tested 536 consecutive smear-positive sputum specimens with a sensitivity of 98.9% for detection of RIF resistance and 94.2% for the detection of INH resistance when compared to results obtained with conventional methods. Although the assay is limited to the detection of known mutations of RIF and INH, the high concordance rate with conventional methods and the rapid time to results makes the MTBDR*plus* assay a useful test for the management of MDR-TB. Therefore, according to different studies conducted in evaluating the assay on sputum of smear-positive specimens, sensitivities for RIF resistance ranged from 95 to 99%, and for INH from 93-99%, while specificities for the detection of resistance to either of the two drugs exceeded 99% (Belay *et al*., 2012; Dhole *et al*., 2013).

2.4.2. GeneXpert (Xpert MTB/RIF) assay

To respond to the urgent need for employing simple and rapid diagnostic tools in high-burden countries, a fully automated (Xpert MTB/RIF) molecular test for TB case detection and drug resistance testing has been developed. The Xpert MTB/RIF purifies, concentrates, amplifies (by RT- PCR) and identifies targeted nucleic acid sequences in the *MTB* genome. The Xpert *MTB*/RIF detects *MTB* and resistance to RIF using heminested RT-PCR assay by amplifying *MTB* specific sequence of the *rpoB* gene, which is probed with molecular beacons for mutations within the RRDR. It provides results from unprocessed sputum samples in 90 minutes, with minimal biohazard. Very little technical training is required to operate the machine. The test correctly detected RIF resistance with a sensitivity of 99.1% and specificity of 100% (Boehme *et al*., 2011).

Based on these excellent results, a WHO expert group recommended in September 2010 that Xpert MTB/RIF should be used as an initial diagnostic test in individuals suspected of having MDR-TB or HIV-associated TB, and that the test may be used as a follow-on test to microscopy where MDR and/or HIV is of lesser concern, especially in smear-negative specimens. In a recent study published in the Lancet on the implementation of the GeneXpert

in 1,033 consecutive culture-confirmed cases of TB in urban health centres in the Western Cape and five other TB endemic countries, GeneXpert had a specificity of 99% and an overall sensitivity of 90.3% for the detection of *MTB.* For RIF resistance detection, the sensitivity of the test was 94.4% and the specificity 98.3% (Boehme *et al*., 2011).

3. MATERIALS AND METHODS

3.1. Study Area

This study was conducted at Karamara General Hospital, located in Jigjiga Town (Annex 1). Jijiga is the capital of the Somali National Regional State which is located at 628 Kms east of Addis Ababa in Fafaan zone, one of the nine administrative zones of the region. The town is located on the main road between Harar and the Somalia city of Hargeisa, and has an elevation of 1,609 meters above sea level with semi-arid climate. There is one public hospital (Karamara) and one private hospital (Dire), two health centers, one maternal & child health (MCH) clinic, and around thirty higher and medium clinics, one university, two high schools, and 10 elementary schools. Based on population projection in 2014/15 the population of Jijiga town was estimated to be 148,000 of whom 78,928 (53.32%) and 69,072 (46.68%) were male and female, respectively. This study was carried out from September 2014 - May 2015.

3.2. Study Design

Questionnaire and laboratory based cross sectional survey design was employed in this study. This design was conducted in health institution in which using pre-tested questioner data for associated risk-factors for drug resistance and sputum specimens for laboratory detection of drug resistant *MTB* were collected from smear-positive PTB patients during the study period.

3.3. Population

3.3.1. Target Population

All TB suspected individuals and those who provided sputum sample for AFB test constituted the target population for this study.

3.3.2. Study Population

Patients included in the study were both new and previously treated cases, in whom PTB were confirmed by smear microscopy, and who were not currently receiving TB treatment.

3.4. Eligibility Criteria

3.4.1. Inclusion Criteria

AFB smear positive individuals and those who gave consent to participate were included in the study. In general, patients were eligible to be included in the study when they were smear-positive PTB patients with new and/or previously treated cases. In this study, previously treated cases defined as newly diagnosed smear-positive cases with history of previous treatment to the standard first line drugs for more than one month, according to WHO/Union definitions of smear positivity.

3.4.2. Exclusion Criteria

Seriously ill individuals, individuals who were less than 18 years of age and those who were not have willing to participate were excluded from the study. EPTB and smear-negative cases and new patients who had already started TB treatment were excluded from the study. Likewise, previously treated patients, who had already started retreatment regimens, were also excluded from the study.

3.5. Sample Size Determination and Sampling Techniques

3.5.1. Sample Size Determination

The required sample size was determined by using single population formula and considering the expected proportion of RIF resistance (6%) (Belay *et al*., 2012), desired precision of 5% at 95 % confidence interval and expected losses of 20%.

The formula which was used to calculate the required sample size was:

$$n = \frac{Z^2 * P * (1 - P)}{d^2}$$

Where, n = sample size

P = expected proportion of RIF resistance= 6%

Z = standard normal value at 95% confidence interval= 1.96

d = absolute precision= 0.05

Thus, using the formula shown above, the sample size was 87. However, by considering an expected loss of 20% (18 participants), the final sample size of the study was adjusted to105 consecutive smear-positive PTB patients from the study area.

3.5.2 Sampling Technique

The study subjects were identified by using consecutive sampling until the required sample size was reached. All individuals who were AFB smear positive and who were willing to participate in the study were recruited as study participants.

3.6. Data Collection

3.6.1. Data Collection Instruments

In this study, two important data collection tools, i.e., structured interviewer guided questionnaire (Annex 2) and molecular method DST (Genotype MTBDRplus assay), were used to gather information related to the objectives of the study.

An interviewer guided structured questionnaire was developed after reviewing different relevant literatures of similar studies (Liu *et al.,* 2013; Ahmed *et al.,* 2014; Berhanu *et al.,* 2014**).** The questionnaire had questions concerning socio-demographic characteristics, life style and medical histories of study participants. The molecular methods used for DST are based on detection of specific mutations associated with drug resistance. Genotype

MDRTBplus assay is a rapid and accurate test used to identify cases with DR-TB and can be done either directly from smear positive sputum samples or from culture isolates of sputum smear negative samples.

3.6.2. Study Variables

Two variables were considered in this survey study, i.e. dependent and independent variables. The dependent variable was drug resistant in MTB (RIF and/or INH resistance) while the independent variables included the following: Age, sex, education status, residence, previous history of anti-TB treatment, degree of smear positivity, HIV status, family history of TB Close contact with TB patient, smoking status, alcohol usage and history of imprisonment.

3.6.3. Data Collection Procedures

3.6.3.1. Sample collection and processing

Sputum specimens were collected from 105 consecutive AFB smear-positive PTB patients among the TB suspects visiting the hospital's TB diagnostic laboratory. Out of the three sputum specimens submitted by each patients meeting the eligibility criteria, the one having a higher bacillary load was enrolled in the study. About 5ml of sputum sample from each participant was collected in 50ml sterile, plastic, screw capped centrifuge tubes and transported to Harar Regional Laboratory in Harar town using a triple package transport system for further analysis (Annex 3). The sputum specimens were then handled in class II biosafety cabinet in a bio-safety level (BSL)-2 laboratory (Stop TB Partnership and WHO, 2008).In this study, all sputum specimens were referred to Harar Regional Laboratory for molecular assays included their TB Laboratory Requesting and Reporting Form (Annex 4) in order to give feedback to the responsible person.

Sputum specimens are viscous materials contaminated with normal flora. Processing involves pre-treatment of the sputum specimens. Sputum specimens were decontaminated by N-acetyl-L-cysteine Sodium Hydroxide (NALC-NaOH) method with a final concentration of 1% (Buyankhishing *et al.,* 2012).During decontamination, equal volume of NALC-NaOH solution

were added into each test tubes containing specimen and gently vortexed each tube at moderate speed for not more than 20 seconds. Each tube was inverted 5 times to ensure that the NALC-NaOH solution contacted the entire inner surface of the tube and cap. Then, the tube was left to stand at room temperature for 15 min for decontamination. Subsequently, specimens were neutralized with pH 6.8 phosphate buffer to the 45 ml mark and recapped tightly and loaded to aerosol free safety centrifuge cups to centrifuge at 3000xg for 15 min at 4 ^{0}C. After centrifugation the supernatant was poured off from each tube. A direct smear was performed from the sediment, stained by ZN staining (Annex 5), and scored according to the bacillary load as described by the national guideline (FMOH, 2013). Finally, the sediments were re-suspended in 1–2 ml sterile phosphate buffer (pH 6.8). Then, the decontaminated samples were used for detection of gene mutations associated with RIF and INH resistance using the Genotype MTBDRplus assay method (Hain Lifesciences). A 0.5 ml of the processed sample were used for DNA isolation in a screw capped tube (Barnard *et al.*, 2012; Hain Lifescience, 2012)

3.6.3.2. GenoType MTBDRplus assay

The molecular assay method used for detection of RIF and INH resistance mutations and identification was performed using GenoType MTBDRplus assay-version 2.0 (Hain Life Science GmbH, Nehren, Germany) according to the manufacturer's instructions. The assay was a rapid and accurate test to identify cases with MDR-TB (FMOH, 2013). Using multiplex PCR, the genes responsible for drug resistance such as *katG, inhA, and rpoB* were amplified and the resulting biotin-labeled amplicons were hybridized to DNA probes bound to membrane on the strip. This was followed by addition of a conjugate and substrate to detect visible band patterns on the strip, which was performed manually using a shaker water bath at 45^{0}C. Then, the strips were allowed to dry and interpreted according to the manufacturer's recommendation (Barnard *et al.,* 2012; Hain Lifescience, 2012).

For each gene, the assay was done to tests for the presence of wild type (WT) and mutant (MUT) probes. Each strip of the assay had 27 reaction zones (Figure 5), including six controls (conjugate, amplification, *MTBC, rpoB, katG* and *inhA* controls), eight *rpoB* WT (WT1–WT8)

and four MUT probes (*rpoB* MUT *Asp516Val*, *rpoB* MUT *His526Tyr*, *rpoB* MUT *His526Asp*, and *rpoB* MUT *Ser531Leu*), one *katG* WT (WT-315) and two MUT probes (*katG* MUT *Ser315Thr1* and *katG* MUT *Ser315Thr2*), and two *inhA* WT (WT-15/-16 and WT-8) and four MUT probes (*inhA* MUT1 *Cys15Thr*, *inhA* MUT2 *Ala16Gly*, *inhA* MUT3A *Thr8Cys*, *inhA* MUT3B *Thr8Ala*) (Yadav *et al*., 2013). This assay included three steps: DNA extraction, multiplex PCR amplification and reverse hybridization. These steps were carried out in three separate rooms with restricted access and unidirectional workflow (Albert *et al*., 2010; Barnard *et al.*, 2012).

DNA Extraction:-This step was carried out with Geno Lyse method (Figure 2), for each participant, 0.5 ml of decontaminated sample was centrifuged for 15 min at 10,000xg, the supernatant was discarded using a micropipette and the pellet re-suspended in 100 µl Lysis Buffer (A-LYS). Each specimen was then incubated for 5 minutes at 95^0C to heat-kill the bacteria. This was then followed by addition of 100µl Neutralization Buffer (A-NB) and centrifugation for 5 min at full speed in a centrifuge with aerosol tight rotor. Once extracted, the DNA was placed in a 4^0C refrigerator until used for amplification. Finally, 5 µl of the DNA supernatant were used for PCR while the remainder was stored (Barnard *et al.*, 2012; Hain Lifescience, 2012).

Amplification process in multiplex PCR:-The amplification reagents used in this assay were obtained as AM-A and AM-B reagent kits consisting of primers, dNTPs, $MgCl_2$ and *Taq* Polymerase. Master mix was prepared in a separate room with BSL-1, while, 45 µl master mix aliquots for amplification were prepared for each specimen by mixing 10 µl AM-A and 35 µl AM-Bin a new 1.5ml conical tube (Figure 3). Then, the master mix aliquot was transferred to each PCR tube containing 5 µl DNA, in a final volume of 50µl for amplification. The reaction was run in an applied Biosystems Gene-amp 2700 Thermal Cycler (Applied Biosystems) using the following thermal cycling conditions. Initial denaturation at 95^0 C for 15 minutes, 20 cycles of denaturation at 95^0 C for 30 sec and annealing at 58^0 C for 2 min; 30 cycles of denaturation at 95^0 C for 25 sec, annealing at 53^0 C for 40 sec and extension at 70^0 C for 40 sec; with a final extension steps 1 cycle at 70^0 C for 8 min (Barnard *et al.*, 2012; Hain Lifescience, 2012).

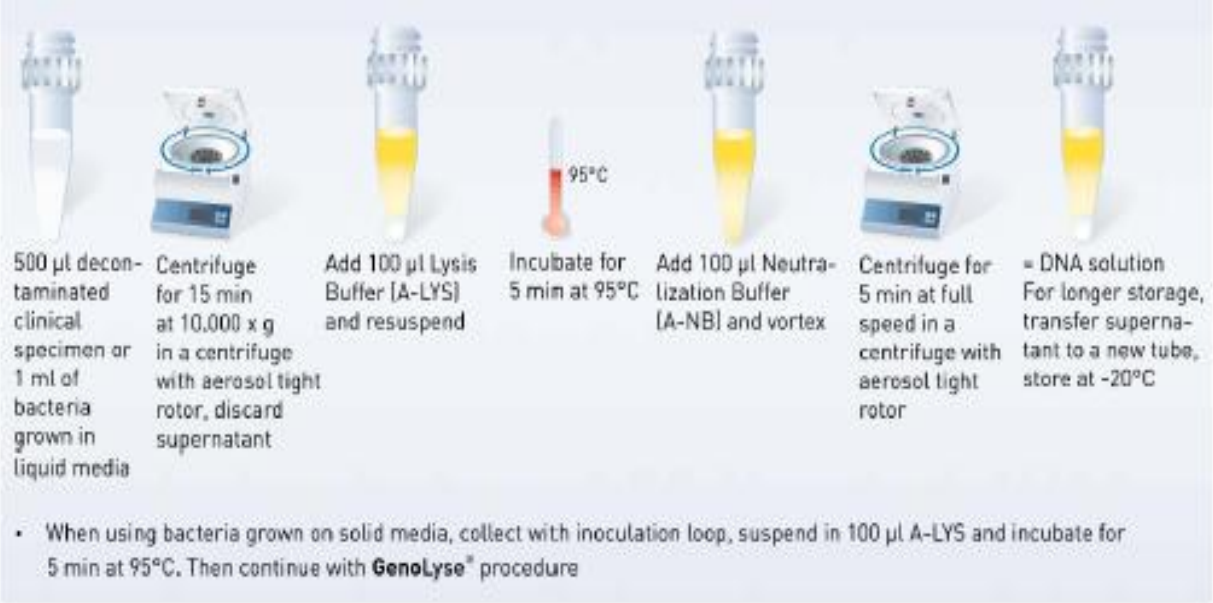

Figure 2. DNA extraction with GenoLyse ® method (Barnard*et al.*, 2012)

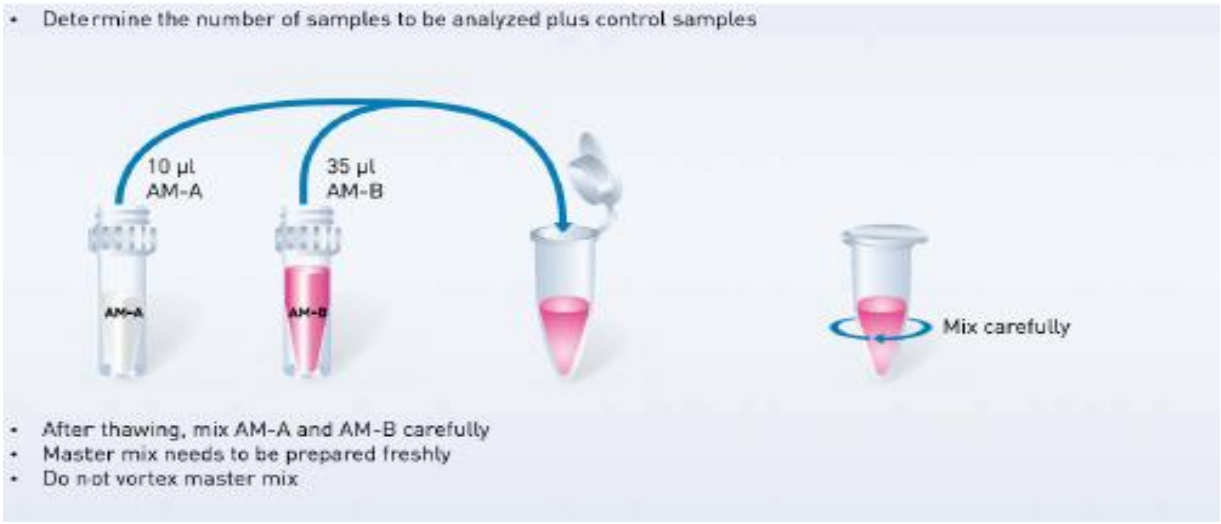

Figure 3. Preparation of master mix using the MTBDRplus® Version 2 kit (Barnard *et al.*, 2012)

Hybridization:-Hybridization and detection procedures were performed using a Twincubator (Hain Lifescience GmbH, Nehren, Germany) with program P1. The hybridization procedure includes the following steps (Figure 4): Aliquots of the hybridization buffer (HYB) and

stringent wash solution (STR) were placed inside a water bath set at 45^0C and allowed sufficient time to reach desired temperature. One ml of diluted Conjugate solution (CON) was prepared for each test by mixing 10 µl CON-C and 990 µl of CON-D. In addition, 1 ml of diluted Substrate solution (SUB) was prepared for each tested specimen by mixing 10 µl of SUB-C and 990 µl of SUB-D.

Furthermore, 20 µl of denaturing solution (DEN) was dispensed into the corner of the required number of test wells in plastic reaction tray. A 20µl of the PCR product was then mixed with the denaturing solution and left to stand at room temperature for 5 min. One ml of pre-warmed HYB was carefully added to each well and carefully mixed without spilling over the solution into neighboring wells. An MTBDRplus nitrocellulose line probe strip was then placed in each well and subsequently kept in a Twincubator (Hain Lifesciences) and incubated at 45^0 C for 30 min. Each well was then aspirated with a fine tipped pipette followed by addition of 1ml of STR. The tray was then placed back onto the Twincubator and incubated at 45^0 C for 15 min. After incubation, the trays contents were poured off and approximately 1 ml of rinse solution (RIN) was added into each well. The tray was placed back onto the Twincubator at room temperaturefor 1 min.

The content of the tray was poured off again and then 1 ml of CON was added into each well. The tray was then placed onto the Twincubator and incubated for 30 min at room temperature. After the incubation step the wells were again emptied and 1ml RIN was added and incubated atroom temperaturefor 1 min. This step was repeated. After the second aliquot of RIN was discarded, approximately 1 ml of distilled water was added to each well and incubated on the Twincubator for 1 min at room temperature. The trays contents were again poured off and 1 ml diluted SUB solution was added to each well. The tray was left in dark place at room $T^0$for 3-20 min depending on color production. Prolonged exposure to substrate can lead to increased background staining which may hinder interpretation of results. Finally, the SUB solution was removed completely and the reaction was stopped by rinsing twice with 1 ml distilled water for 1 min. then, the nitrocellulose line probe strips were removed from the tray and dried on absorbent paper(Barnard *et al.,* 2012; Hain Lifescience, 2012).

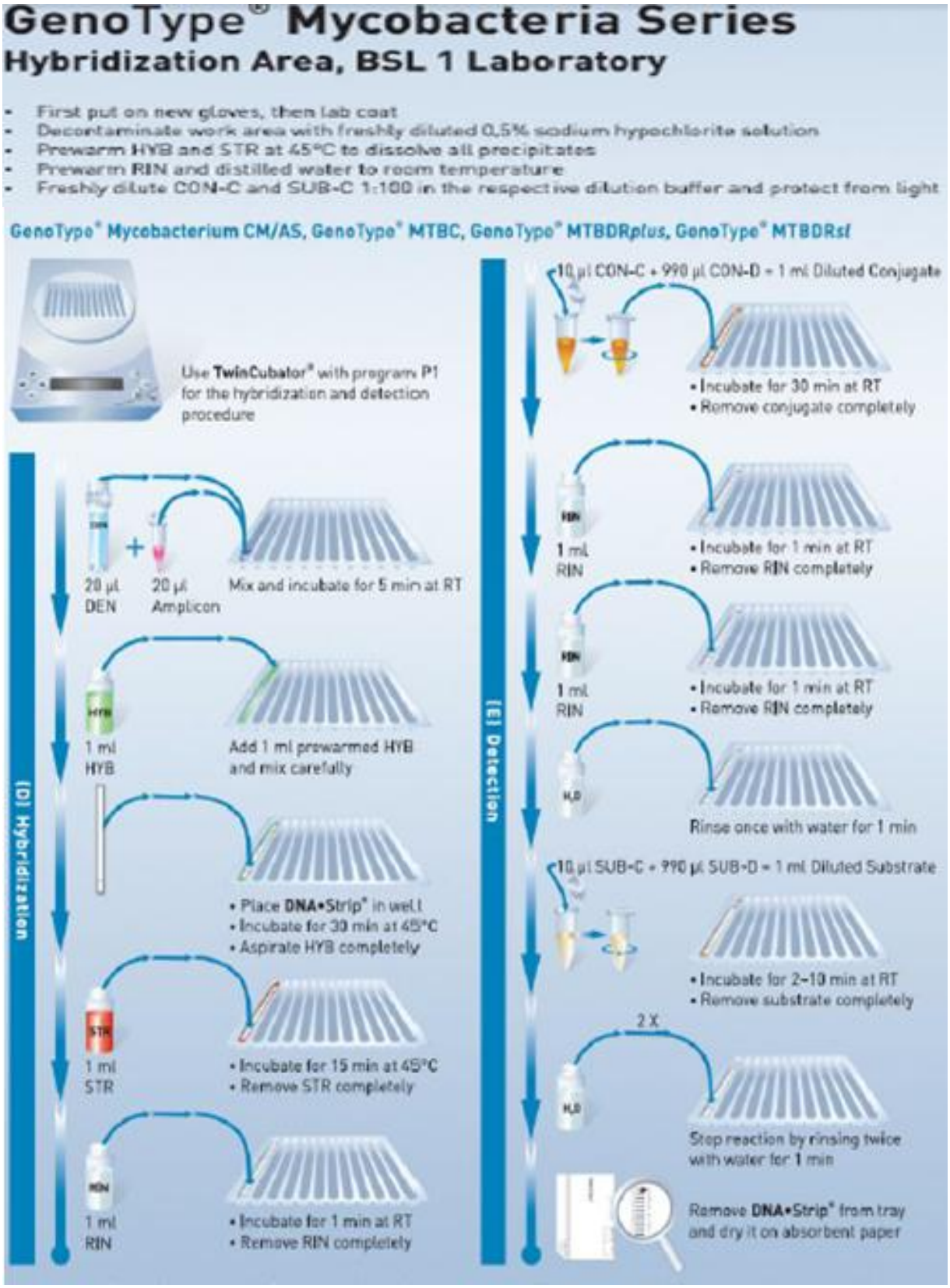

Figure 4.Work-flow diagram illustrating the different steps of the Hybridization and Detection procedure using the Twincubator.

Interpretation of results: - The evaluation sheet supplied in the kit was filled out with details of each specimen. The nitrocellulose line probe strip for each specimen was pasted on to the line sheet lining up the Conjugate Control and Universal Control lines that correspond on the evaluation sheet. The columns corresponding to genes and mutations were marked with + or - according to the result. If any WT band from each gene was missing, the specimen was considered to be resistant to the drug. If a MUT probe was positive, then the specimen was considered resistant to the drug to which the gene conferred resistance. During interpreting the result, a specimen was considered sensitive if all wild type probes test positive and when there was no hybridization with mutation detection probes. In normal situations, if a MUT was apparent, the WT probe in the region was normally absent (Figure 5 and 6). In some circumstances, specimens may have a mixed population of a susceptible and a resistant strain of *MTB* (Barnard *et al.,* 2012). This result would have all WT probes, as well as a MUT probe. Incomplete amplification of RIF and/or INH genes was considered as an invalid result. Specimens failing the assay were rechecked for the presence of MTB*C* specific DNA and resistance to RIF and INH.

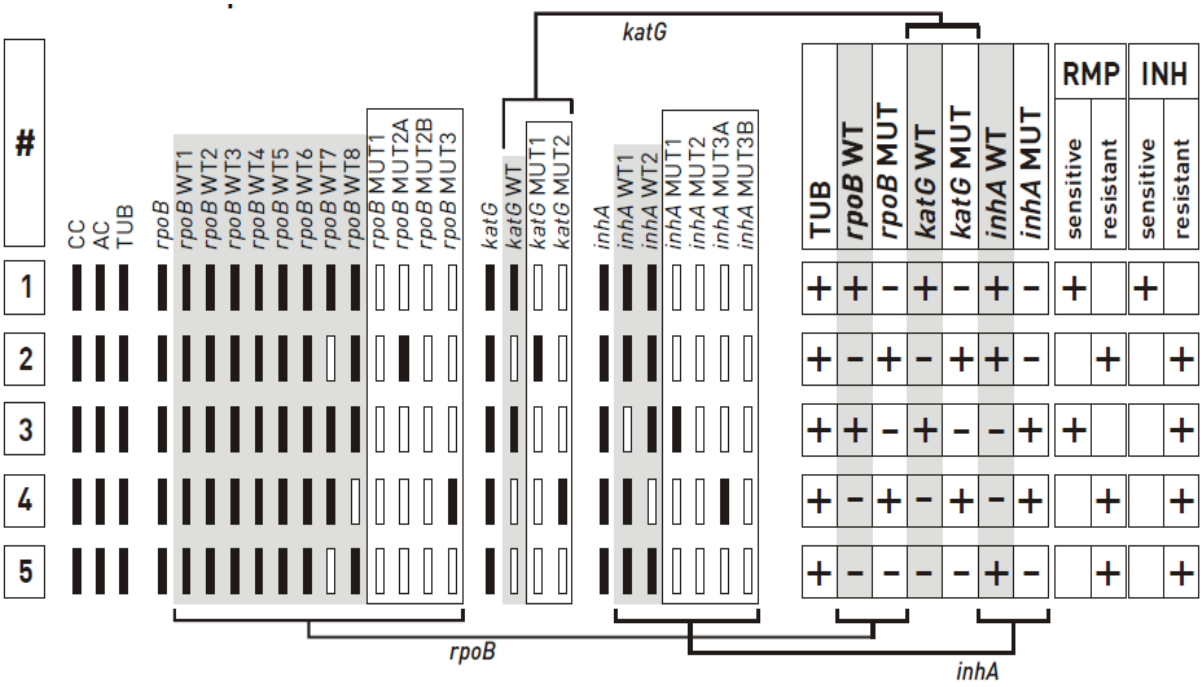

Figure 5.Example of banding patterns and their evaluation with respect to RIF and/or INH resistance (Hain Lifescience, 2012)

3.7. HIV Testing

All study participants were requested for HIV testing after counseling. Five ml of venous blood was drawn aseptically from each participant and checked for sero-status using rapid test kit (KHP, Stat pack, and Unigold) as recommended by the national guideline (FMOH, 2013).

3.8. Quality Control

It is extremely important to perform a quality control of molecular DST periodically. If the batch Quality Control fails, all the results obtained within that batch, as well as the new batch of a reagent should be thoroughly reviewed and the testing should be repeated. The reference strain of *MTB* H37Rv (American Type Culture Collection 27294), which was susceptible to all standard anti-TB drugs, was used in each batch test (Siddiqi and Rusch-Gerdes, 2006). The strain was supplied by the GenoType MTBDRplus assay manufacturer (Hain Lifescience GmbH, Nehren, Germany) with all other required.

3.9. Data Quality Management

To assure quality of data, the questionnaire was prepared in English (Annex 2.) and one BSc nurse with good language ability of English and Somali translated the English version into Somali. The translated Somali version of the questionnaire was also translated back to English by other BSC nurses looking for a possible gap in the contents of the original and the second translated versions. The same procedure was followed to develop the Amharic version of the questionnaire. Based on the exercise, wording and ways of questioning to avoid vagueness was modified. In addition, thorough training for data collectors was given for one day by the principal investigator. Furthermore, the principal investigator made frequent checks on the data collection process to ensure the completeness & consistency of the gathered information. Any error, ambiguity, incompleteness encountered was addressed on the following day before starting next day activities.

3.10. Data Processing and Analysis

In this study, primary data obtained from the assay was collected using standard formats recommended by the manufacturer. The investigator used to check the data, sort, and review it manually for errors and inconsistencies. Data entry and analysis was performed using SPSS Version 16 statistical software (SPSS Inc., Chicago, IL, USA). A bivariate logistic regression analysis was done to select the variables to be entered into the final logistic multivariable analysis. Explanatory variables (with P-value<0.25) were entered into the final logistic regression (multivariate) model based on the likelihood ratio. Aassociation between the independent variables and the outcome variable were assessed using AOR and 95% CI for the AOR and P-values less than 0.05 as cut-off point for statistical significance.

3.11. Ethical Clearance

Ethical clearance was obtained from Haramaya University, Postgraduate Program Directorate' Ethical Review Committee. Permission was also obtained from the Somali Regional Health Bureau and from Harar Regional Laboratory. Informed oral consent was obtained from the study participants. In addition, the entire outcomes (drug resistance pattern) of the study were submitted to the physicians and to the responsible person in the study area for further management of the patients. Confidentiality of the participants was kept by the investigator.

3.12. Operational Definitions

The following definitions were used based on the National TB guide line of Ethiopian federal ministry of health (FMOH, 2013). A smear-positive case defined as at least two smears of scanty grade (1 to 10 AFB per 100 fields) or one or more smears of 1+ or more (10 to 99 bacilli per 100 fields) (FMOH, 2013).New case means a patient who has never had treatment of TB or has been on treatment for less than four weeks and previously treated case is a patient previously treated for TB undergoing treatment for a new episode, usually of bacteriological active positive TB. Relapse defined as a patient who was previously treated for TB and whose most recent treatment outcome was “cured” or “treatment completed”, and who is

subsequently diagnosed with recurrent episode of TB. Any resistance refers to resistance to at least one anti-TB drug, while mono-resistance is a resistance to only one first-line anti-TB drug. MDR: resistance to at least both INH and RIF (FMOH, 2014).

4. RESULTS

4.1. Characteristics of the Study Participants

In the present study, a total of 105 consecutively registered smear-positive PTB patients who agreed to participate in the study were enrolled. Of the 105 study participants' specimens, 98 (93.3 %) gave interpretable results. The rest were excluded because the DNA of *MTBC* was not detected in the specimens using Genotype MTBDRplus assay. The majority of the patients, 58(59.2 %) were males and 40(40.8 %) were females. The mean and median age was 35 and 33 years, respectively. Of the total, 67 (68.4 %) were new smear-positive PTB patients and 31 (31.6 %) were previously treated smear positive patients.

The study participants comprised of 28 (28.6%) rural and 70 (71.4%) urban dwellers and their educational status showed that39 (39.8%) were illiterate, 49 (50%) were able to read and write, and 10 (10.2%) were college graduates and above. Alcohol consumption was reported by 9 (9.2%) of the study participants, while cigarette smoking was reported in 15 (15.3%). History of imprisonment was indicated by 9 (9.2%) of the respondents. The smear of 7 (7.1%) patients were scanty; 51 (52.1%) were 1+; 23 (23.5%) were 2+; and 17 (17.3%) were 3+ in AFB smear microscopic examination. HIV status data of all study participants were collected either by *in-situ* testing or from their medical record. Of the total, 8 (8.2 %) were co-infected with HIV while 90 (91.8 %) were without HIV co-infection (Table 2).

Table 2. Distribution of the study participants (n= 98) by socio-demographic characteristics, life style and HIV status vis-a-vis their previous history of treatment.

Variables	New cases (N=67),F (%)	Previously treated cases (N=31) F (%)	Total(N= 98), F (%*)
Sex			
Female	30 (75%)	10 (25%)	40 (40.8%)
Male	37 (63.6%)	21 (36.2%)	58 (59.2%)
Age			
18-30	30 (71.4%)	12 (28.6%)	42 (42.9%)
31-50	31 (70.55)	13 (29.5%)	44 (44.9%)
>50	6 (50%)	6 (50%)	12 (12.2%)
Mean (SD)			**35 (12.53)**
Educational status			
Illiterate	26 (66.7%)	12 (33.3%)	39 (39.8%)
Read and write	36 (73.5%)	14 (26.5%)	49 (50 %)
College and above	6 (60%)	4 (40%)	10 (10.2%)
Residence			
Rural areas	18 (64.3%)	10 (35.7%)	28 (28.6%)
Urban	49 (70%)	21 (30%)	70 (71.4%)
Degree of smear positivity			
Scanty	7 (100%)	-	7 (7.1%)
+1	33 (64.7%)	18 (35.3%)	51 (52.1%)
+2	16 (69.6%)	7 (30.4%)	23 (23.5%)
+3	13 (76.5%)	6(23.5%)	17 (17.3%)
HIV status			
Non-reactive	65 (71.4%)	26 (28.6%)	91 (92.9)
Reactive	3 (37.5%)	5 (62.5%)	8 (8.2%)
Family history of TB patients			
No	57 (73.1%)	21 (26.9%)	78 (79.6%)
Yes	10 (50%)	10(50%)	20 (20.4%)
Close contact to TB patients			
No	58 (69%)	26 (31%)	84 (85.7%)
Yes	9 (64.3%)	5 (35.6%)	14 (14.3%)
History of Imprisonment			
No	61 (68.5%)	28 (31.5%)	89 (90.8%)
Yes	6 (66.7%)	3 (33.3%)	9 (9.2%)
Alcohol usage			
No	62 (66.7%)	31 (33.3%)	93 (94.9%)
Yes	5 (100%)	-	5 (5.1%)
Smoking Status			
No	58 (68.2%)	26 (31.8%)	84 (85.7%)
Yes	9 (44.4%)	5 (55.6%)	14 (14.3%)

N= total number, F= Frequency, SD= Standard Deviation, - %* = (F/N) 100

4.2. Genotype MTBDRplus Assay Result of the Study Participants

A molecular DST called Genotype MTBDRplus assay was performed on all specimens using the methodology described previously. Strips were evaluated and interpreted using the evaluation sheet included in the kit (Figure 6). Examples of hybridized strips and their interpretation can be seen in Figure 5 and 6. Overall, a total of 105 smear-positive specimens were processed and valid results were obtained for 98 of them. Of the 98 samples, 80 (81.6%) were sensitive to both drugs, 18 (18.4%) were resistant to RIF and/or INH and 10 (10.2%) of the specimens were MDR-TB cases. In this study, among the total 67 new cases observed, 3 (4.5%) showed MDR-TB. On the other hand, of the total 31cases characterized previously treated, 7 (22.6%) were MDR-TB cases. The prevalence of any resistance to RIF and INH were 10 (10.2%) and 18 (18.4%), respectively (Table 3). While there was no mono-resistance to RIF, mono-resistance to INH was 8 (8.2 %); 5 (7.5%) among new cases and 3 (9.7%) among previously treated cases. Specimens of 59 (88.1%) new patients and 21(67.7%) of previously treated patients were fully sensitive to both anti-TB drugs tested. The proportions of patients showing resistance to any of the drugs among new patients and previously treated patients were, 8/67 (11.9%) and 10/31 (32.3%), respectively, among new patients and previously treated cases.

Table 3: GenoType MTBDRplus assay results among new and previously treated cases in Karamara General Hospital, Jigjiga town.

Resistant status	New cases (N=67) F (%)	Previously treated cases (N=31) F (%)	Total (N=98) F (%)
Susceptible	59 (88.1%)	21 (67.7%)	80 (81.6%)
Any resistance	8 (11.9%)	10 (32.3%)	18(18.4%)
RIF	3 (4.5%)	7 (22.6%)	10 (10.2%)
INH	8 (11.9%)	10 (32.3%)	18 (18.4%)
Mono-resistance			
RIF	0	0	0
INH	5 (7.5%)	3 (9.7%)	8 (8.2%)
Multidrug resistance	3 (4.5%)	7 (22.6%)	10 (10.2%)

RIF= Rifampicin, INH= Isoniazid, N= total number, F=Frequency

4.3. Association between Risk-factors and Drug Resistance Patterns

Data was collected from each study participants using interview guided structured questionnaire and analyzed using SPSS version 16. The association between risk factors and resistance to any of the anti-TB drugs (i.e. RIF and INH) was assessed by bivariate logistic regression. Those independent variables with p-value less than 0.25 were subsequently selected for multivariate logistic regression analysis (Table 4). Thus, on the basis of the outcomes of the bivariate analysis, the risk factors (independent variables): age, educational status, history of imprisonment and alcohol usage were excluded from the final (multivariate) regression analysis (p-value ≥0.25).

In the present study, multivariate logistic regression (Table 4) analysis was performed to determine the association between risk factors and any drug resistance among study participants. Therefore, previous treatment history of TB (AOR= 6.86, 95% CI: 1.41 – 33.32, P-value: 0.017), close contact with TB patients (AOR= 12.41, 95% CI: 2.06 – 74.86, P-value: 0.006) and smoking cigarette (AOR= 9.66, 95% CI: 1.06 – 83.30, P-value: 0.045) were significantly associated with the development of any drug resistance. Previously anti- TB treated patients had 6.8 times more likely to develop both RIF and/or INH drug resistant TB than new cases. Similarly, participants with cigarette smoking habit and those who had close contact with TB patients had 9.66 and 12.4 odds ratio, respectively, than participants who did not smoke cigarettes and those who did not have close contact with TB patient in developing drug resistance. However, in the present study factors such as sex, residence, degree of smear positivity, HIV status, and family history of TB were not significantly associated with the development of RIF and/or INH resistant specimens (P>0.05).

Table 4. Bivariate and multivariate analysis for selected characteristics of study participants and their association with any resistance to RIF and/or INH based on GenoType® MTBDRplus

Variables	Any drug resistance (%)	COR (95% CI)	P-value	AOR (95% CI)	P-value
Sex					
Female	5/40 (12.5%)	R		R	
Male	13/58 (22.4%)	2.02 (0.66 – 6.21)	0.219	1.36 (0.27 - 6.85)	0.707
Degree of S+					
Scanty	1/7 (14.3%)	R		R	
+1	11/51 (21.6%)	2.67 (0.14–49.76)	0.511	5.06 (0.09 - 227.72)	0.427
+2	5/23 (21.7%)	4.4 (0.52 – 36.94)	0.172	3.02 (028 - 32.62)	0.362
+3	1/17 (5.9%)	4.44 (0.47–42.18)	0.194	10.82 (0.88-133.87)	0.630
Residence					
Rural	8/28 (28.6%)	R		R	
Urban	10/70 (14.3%)	0.42 (0.15 – 1.20)	0.105	0.30 (0.08-1.21)	0.090
HIV status					
No	15/91 (16.5%)	R		R	
Yes	3/8 (37.5%)	3.00 (0.65–13.92)	0.161	1.87 (0.15-23.33)	0.626
Previous Rx history of TB					
No	8 /67 (11.9%)	R		R	
Yes	10/31 (32.3%)	4.71 (1.61–13.80)	0.005	**6.86 (1.41-33.32)**	**0.017***
Family history of TB					
No	9/78 (10.3%)	R		R	
Yes	9/20 (45%)	4.53 (1.49–13.81)	0.008	2.79 (0.56-14.04)	0.212
Close contact with TB Pts.					
No	8/84 (9.5%)	R		R	
Yes	10/14 (71.4%)	9.87 (2.83–34.36)	0.000	**12.41 (2.06-74.86)**	**0.006***
Smoking status					
No	13/84 (15.5%)	R		R	
Yes	7/14(50%)	6.64 (1.95–22.58)	0.002	**9.66 (1.06-83.30)**	**0.045***

R= Reference value, F= Frequency, INH= Isoniazid, RIF= Rifampicin, AOR= Adjusted odds-ratio, COR= Crude odds-ratio, CI= Confidence Interval, S+= Smear positive, Pts= Patients, Rx= Treatment, '*' significant (P< 0.05)

4.4. Frequency of Gene Mutations Associated with Drug Resistant Specimens

Drug resistance was reported using the assay when either an absence in one of the WT probes, or the presence of a MUT probe is observed. The molecular method DST results showed that18 of the specimens had mutations conferring resistance to both RIF and INH or INH only (Table 5). Mutations conferring resistance to both INH and RIF were detected in 18 and 10 of the specimens, respectively. *Mycobacterium tuberculosis* strains obtained from10 of the samples showed mutations in both *rpoB* gene and *katG* gene or *inhA* promoter region indicating that they were MDR. Among the total 10 RIF resistant specimens, the distribution of mutant genes in various regions showed that 8 were because of the absence of *rpoB* WT8 and presence of *rpoB* MUT3 and the amino acids changed were *Ser531Lue*. Whereas, the remaining were;1was due to absence of *rpoB*WT7 and presence of *rpoB* MUT2B, with an amino acid change of*His526Asp* and 1was due to deletion in *rpoB* WT7/*rpoB*MUT2A, with amino acid change of *His526Tyr* (Table 5). All mutations in the *rpoB* gene, that indicated RIF resistance, were also had mutation in either *katG* gene or *inhA* promoter region that shown INH resistance.

Mutations associated with INH resistance were more often encountered as compared to those seen in RIF resistance specimens. Of the total 18 INH resistant specimens, 15 had mutations in the codon 315 of *katG* (*katG* WT/*katG* MUT1) gene with a change in amino acid*Ser315Thr1*, indicating high level resistance, while 3 of the specimens had mutation in the codon15 of *inhA* promoter gene (*inhA* WT1/*inhA* MUT1), with amino acid change of *Cys15Thr*, indicating low level resistant. There was no specimen that showed mutations at both *katG* and *inhA* genes. All *katG* gene mutations were as a result of the absence of WT probes and the presence of mutant probes, as was the case with the *inhA* gene mutations (Table 5).

Table 5. Determination of frequency of gene mutations associated with resistance to INH and RIF by GenoType MTBDRplus

Anti-TB drugs	No. of resistant specimens	Patterns of gene mutations (wild-type/mutant)	Amino acid Changes	Frequency F (%)
Isoniazid	18	Δ*katG* WT/*katG* MUT1	*Ser315Thr1*	15 (83.3%)
		Δ*inhA* WT1/*inhA* MUT1	*Cys15Thr*	3 (16.7%)
Rifampicin	10	Δ*rpoB* WT8/*rpoB* MUT3	*Ser531Leu*	8 (80%)
		Δ*rpoB* WT7/*rpoB* MUT2B	*His526Asp*	1 (10%)
		Δ*rpoB* WT7/*rpoB* MUT2A	*His526Tyr*	1 (10%)

Δ= deletion, F= Frequency of specimens, WT=wild-type, MUT= mutant

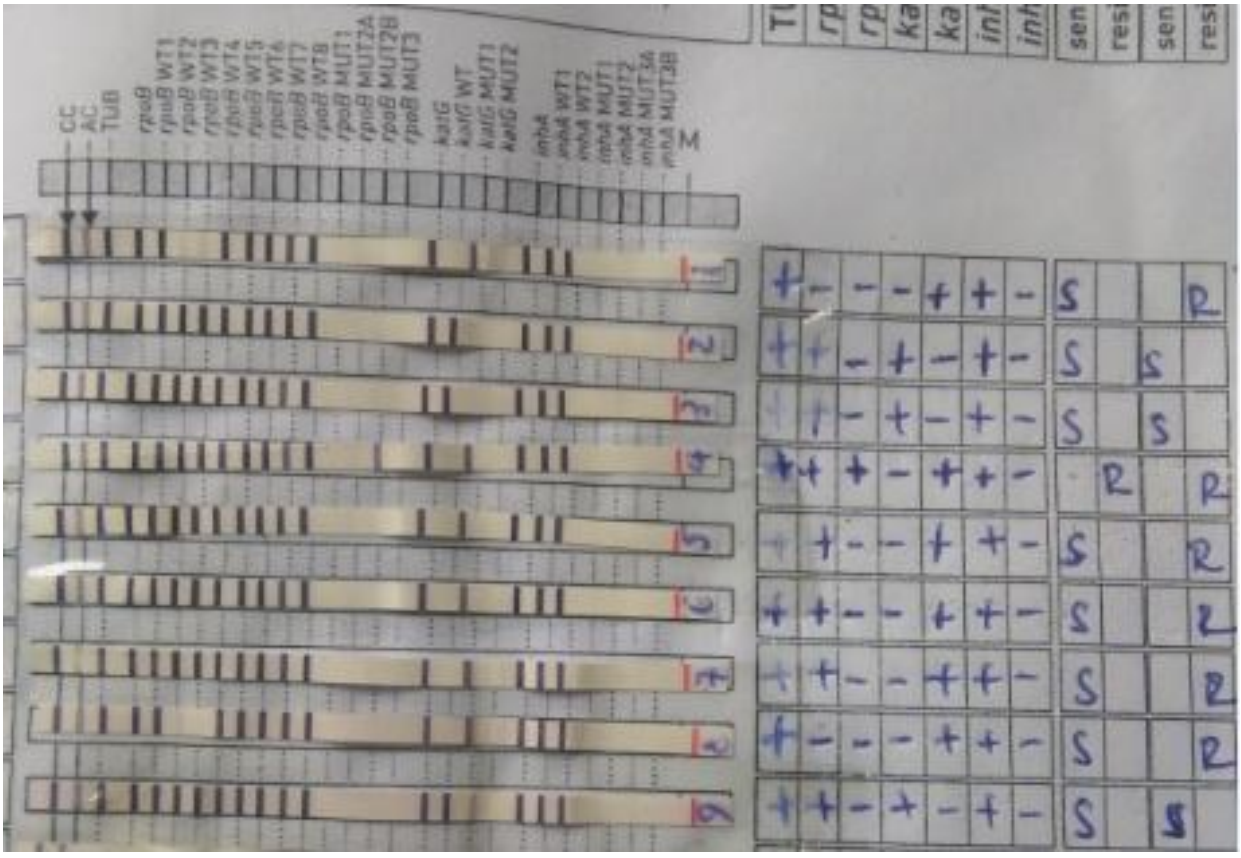

Figure 6. Banding patterns of genes showing mutation and their evaluation with respect to RIF and /or INH resistance

5. DISCUSSION

Rapid identification of drug resistance, particularly MDR-TB is most important to help reduce the spread of disease (WHO, 2008). The MTBDRplus assay has been widely used in clinical routines for identification of *MTB* complex and detection of RIF and INH resistance. The purpose of the study was to identify drug resistance TB including MDR-TB and to determine the frequency of mutations conferring resistance to RIF and INH in clinical specimens in the study area.

In the present study the overall prevalence of MDR-TB cases were 10.2%. According to the review paper by Fantahun *et al.* (2014), the proportion of MDR-TB cases varies from place to place in Ethiopia. The finding from the present study is comparable to the studies conducted by Hussein *et al.* (2013) and Ali *et al.* (2012). However, the studies conducted by Mulualem *et al.* (2010) and Dereje *et al.* (2012) reported significantly higher rate of MDR-TB cases. On the other hand, studies conducted in other parts of the country reported lower prevalence of MDR-TB cases (Ahmed *et al.*, 2014; Fantahun *et al.*, 2013). In the present study, the prevalence of MDR-TB among new and previously treated cases was 4.5% and 22.6%, respectively. This is a higher rate compared to the second round drug resistance survey performed in Ethiopia from 2011 to 2013, where MDR-TB prevalence was shown to be 2.8% and 18.6% among new and previously treated TB patients, respectively (FMOH, 2014). Similarly, this study has also shown higher prevalence of MDR-TB than global MDR-TB report by WHO, in which 3.6% of newly diagnosed and 20% of retreatment cases were estimated to have MDR-TB (WHO, 2013).

Among new patients, 4.5% were MDR-TB cases (Table 3). This result was higher than previous studies done in Ethiopia that range from 0.9% to 2.3% (Desta *et al.*, 2007; Mulualem *et al*., 2010; Gemeda *et al*., 2012; Birhanu *et al*., 2014; Ahmed *et al.*, 2014) and other studies conducted in African countries such as in Kenya (0.54%) (Ogaro *et al.*, 2012), in Madagascar (0.2%) (Wright *et al.*, 2009) and in Tanzania (0.4%) (Willy *et al.*, 2008). Studies in other parts of the world also reported low rate of MDR-TB among new cases (Chacón *et al.*, 2009; Dye, 2009). However, a higher rate of MDR-TB in new cases was observed in other studies

conducted: 7.7% in Swaziland (Sanchez-Padilla *et al.,* 2012), 5.8% in Mozambique (Nunes *et al.,* 2005), and 5.2% in Somalia (Sindani *et al.,* 2013). This indicated that there was high transmission of drug resistance in the community.

Similarly, in the present study, MDR-TB among previously treated cases was observed to be higher (22.6%) than studies conducted in eastern Amhara region of Ethiopia (18.5%) (Ahmed *et al.,* 2014), in Madagascar (3.4%) (Wright *et al.,* 2009), in Kenya (8.5%) (Ogaro *et al.,* 2012) and in Uganda (12.7%) (Temple *et al.,* 2008). But, in this study, MDR-TB was lower than that reported by Dereje *et al.* (2012) (46.3%). In studies conducted by Liu *et al.* (2013) in northeastern China and Choi *et al.* (2007) in Korea, the prevalence of MDR-TB was greater than the present study among previously treated cases. In this study, there was a higher proportion of MDR-TB than that in previous reports in other parts of Ethiopia. This may possibly be due to ineffective functioning of TB Control Programs in the study area which included irregular supply of anti-TB drugs, less-organized patient diagnosis, treatment, and follow-up and improper patient adherence to treatment that may contribute to the high prevalence of MDR-TB cases in the study area.

In the present study, it was observed that drug resistance was significantly associated ($P<0.05$) with previous history of TB treatment (Table 4). In agreement with this finding, previous studies in Ethiopia reported that previous history of treatment was the most significant risk factor for drug resistance (Mulualem *et al.,* 2010; Dereje *et al.,* 2012; Hirpa*et al.,* 2013; Asres*et al.,* 2013; Ahmed *et al.,* 2014). Similar reports were also documented in studies conducted in East Africa, India and China (Liu*et al.,* 2013; Kidenya *et al.,* 2014). The association of previous history of treatment with anti-TB to DR-TB in this study might be explained by inadequate or irregular drug supply, inappropriate chemotherapy regimens, and lack of supervision of treatment in hospitals. Compared to other studies, in the present study close contact with TB patients and smoking cigarette were also significantly associated with the development of drug resistance.

Rifampicin resistance is often considered as a surrogate marker for checking MDR-TB (Ramaswamy and Musser, 1998). This hypothesis is supported by the finding in the present

study that 100% of the RIF resistant specimens were MDR (Table 3). In this study, 10 (10.4%) *rpoB* gene mutations which indicated RIF resistance were detected. Previous studies reported that 95% of RIF-resistant *MTB* worldwide have mutations within the 81-bp core region of the *rpoB* gene, mainly in the region of codon 507-533 (Zhang Ying *et al.,* 2005; Vilcheze and Jacobs, 2007; Riccardi *et al.,* 2009). In the present study mutations in this region was detected in 100% of RIF resistant specimens. Of the total *rpoB* gene mutations detected, the most frequently mutated codon in the study was codon 531 (80%) at position *Ser531Leu*, which is similar to those reported in previous studies (Kozhamkulov *et al.*, 2011; Belay *et al.*, 2012; Poudel *et al.*, 2012). The remaining 20% of the mutations were at the position of codon-526 (an amino acid change of *His526Asp* and *His526Tyr*).

In this study, the percentage of gene mutation at position *Ser531Leu* (80%) was higher than that reported by Belay *et al.* (2012), which was 73 % at that position; whereas study in western Poland detected slightly higher proportion (82.7%) (Kozhamkulov *et al.*, 2011). In the present study it was observed that there was no mutation detected at the WT probes alone, that is, all mutations at the WT were also detected at the MUT probes. This is different from the previously reported gene mutation distribution, in which 60% and 20% of the mutations were only detected at the WT probes by Zhang *et al.* (2011)and Belay *et al.* (2012), respectively. This reflects the difference in the distribution of gene mutations associated with RIF resistance in different geographical locations.

Previous studies have already indicated that INH resistance was mediated by mutations in several genes, most commonly *katG*, particularly in codon 315, and the promoter region of *inhA* (Riccardi*et al.,* 2009; Zhang and Yew, 2009). Many researchers have also shown that 40-95% of INH resistance may be defined as high-level drug resistance due to the *katG* gene mutations while 8% to 43% of INH resistance may be defined as low-level drug resistance mainly caused by mutations in the promoter region of *inhA* gene (Vilcheze and Jacobs, 2007; Riccardi *et al.,* 2009; Zhang and Yew, 2009). In the present study, 83.3% of INH resistances were attributed to *katG* mutations which confer high level resistance to INH and in addition to a low-level drug resistance observed in 16.7% of INH resistance cases (Table 5), which were within the reported ranges by previous studies.

Furthermore, several studies have also shown that 75-90% *katG* gene mutations are recognized as mutations in the 315th codon of the *katG* gene, which mainly results in *Ser315Thr1* and *Ser315Thr2* changes (Vilcheze and Jacobs, 2007; Riccardi *et al.,* 2009; Poudel *et al,* 2012). The present study revealed the detection of *katG* gene mutation responsible for the *Ser315Thr1* amino acid substitution observed in 83.3% of INH resistant *MTB*, which is comparable to the reports from Nepal (81.2%), Pakistan (84%), Lithuania (85.7%), and Germany (88.4%) (Bakonyte *et al.,* 2003; Sajduda *et al.,* 2004; Poudel *et al,* 2012). One study conducted in Ethiopia has even shown higher prevalence of *katG* gene mutations (94%) among INH resistant *MTB* (Belay *et al.,* 2012). The same study also reported that 100% of INH resistance was due to *katG* gene mutations causing amino acid substitutions at the position *Ser315Thr1*. Another study showed that 8% to 43% of INH resistance is defined as low-level drug resistance mainly caused by the mutations in the promoter region of *inhA* gene (Zhang *et al.,* 2011). The results of this study, also reveal a 16.7% low-level drug resistance, which is within the range reported by Zhang *et al.* (2011) but higher than that reported by Belay *et al.* (2012) from Ethiopia.

6. SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

6.1. Summary

Efforts to control TB are hampered by increasing resistance of *MTB* strains to the most effective anti-TB drugs. Drug resistant TB, including MDR-TB, has emerged as a serious threat to global health. Based on the second round drug resistance survey performed in Ethiopia (2011-13), MDR-TB prevalence was reported as 2.8% in new and 18.6% in previously treated TB patients. Molecular methods that target drug resistance mutations are a suitable approach for a rapid DST to detect drug resistant *MTB.* The primary objective of this study was to investigate gene mutations associated with RIF and INH resistance of MTB and to determine the associated risk factors related to drug resistance TB among smear-positive PTB patients attending Karamarada General Hospital, located in Jigjiga Town.

Institutional based cross sectional study design was employed. Structured interviews guided questionnaire and Genotype MTBDRplus assay was employed as data collection tools. Patients included in the study were both new and previously treated cases, in whom TB were confirmed by smear microscopy, and who were not currently receiving TB treatment. The study subjects were identified by using consecutive sampling. Sputum specimens were collected from 105 consecutive AFB smear-positive PTB patients in 50ml sterile, plastic, screw capped centrifuge tubes and transported to Harar Regional Laboratory for further analysis. Sputum specimens were decontaminated by NALC-NaOH method with final concentration of 1% and then used for detection of gene mutations associated with RIF and INH resistance using Genotype MTBDRplus assay according to the manufacturer's instructions.

Overall, a total of 105 smear-positive specimens were processed and valid results were obtained for 98 of them. Among the patients, 58 (59.2 %) were males and 40 (40.8 %) were females. Of the total, 67 (68.4 %) were new cases and 31 (31.6 %) were previously treated cases. Of the 98 samples, 80 (81.6%) were sensitive to both drugs, 18 (18.4%) were resistant to RIF and/or INH and 10 (10.2%) of the specimens were MDR-TB cases. The prevalence of

MDR-TB among new cases and previously treated cases were 3 (4.5%) and 7 (22.6%), respectively. While there was no mono-resistance to RIF, mono-resistance to INH was 8 (8.2%). In the present study, previous treatment history of TB, close contact with TB patients and smoking cigarette were significantly associated with the development of drug resistance. Mutations conferring resistance to INH and RIF were detected in 18 (18.2 %) and 10 (10.2 %) of the specimens, respectively.

Among the total 10 RIF resistant specimens, the distribution of mutation genes in various regions showed that 8 were because of the absence of *rpoB* WT8 and presence of *rpoB*MUT3 and the amino acids changed were *Ser531Lue*. Whereas, the remained were; 1 were due to deletion of *rpoB* WT7/*rpoB*MUT2B, with an amino acid change of *His526Asp* and 1 was also due to deletion in *rpoB* WT7/*rpoB* MUT2A, with amino acid change of *His526Tyr*. Of the total 18 INH resistant specimen, 15 specimens had mutation in the *katG* (codon 315) gene with amino acid change of *Ser315Thr1*, indicating high level resistance, while 3 of the specimens had mutation in the *inhA* promoter gene, with amino acid change of *Cys15Thr*, indicating low level resistance. There was no specimen that showed mutations at both *katG* and *inhA* genes. Among mutations associated with the resistance to RIF and INH, the majority were in codon 531 of the *rpoB* gene and codon 315 of the *katG* gene.

6.2. Conclusions

Studies on anti-TB drug resistance are an essential management tool for evaluating the performance of NTPs. In MDR-TB high-burdened country, the molecular assays performed extremely well in the detection of *MTBC* and MDR-TB as compared to conventional culture and DST. Its application will help better solve the long-standing problem of laboratory diagnosis of drug resistance in Ethiopia, particularly in the study area. In the present study, relatively high prevalence of MDR-TB were observed in the study area compared to the second round national drug resistance survey among the both new and previously treated participants. This situation might threaten efforts of TB control activities and further aggravate development of MDR-TB.

Previous history of anti-TB treatment, close contact with TB patients and smoking cigarette were important risk-factor with the development of drug resistance. Among mutations associated with the resistance to RIF and INH, the majority were in codon 531 of the *rpoB* gene and codon 315 of the *katG* gene.

6.2. Recommendations

- The study found high levels of drug resistant TB in the study area which calls for urgent concerted efforts to prevent transmission of drug resistant TB and effectively treat patients with MDR-TB as a crucial protection of public health and TB control.
- The high prevalence of drug resistance in this study area may limit the success of TB control program. So the study underscores the importance of establishing advanced diagnostic facilities for early detection of MDR-TB.
- This study observed associated risk factors with the development of drug resistance TB. Therefore, it is essential to address the problems of development of drug resistant TB by establishing good TB control programs (DOTS and DOTS plus).
- Strengthening of laboratories for TB culture and DST should be promoted in the region.
- Studies are required to understand the mechanism of resistance to evaluate GenoType®MTBDRplus assay for the diagnosis of INH and RIF resistance from direct sputum specimens of TB patients in Ethiopia.
- The study calls for strengthened efforts on the implementation of the comprehensive framework of DOTS-Plus strategy that adds components for MDR-TB diagnosis, management and treatment integrated within the DOTS programs.
- Prevention and control of drug-resistant TB should be emphasized by routine and quality-assured DST for those patients at high risk of resistance and proper administration of anti-TB drugs according to the recommended national guidelines.
- Further studies on a large scale to determine the extent of drug resistance to all first line drugs (INH, RIF, EMB, PZA, and STR).

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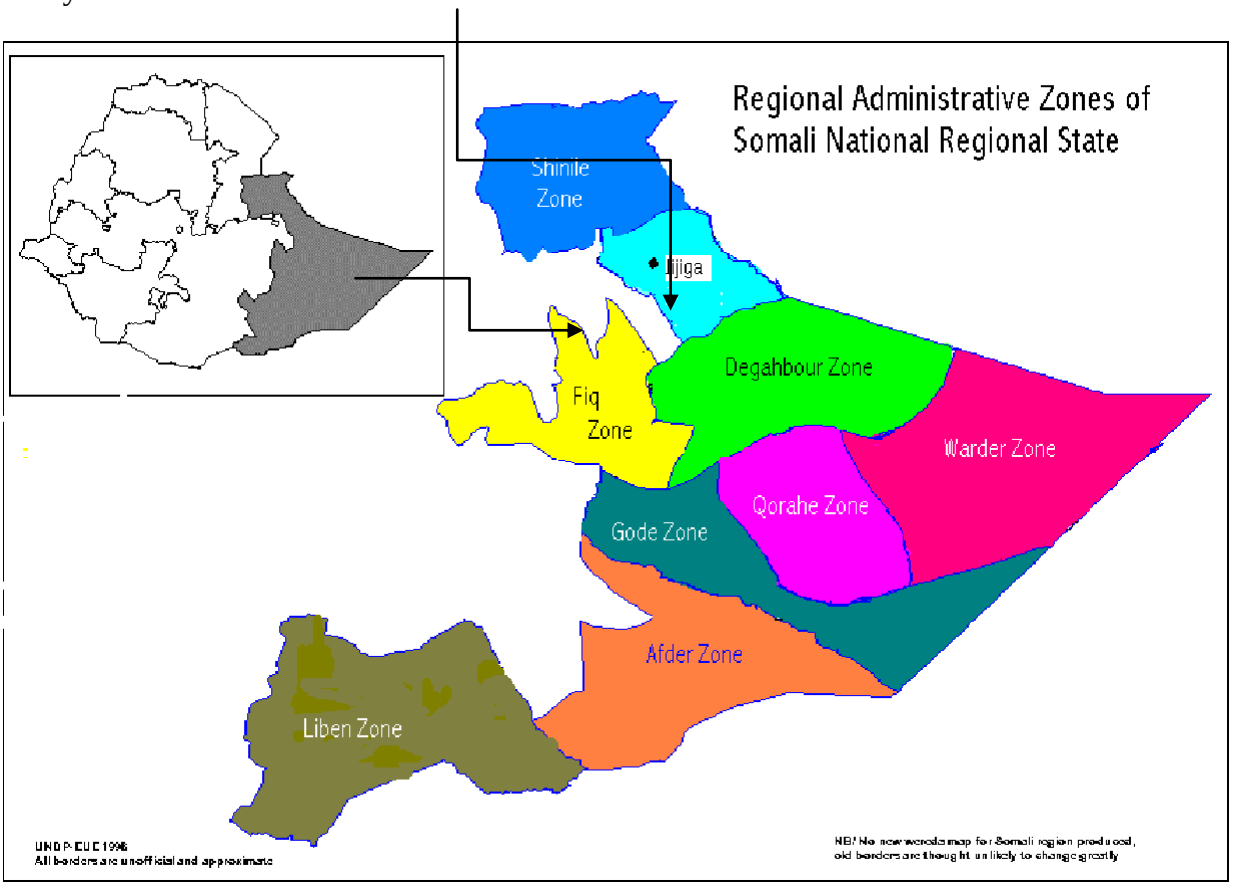
8. APPENDIX

Annex 1. Ethiopia Somali Regional State Map

Study area: Fafan Zone



Annex 2. Questionnaire

HARAMAYA UNIVERSITY

POSTGRADUATE PROGRAM DIRECTORATE

COLLEGE OF COMPUTITIONAL AND NATURAL SCIENCES

DEPARTMENT OF BIOLOGY

Questionnaire Developed for Study on Molecular Detection of Multidrug Resistant Tuberculosis (MDR-TB) and Its Associated Risk-Factors among Smear-positive Pulmonary Tuberculosis Patients in Jigjiga Town, Somali Region of Ethiopia

Introduction: Good morning/afternoon, my name is _____and I am laboratory technician working in this hospital. I am also part of a team carrying out a study conducted by a Biotechnology postgraduate student in Haramaya University. The purpose of the study is molecular detection of MDR-TB and its associated risk-factors among smear-positive pulmonary TB patients.

Confidentiality and consent: You have been selected to participate in this study. I am going to ask you some very personal questions. You can refuse to answer any questions or series of questions if you are uncomfortable. Your participation is purely voluntary, and you can withdraw any time after you get involved in the study without compromising the services you ought to get from the hospital However, I would like to assure you that all that is said during the interview will be strictly confidential and the information collected from you will be used only in scientific reports without mentioning your personal identification including your name. There is no harm to you in participating or no incentive paid but your honest answer to these questions will help us better understand the situation. In addition the Information gathered from the study will be used to improve programs that promote the wellbeing of women. So we hope you will give accurate answers! We appreciate your help in responding to these questions. Do you have any questions?

Would you be willing to participate?"

Yes_____ (continue) No_____ (Thank and stop)

Questionnaire code_____ Name of the health facility: _____

General instruction

i. For all questions that have a pre-coded response, it is important to follow the following instructions while you are interviewing the respondents and recording their responses

- ✓ Ask each question exactly as written on the questionnaire
- ✓ Circle the responses that best match with the answer of the respondent
- ✓ Do not read the pre coded responses for the respondents, listen only the response of the respondents.

Participant Id. No.: _____ Date of interviewing: ____/____/_____E.C. (Day/Mo/Yr)

1. Socio-demographic Data

S. No.	Questions	Possible answer	Remark
1.1.	Sex	1. Female 2. Male	
1.2.	How old are you? (in full year)	1. _____Years 88. Don't know	
1.3.	Where is your residence/Inhabitant area	1. Rural area 2. Urban	
1.4.	What is your educational status	1. Illiterate 2. Read and write 3. College or above	

2. Medical record and histories given by the study participants

2.1.	HIV sero-status	1. Non-reactive 2. Reactive 3. Unknown	
2.2.	Degree of smear positivity	1. Scanty 2. 1+ 3. 2+ 4. 3+	
2.3.	Do you have previous history anti-TB treatment?	1. No 2. Yes	
2.4.	Do you have history of TB in your family member?	1. No 2. Yes	
2.5.	Have you ever had close contact with active TB patient?	1. No 2. Yes	
2.6.	Do you have cigarette smoking habit	1. No 2. Yes	
2.7.	Do you have history of alcohol consumption?	1. No 2. Yes	
2.8.	Do you have a history of imprisonment?	1. No 2. Yes	

Annex 3. National Standard Operating Procedures for Biological Sample Transportation

PURPOSE

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

GENERAL CONSIDERATION

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

MATERIALS

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

SAMPLE TYPE: Sputum

AMOUNT: 3-5 ml*

COLLECTION:

- Two purulent /muco purulent early morning and spot sputum specimen for culture and DST.

STORAGE: Store the sputum specimen at 2 to 8oC up to 5 days

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

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

SPECIMEN REJECTION:

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

SAFETY PRECAUTIONS

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

PROCEDURES: SPUTUM SPECIMEN COLLECTION PROCEDURE

Instruct the patient

- To collect in a separate, ventilated room or preferably outdoors/ produce sputum in sputum coughing designation area/
- To Keep both hands on hips, cough forcibly and collect sputum in the mouth
- To spit the sputum carefully into a wide-mouthed, leak proof container
- To collect 3–5ml in volume, although smaller quantities are acceptable if the quality is satisfactory.

SPUTUM SAMPLE PACKAGING AND SHIPMENT

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

Triple Packaging Materials

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

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

Annex 4. Tuberculosis Laboratory Requesting and Reporting Form

1. PATIENT IDENTIFICATION:

Patient Full Name: _____Age (Yrs): _____Sex (M/F): _____

Region:_____Zone/Subcity:_____Woreda:_____Kebele:_____

House No: _____ Tel.: _____

Referring Health Facility: _____ Co-infection: _____

2. B DISEASE TYPE & TREATMENT HISTORY:

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

3. REQUEST FOR TESTING AT TB LABORATORY:

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

4. LABORATORY RESULT:

Sample Number: _____ Date specimen received: ____/___/____ (Ethiopian Calendar)

Date of result: ____/____/___ Examined by (name and signature): _____

Microscopic examination result:

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

☐ Ziehl-Neelsen (ZN) ☐ Fluorescence
☐ Direct Smear ☐ Concentrated Smear

Xpert MTB/RIF test result (*to be completed in the laboratory)*

Date sample collected: ____/___/___ Date of result: ____/___/___

Examined by (name and signature): _____

M. tuberculosis: ☐Detected ☐Not detected ☐ Invalid / No result / Error

Rifampicin resistance:☐ Detected☐ Not detected ☐Indeterminate result

TB Culture result:

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

[1] Non-tuberculous mycobacteria,C= colonies

Drug susceptibility test (DST) and line probe assay (LPA) results

Date Sample Collected	Method[a]	Laboratory Serial number(s)	Results[b] (mark for each drug)								
			H	R	E	S	Amk	Km	Cm	FQ	Other( )

a=Method, Specify: solid media DST; liquid media DST; direct LPA; indirect LPA
b=Results codes: R = Resistant; S = Susceptible; C = Contaminated ; ND = Not done

Date reported: ____/____/____ (Ethiopian Calendar)

Name/ Signature: _____Reviewed by: _____

Annex 4. Acid Fast Bacilli Smear Preparation and Staining Procedure

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

Overview of smear preparation

1. Label each slide with the correct number (serial and order number)
2. Smear sputum onto slide
3. Allow smear to air dry
4. Heat fix smear

Preparing sputum smears

I. Numbering the slides

1. Select new, clean, grease-free, unscratched slides which are free fromfingerprints.
2. Using a pencil number of the sputum specimen on the frosted end of the slide

II. Sputum smearing

1. Using the end of an applicator stick or wire loop, pick up purulent particles of sputum.
2. Prepare the smear in an oval shape in the center of the slide.
3. For good spreading of sputum, firmly press the stick perpendicular to the slide and move in small concentric circles or coil-like patterns.
4. Place the used stick into a discard container.
5. Use a separate stick for each specimen.
6. Alternatively, if a wire loop is used instead of a broken stick, dip the wire loop in a sand-alcohol bottle. After each smear is completed, heat the wire loop in a flame until red-hot.
7. Thorough spreading of the sputum is very important; it should be neither too thick nor too thin.

III. Air drying of smear

1. Allow the smear to air drycompletely at room temperature.
2. Do not dry smears in direct sunlight or over a flame.

IV. Heat fix smear

1. After the slide is completely dry, use forceps to hold the slide upwards.
2. Pass the slide over the flame 2–3 times for about 2–3 seconds each time.
    - Do not heat the slide for too long or keep it stationary over the flame, orelse the slide will be scorched.

Overview of ZN staining procedure:

1. Arrange slides in serial order on staining bridge, with smear side up
2. Flood slides with filtered Carbol fuchsin stain
3. Gently heat to steam
4. Keep the staining reagent for at least 5 minutes after heating
5. Rinse with water and drain
6. Apply decolorizing solution for 3 minutes
7. Rinse with water and drain
8. Apply methylene blue counter stain for NOT MORE THAN 1 minute
9. Rinse with water and drain
10. Air dry on a slide rack
11. Then look AFB using oil immersion objective (100 times) under microscope

WHO/IUATLD Grading Scale

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